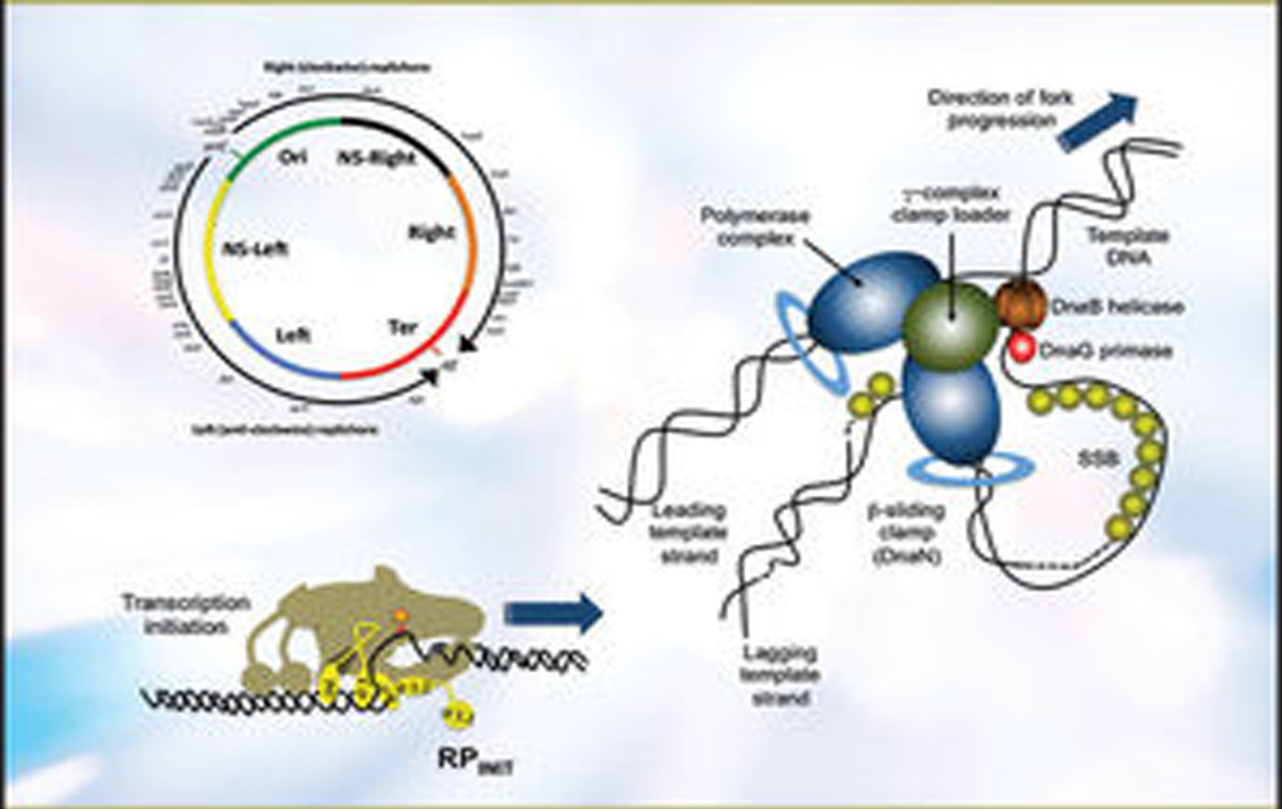
CHARLES J. DORMAN

STRUCTURE AND FUNCTION OF THE BACTERIAL GENOME



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Structure and Function of the Bacterial Genome

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This book is dedicated to my wife, Dr Niamh Ní Bhriain and to our sons, Andrew Dorman and Matthew Dorman.

Contents

Preface

The bacterial genome is the software that contains the information for running the cell. The information resides in the genetic material of the bacterium, metaphorically, in both digital (nucleotide sequence) and analogue (DNA topology) forms. From this information emerges the operational hardware, in the form of RNA and proteins, which compose and regulate the pathways for the building of the cell, for the operation of the processes that maintain cell function and which repair and replicate the cell and its contents. Expressing the information that is contained in the cellular software requires sophisticated molecular machines and involves complex processes. Over many decades, molecular microbiology has revealed the details of these machines and processes and has greatly deepened our understanding of the genome itself. By now, the breadth and depth of the information available about these topics can be intimidating, even to experienced investigators. This book will attempt to organize its most important features in ways that allow the reader to grasp the 'big picture'.

Specialists who focus on 'the nucleoid', 'the cell cycle', 'bacterial metabolism', 'gene regulation', 'transport', etc. have often studied these bacterial cellular systems and processes in isolation from one another for the very good reason that each is an enormous subject, capable of occupying a whole scientific career. This compartmentalisation of information is valuable because it helps to organize information in discrete packets under clear headings. It is also consistent with the idea of science as an organized body of knowledge. However, even a cursory reading of the literature under the standard headings will reveal that there is tremendous overlap between distinct cellular systems in terms of their components and their governance architecture.

Recently, there has been a great growth in the quantity of data coming from whole-genome studies of bacterial cells. Experienced investigators may feel that there is just too much information to absorb and the literature is now too vast to read and assimilate. Students entering the field are at an even greater disadvantage and can be forgiven for being discouraged by the mountain of facts. If the discipline of molecular microbiology is to thrive in the future, it is essential that new entrants to the field, and established investigators, have the ability to navigate the sea of information safely. It is the purpose of books like this one to be islands of meaning in this sea of information. This will be done by highlighting the most important components and processes of the bacterial cell, by providing context for cellular operations and by pointing out connections between the different systems and operations. The objective is to provide the reader with a unified picture of the bacterial genome at the structural and functional levels.

Detailed descriptions of the genome's container (the cell envelope) and of the metabolic processes and components that build and maintain it are not within the scope of this book. It will not be possible to provide comprehensive listings of all of the genes and gene products involved in every process that is included because there are just too many, and to attempt this would simply confirm in the reader's mind the impression that this is all just too complicated. Similarly, organism-to-organism comparisons will only be made to illustrate important principles; for the most part the narrative will be concerned with the model bacterium *Escherichia coli* and its close relatives. To attempt wider coverage of the prokaryotic world would simply create an unwieldy book and would defeat its main purposes.

The material in the book is based on the lectures I have delivered to my Junior and Senior Sophister (penultimate and final year) BA students in the Microbiology Moderatorship at Trinity College Dublin over the past 25 years. Although the Microbiology Moderatorship degree at Trinity College is an undergraduate degree, in terms of international comparisons, its advanced content and the demands it places on students are equivalent to taught MSc degrees elsewhere. The lectures seek to introduce the students to the complexities of global gene regulation in model bacteria and how these relate to the structure of the bacterial nucleoid. They are informed by my research in these fields since 1981 and by a close reading of the development of understanding in those fields over the intervening years. This work has been supported by grants from Science Foundation Ireland since 2003. I am grateful to Steve Busby and Jayaraman Gowrishankar for comments on the draft manuscript; the responsibility for any remaining errors of fact or of omission is mine alone.

Dublin 2019 *Charles J. Dorman*

1

The Bacterial Genome – Where the Genes Are

1.1 Genome Philosophy

The genome of a bacterium consists of its entire collective of genes, and these can be located in a chromosome (or chromosomes) and on extra-chromosomal autonomous replicons such as plasmids. Chromosomes and plasmids replicate, copying the genes that they carry, with the replicon copies being segregated into the daughter cells at cell division. This process drives the vertical transmission of genetic information from one generation to the next and its fidelity determines the stability of the genetic information in the genome. If non-lethal errors occur during the replication of the genome the resulting mutations will be transmitted to the next generation.

The vertical transmission of genetic copying errors is one of the driving forces of evolution in all types of organisms. Bacterial cells are also prone to the evolutionary influence that is horizontal gene transfer (HGT). Here, foreign genes are transmitted to the bacterium, adding to its genetic complement. Evolution through HGT is much less subtle than evolution through the vertical transmission of copying errors which often involves the gradual accumulation of single nucleotide changes to the genome over many generations. Other types of mutation that are transmitted vertically are inversions, insertions, and deletions of genomic DNA. In HGT a bacterium can acquire entire clusters of genes *en bloc*, resulting in the acquisition of novel capabilities in a single generation. Examples include the arrival of genes that make the bacterium resistant to an antibiotic or to a heavy metal that previously could kill it, or genes that allow the organism to colonise a niche in the environment from which it had previously been excluded.

HGT played a key role in the early research work that led to the mapping of the bacterial genome and to our understanding of the locations of its genes. Among the autonomously replicating plasmids found in bacteria are elements that can promote their own transfer from cell to cell. The fertility, or 'F', factor of *Escherichia coli* was among the first to be studied. F encodes proteins that can build a connecting bridge between the F^+ (or male) cell and one that is F^- (female). F transfers one of its DNA strands to the F^- cell in a process called conjugation. This is the bacterial equivalent of sex. It resembles sex in higher organisms in that the participants are male (F^+) and female (F^-) but it differs from conventional sex because the process converts the female into an F^+ male. The F plasmid has segments of DNA that are identical in sequence, or almost identical, to DNA segments found in the chromosome. Usually these are mobile genetic elements called insertion

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sequences (IS). The homologous recombination machinery of the cell can recombine the F-associated region of DNA sequence identity with a chromosomal counterpart, causing F to become fused with that part of the chromosome (Hadley and Deonier 1980). Where this happens is determined by the location of the IS element, and these mobile elements can be found at sites distributed around the chromosome.

Once F has become one with the chromosome, it is replicated as a part of that molecule. It can still engage in conjugation, however. When this happens, the DNA that is transferred to the F^- female bacterium consists of chromosomal DNA with F DNA in the vanguard. Strains that can act as DNA donors in these matings are called 'Hfr' (high frequency of recombination) (Reeves 1960) and homologous recombination between the incoming, horizontally transferred DNA and the resident chromosome allows the order of the genes on the chromosome to be determined. Experiments, in which chromosomal gene transfer mediated by the F plasmid was monitored as a function of time, allowed a rudimentary genetic map of the *E. coli* chromosome to be assembled (Bachmann 1983; Brooks Low 1991). Because the mating experiments were allowed to proceed for fixed periods of time before the deliberate breakage of the conjugation bridges by mechanical shearing, these early genetic maps were calibrated in 'minutes'. It was discovered that it took 100 minutes to transfer the entire *E. coli* chromosome from one cell to another by conjugation (Bachmann 1983; Brooks Low 1991). Similar experiments were performed for other bacterial species, including the pathogen *Salmonella*, giving rough approximations of the physical scale of bacterial genomes (Sanderson and Roth 1988). Hfr strains could also mediate gene transfer between *E. coli* and *Salmonella* (Schneider et al. 1961). When the F plasmid is excised from the chromosome, genes that had been adjacent to the plasmid can be removed too, becoming part of the autonomously replicating episome. The plasmids are called F-prime (F′) and have proved to be very useful in genetic analysis. The chromosomal gene 'cargo' can be transferred to F-minus strains by conjugation and this phenomenon can be exploited in genetic complementation experiments. Work of this type provided useful information about gene order and the position and nature of genetic mutations. F-primes have been used to investigate plasmid stability, incompatibility, and DNA replication: for example, the F′-*lac* episome was used extensively to study plasmid replication in *E. coli* (Davis and Helmstetter 1973; Dubnau and Maas 1968). Experiments with *E. coli* mutants deficient in Hfr recombination led to the discovery of important genes involved in homologous recombination: for example, *recA* (Clark and Margulies 1965), *recB*, and *recC* (Barbour and Clark 1970; Willetts et al. 1969; Youngs and Bernstein 1973).

HGT also provided a means for more refined mapping of genomes. Bacteriophages (often abbreviated to 'phages') are viruses that replicate in bacterial cells. Some phages package bacterial DNA in their viral heads as they exit the bacterial host and this DNA is transferred to the next bacterium that they manage to infect in a process known as transduction. The length of the DNA segment that a phage head can accommodate is finite and known in the cases of the viruses most commonly used for generalised transduction in *E. coli* (P1, 100–115 kb) and *Salmonella* (P22, 42 kb) (Sternberg and Maurer 1991). Therefore, genes that are co-transduced must be within a distance of one another that is compatible with being co-packaged by the phage. Very sophisticated experiments with transducing phage allowed not only gene-to-gene distance relationships to be determined but also the

measurement of the physical relationships between features of individual genes, such as their regulatory elements.

Thus, HGT has driven bacterial evolution and microbial geneticists have exploited it to assemble the first genetic maps of bacterial genomes. Genetic engineers have also used HGT to build novel variants of bacterial genomes in the lab. Cloning experiments using vectors based on natural or engineered plasmids rely on the HGT process known as transformation to move new DNA sequences into bacterial cells. A bacterium that is susceptible to transformation is said to be 'competent' and competence can be induced chemically or by electric shock (Hanahan et al. 1991). In addition, many bacterial species are naturally competent and therefore open to the uptake of foreign DNA from the environment. Knowledge and application of the HGT processes of transformation, transduction, and conjugation have revolutionised our understanding of bacterial genomes in a matter of decades. Genome sequencing has extended and deepened this knowledge.

Foreign DNA entering bacterial cells may undergo surveillance. Much attention has been focused on clustered regularly interspaced short palindromic repeat (CRISPR) systems both in their natural roles as systems that identify and destroy 'non-self' DNA, and as a result of their promise as agents of genome editing (Barrangou et al. 2007; Brouns et al. 2008; Garneau et al. 2010). Restriction endonucleases and their associated DNA methylases represent another mechanism for defending the bacterial cell from foreign DNA. Here, the methylases chemically modify the newly synthesised DNA of the bacterial genome so that it matches the 'approved' pattern: incoming DNA that lacks this methylation pattern is cut into pieces by the restriction endonucleases. These DNA surveillance mechanisms help to control the access of foreign DNA to established genomes.

The concepts of 'foreign DNA' and 'established genomes' can also be expressed by the terms 'accessory genome' and 'core genome', respectively. 'Accessory' implies that portion of the genome is not essential for the life of the bacterium, and that may be true in the artificial environment of the laboratory. The name also suggests that some form of value is added to the life of the organism, but that this is conditional. In fact, the same can be said of any gene or portion of the genome. In some cases, the essential nature of a genome component is made obvious because the bacterium dies if this component is eliminated. However, this can apply to a portion of the accessory genome just as much as to one of the core genome, depending on the circumstances of the bacterium. A facile example concerns the presence in a bacterium of a gene encoding resistance to penicillin. The gene is not a part of the core genome (it may even be located on a plasmid and not on the chromosome) and it is not essential unless penicillin-class antibiotics appear in the neighbourhood. In the absence of this gene during periods of cell wall peptidoglycan synthesis, the bacterium dies if penicillin-class antibiotics are introduced; despite being a part of the accessory genome, the resistance gene is now an essential gene.

The accessory genome is distinguished from the core genome in being of more recent arrival in the cell. It is not a monolithic entity but a mosaic of imported genetic components that have arrived through HGT, possibly over a very long period of time. In this context, it is very important to realise that genome evolution is not only a process of gene acquisition: gene loss is equally important. A gene may be lost safely if another member of the genome can supply its function, if the cell can acquire the lost product from an exogenous source, or if the selective pressure to retain the gene has been removed.

The core genome consists of those genes that are essential for the life and reproduction of the cell and that are widely shared by other organisms, even those that are only distantly related. Thus, the analysis of genome composition using computers to compare and contrast the genes possessed by thousands of bacteria has helped to identify those genes that are truly almost universally present in bacteria. These designations of essentiality have been supported in some cases by experiments in which the genes have been removed and the impact of their loss on the survival of the bacterium has been measured (Baba et al. 2006; Gerdes et al. 2003; Goodall et al. 2018; Rousset et al. 2018). Essential genes include those whose products replicate the genome, transcribe the genes, translate the messages, and operate the principal metabolic pathways of the cell. In many cases, redundancies are revealed where more than one gene can contribute to an essential process. For example, *E. coli* has seven operons that contribute to the building of ribosomes, so losing one is not life threatening. On the other hand, the loss of even an apparently redundant gene might impose a fitness cost when a bacterium that has lost this gene competes with one that has not (Condon et al. 1995a).

An essential gene may cease to be essential if another microbe can supply the missing function. This phenomenon is easily illustrated in the laboratory by cross feeding of the mutant by a strain lacking the mutation, but it is not confined to metabolic functions. For example, the absence of an apparently essential virulence gene in one pathogen during infection can be compensated by a function encoded by a second, co-infecting pathogen (Ibberson et al. 2017).

Loss of competitive fitness arises when a change to the genome (a mutation) renders the bacterium unable to compete with an otherwise genetically identical counterpart. While this can result from the loss of a gene it can also be caused by gene acquisition. Indeed, the negative effect even may arise simply due to the process of expressing the new gene, and not to the effect on the cell of the new gene product (Stoebel et al. 2008a). This illustrates the subtle nature of the causes of competitive fitness differences and their relationships to genome composition and structure.

We will begin by considering genome composition and structure in the model bacterium *E. coli* and some others where useful data are available. This survey will provide information about any discernable rules governing these important aspects of microbial cell biology.

1.2 The Bacterial Chromosome

E. coli K-12 has played a central role in the history of bacterial genetics and bacterial physiology. The original K-12 isolate came from a stool sample from a human patient suffering from diphtheria and was cultured in Palo Alto, California, USA, in 1922 (Bachmann 1996). This isolate was the ancestor of W1485 from the Joshua Lederberg laboratory, the isolate that was named MG1655 by Mark Guyer (hence 'MG'). The first *E. coli* chromosome to be sequenced came from this intensively studied MG1655 strain (Blattner et al. 1997). However, this was not the first bacterial chromosome to have its complete nucleotide sequence determined: that honour belongs to *Haemophilus influenza* (Fleischmann et al. 1995).

The Blattner lab chose MG1655 because it has undergone relatively little genetic manipulation and is considered a good representative of wild-type *E. coli*. It has been cured of bacteriophage lambda and of the F plasmid and has few genetic lesions. An *ilvG*

mutation deprives it of acetohydroxy acid synthase II, making it prone to valine-dependent isoleucine starvation (Lawther et al. 1981, 1982) and there is an IS*5* insertion in the *rfb* locus that interferes with O-antigen synthesis (Liu and Reeves 1994). If this mutation is repaired, the bacterium has its lipopolysaccharide expression reinstated and it becomes pathogenic in an infection model based on the worm *Caenorhabditis elegans* (Browning et al. 2013). Strain MG1655 displays mild starvation for pyrimidine arising from poor expression of its *pyrE* gene: the cause is a frameshift mutation at the end of the *rph* locus (*rph-1*) (Jensen 1993). Interestingly, genome sequence analysis shows that MG1655 is closely related to NCTC 86, the bacterium originally named *Bacillus coli* by Theodor Escherich in 1885, isolated before the antibiotic era (Dunne et al. 2017).

The *E. coli* K-12 chromosome is a single, covalently closed, circular, double-stranded DNA molecule of 4 639 221 bp (Blattner et al. 1997). Although chromosome circularity is the norm in *E. coli*, cells in which the chromosome is artificially linearised (with the ends closed by hairpin turns) are viable, show few alterations in gene expression, have normal nucleoid structure, and do not display growth defects (Cui et al. 2007). Thus, the circular nature of the chromosome is not essential for its functionality or for its ability to be replicated and to be segregated at cell division.

The *E. coli* chromosome was visualised originally in the early 1960s by autoradiography of cells fed with tritiated thymidine in a classic experiment that also revealed the existence of the moving replication fork (Cairns 1963a,b). The chromosome undergoes bi-directional replication from its *oriC* locus (Kaguni 2011), creating two replichores: Left and Right (Figure 1.1) (Lesterlin et al. 2005; Wang, X., et al. 2006). Through a process of

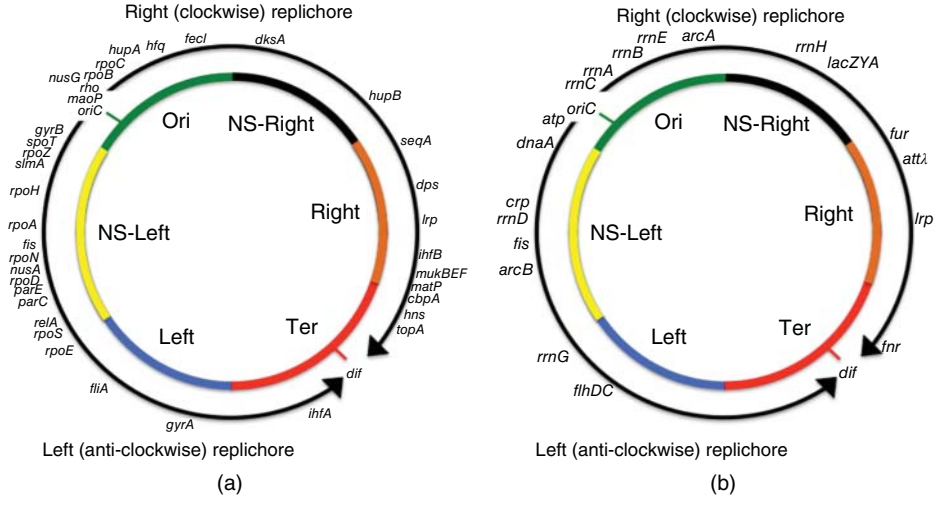

Figure 1.1 The macrodomain structure of the *E. coli* chromosome. Shaded segments represent the Ori, Right, Ter, and Left macrodomains, and the Left and Right non-structured regions. The curved arrows outside the circular chromosome represent the Left (anticlockwise) and Right (clockwise) replichores. (a) The positions of genes that encode NAPs, chromosome organisation factors, topoisomerases, proteins involved in the process of transcription, the Hfq RNA chaperone are indicated around the periphery of the chromosome. (b) The positions of the seven *rrn* operons and genes encoding transcription regulators that are discussed in the text are shown. The positions of the *lac* operon and the bacteriophage lambda attachment site (*attλ*) are also indicated. (*See colour plate section for colour representation of this figure*)

semi-conservative DNA replication, the bacterium acquires a second copy of its chromosome prior to cell division. In rapidly growing bacteria, one or more additional rounds of chromosome replication are initiated before the first one is completed, creating multiple copies of those chromosomal sequences that lie closest to *oriC* (Figure 1.1) (Cooper and Helmstetter 1968). Genes in the *oriC*-proximal zones of the *E. coli* chromosome will be present in higher copy numbers than genes in Ter, the region of the chromosome where replication terminates. In slower-growing bacterial populations, gene copy numbers are more in balance around the chromosome with only a twofold difference in copy number between genes close to *oriC* and those near Ter.

Most of our knowledge about chromosome replication and segregation comes from studying a handful of model organisms: *E. coli*, *Caulobacter crescentus*, *Vibrio cholerae*, and *Bacillus subtilis*. The focus in this chapter will be on *E. coli*, with comparisons to other organisms where this is useful.

1.3 Chromosome Replication: Initiation

Chromosome replication, segregation, and cell division are complex processes that must be coordinated to ensure the successful replication of the cell (Reyes-Lamothe et al. 2012). The nutritional status of the cell and its metabolic flux are very influential in achieving this coordination and they have a direct bearing on the growth rate of the culture (Wang and Levin 2009).

Replication of the *E. coli* chromosome begins at a specific site, *oriC*, which has a number of important DNA sequence elements called DnaA boxes that make up the DnaA Oligomerisation Region, DOR (Figure 1.2) (Fuller et al. 1984; Jameson and Wilkinson 2017; Katayama et al. 2017). These boxes are bound by DnaA, an adenosine triphosphate (ATP)-dependent initiator protein (Schaper and Messer 1995; Sutton and Kaguni 1997), which then forms a right-handed helical protein oligomer along the DNA that unwinds the duplex at an A+T-rich element known as the DNA Unwinding Element, DUE (Bramhill and Kornberg 1988a; Kowalski and Eddy 1989) (Figure 1.2). The DnaA oligomerisation process is assisted by another protein called DiaA (Ishida et al. 2004). The DUE has an A-rich and a T-rich DNA strand; once it is unwound, the T-rich strand binds to the DnaA oligomers at the DOR. A helicase loader known as DnaC then loads the DnaB helicase onto the single-stranded DNA (Koboris and Kornberg 1982). This helicase then recruits in turn the DnaG primase and DnaN, the DNA polymerase beta-clamp (Fang et al. 1999). When fully assembled, this complex is known as the replisome (Figures 1.3 and 1.4).

In *oriC* of *E. coli*, the DnaA boxes are of variable affinity for the DnaA protein (Blaesing et al. 2000) (Figure 1.2). Boxes with high affinity bind DnaA that is in a complex with either ATP or ADP, whereas weak boxes bind only DnaA that has bound ATP (Grimwade et al. 2007). Binding of DnaA to *oriC* is cooperative, with DnaA-ATP that has bound to strong boxes facilitating the subsequent binding of DnaA-ATP to the weaker sites, promoting the formation of the DnaA oligomer at the origin of replication (Miller et al. 2009; Kaur et al. 2014). The activity of DnaA may also be controlled by reversible acetylation at lysine residues: of the 13 lysine amino acids in DnaA, acetylation of residues K178 and K243 seems to be especially important in promoting the initiation of chromosome replication (Li et al. 2017; Zhang, Q., et al. 2016).

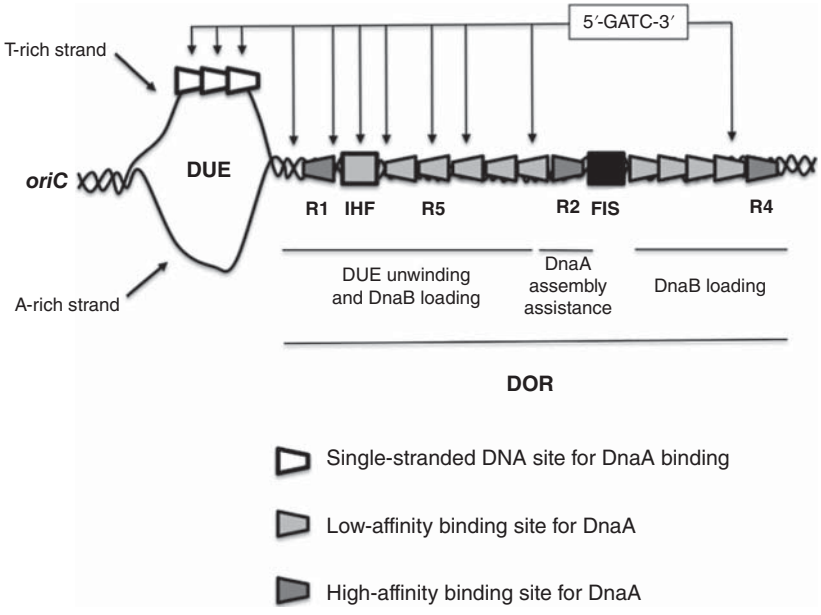

Figure 1.2 Structure of *oriC* on the *E. coli* chromosome. The ATP-dependent DnaA protein binds to sites throughout *oriC* and oligomerises in the DnaA Oligomerisation Region (DOR), driving DNA unwinding at the A+T-rich DNA Unwinding Element (DUE). Single-stranded T-rich DNA in DUE binds to the DnaA oligomers at DOR. High-affinity sites bind DnaA-ATP or DnaA-ADP; low affinity sites bind just DnaA-ATP. Binding sites for the NAPs FIS and IHF are also shown: FIS and IHF modulate the process of replication initiation negatively and positively, respectively. The Dam methylase methylates *oriC* at several 5′-GATC-3′ sites (indicated by vertical arrows): hemimethylated sites bind SeqA, excluding DnaA and preventing untimely re-initiation of chromosome replication.

The availability of DnaA-ATP is a rate-limiting factor for the initiation of chromosome replication. A protein called Hda converts active DnaA-ATP into inactive DnaA-ADP through ATP hydrolysis (Kato and Katayama 2001). This conversion also requires DnaN, the DNA polymerase beta-clamp, linking ATP hydrolysis to the elongation phase of DNA synthesis (Takata et al. 2004).

The nucleoid-associated proteins (NAPs) Integration Host Factor (IHF) and the Factor for Inversion Stimulation (FIS) are DNA-binding and -bending proteins that are thought to play important architectural roles at the origin of replication (Figure 1.2) (Kasho et al. 2014; Ryan et al. 2004). IHF has a positive role at *oriC* where it binds to a specific DNA sequence, introducing a DNA bend that encourages DnaA binding and oligomer formation; it can also redistribute DnaA on supercoiled DNA (Grimwade et al. 2000). While some work has not found a major role for FIS in regulating events at *oriC* (Weigel et al. 2001) data from other investigations show that, in contrast to IHF, the role of FIS is inhibitory to DNA replication: when it binds to *oriC* it interferes with the binding of IHF and DnaA, blocking unwinding of the DUE sequence (Ryan et al. 2004).

The many 5′-GATC-3′ sites found throughout *oriC* (Figure 1.2) are hemimethylated in the period immediately following the initiation of chromosome replication (Lu et al. 1994).

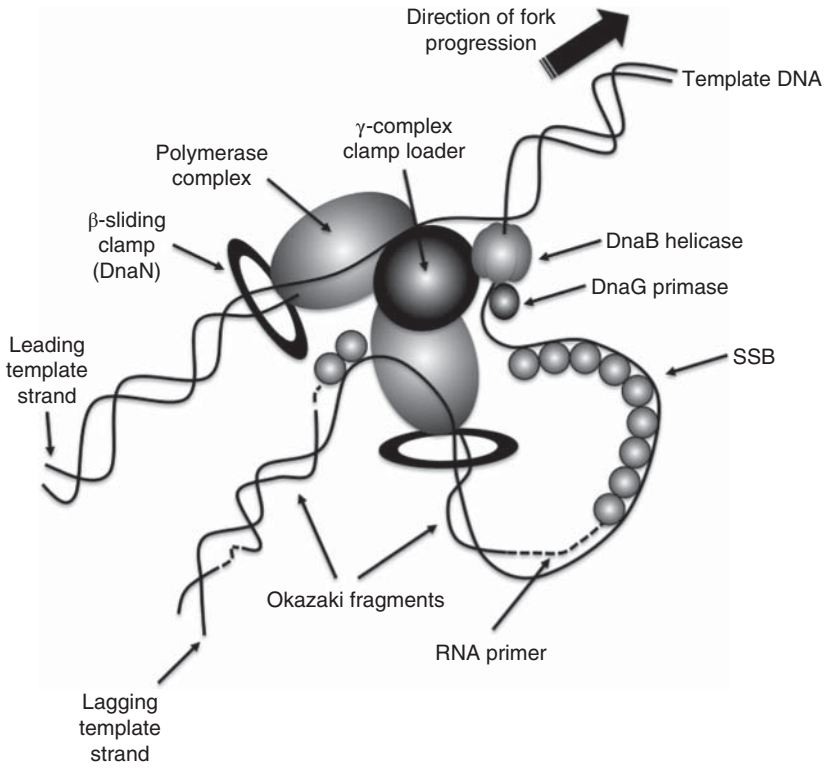

Figure 1.3 Structure of the *E. coli* replisome in chromosome replication. The replisome is made up of the two cores of DNA Polymerase III, a gamma (γ) complex (or clamp loader) and the beta clamp together with a hexameric helicase, the DnaG primase, and the single-stranded binding protein, SSB. The DnaB helicase uses energy from ATP hydrolysis to translocate along the lagging strand, unwinding the DNA duplex. The two Polymerase III cores, linked by the tau subunits (Figure 1.4), are each dedicated to coordinated and simultaneous replication of the leading and lagging template strands of the replication fork. The ring-like beta (β) clamp (DnaN), or processivity factor, encircles DNA and is attached to the replisome via the alpha (α) subunit. The β clamp stabilises the moving replication machine on its template, allowing it to operate with a high degree of processivity. A single-stranded DNA bubble is formed by the unwinding action of the replisome and SSB protein coats the ssDNA. The DnaG primase interacts with the helicase to generate RNA primers that are used to prime Okazaki fragment synthesis.

The SeqA protein binds to these hemimethylated sites, preventing immediate and untimely re-initiation of chromosome replication by DnaA: SeqA also downregulates the expression of the negatively autoregulated *dnaA* gene (Campbell and Kleckner 1990; Waldminghaus and Skarstad 2009). Dam-mediated methylation of the 5′-GATC-3′ sites is inhibitory to SeqA binding and re-admits DnaA to *oriC* (Lu et al. 1994).

E. coli uses clusters of DnaA binding sites that are located outside *oriC* to modulate the initiation of chromosome replication (Figure 1.5). One of these is the 183-bp *datA* site, located next to the *vjeV* gene on the *E. coli* chromosome. The *datA* site is made up of five high-affinity DnaA binding sites (Kitagawa et al. 1996); *datA* also binds IHF (Nozaki et al. 2009). The interaction of IHF with *datA* occurs immediately after the initiation of

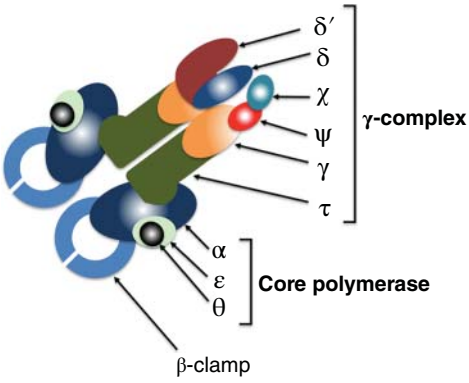

Figure 1.4 The structure of the DNA polymerase III subassembly (Pol III*). The core is made up of the alpha (α), epsilon (ε), and theta (θ) subunits and the holoenzyme contains two cores. The tau (τ) subunit (two copies) links the cores together, ensuring simultaneous replication of the leading and lagging strands. The function of the cores is DNA synthesis on the leading and lagging strands (Figure 1.3). The clamp loader (or gamma complex) is made up of the chi (χ), delta (δ), delta-prime (δ′), gamma (γ), and psi (ψ) subunits. The gamma subunit loads the beta clamp onto the DNA that is primed for de novo DNA synthesis. The arrival of the beta (β) clamp (processivity factor) converts the Pol III* subassembly into the Pol III holoenzyme (Figure 1.3). (*See colour plate section for colour representation of this figure*)

chromosome replication and this facilitates the binding of DnaA-ATP to *datA* (Nozaki et al. 2009). DnaA-ATP bound to *datA* undergoes ATP hydrolysis, reducing the size of the pool of DnaA-ATP that is available for binding to *oriC* (Ogawa et al. 2002). This IHF-dependent process has a negative influence on the frequency with which chromosome replication is initiated at *oriC* (Kasho and Katayama 2012).

Conversion of DnaA-ADP to DnaA-ATP has been associated with two so-called DnaA Reactivation Sites, DARS1 and DARS2 (Fujimitsu et al. 2009) (Figure 1.5). DARS1 is 103 bp in length, has three DnaA binding sites, and is located upstream of *uvrB* in *E. coli*. The DARS2 site is more sophisticated. It is 455 bp in length and is located upstream of the *mutH* gene in *E. coli*. DARS2 binds IHF and FIS in addition to DnaA. Binding of these NAPs to DARS2 stimulates the conversion of DnaA-ADP to DnaA-ATP. IHF binding is cell cycle determined while FIS binding is growth phase determined: FIS binds in rapidly growing cells and this is consistent with the observation that FIS stimulates DNA replication in rapidly growing *E. coli* (Flåtten and Skarstad 2013; Kasho et al. 2014). The chromosomal locations of *datA* and the DARS elements seem to be important for their function: if they are repositioned, the chromosome replication control is disrupted (Frimodt-Møller et al. 2016).

The *oriC* locus is found between two highly conserved genes, *mioC* and *gidA* (Figure 1.6). The *mioC* gene is transcribed towards *oriC* while *gidA* is transcribed away from it. The two genes exhibit reciprocal transcription patterns that are functions of the cell cycle: *mioC* transcription is maximal midway through chromosome replication while *gidA* transcription is minimal at that point; maximal expression of *gidA* coincides with the onset of septation and cell division (Lies et al. 2015). MraZ, a protein possibly involved in cell division control, binds and represses the *mioC* promoter (Eraso et al. 2014) and this promoter is also stringently regulated, linking *mioC* transcription to growth rate

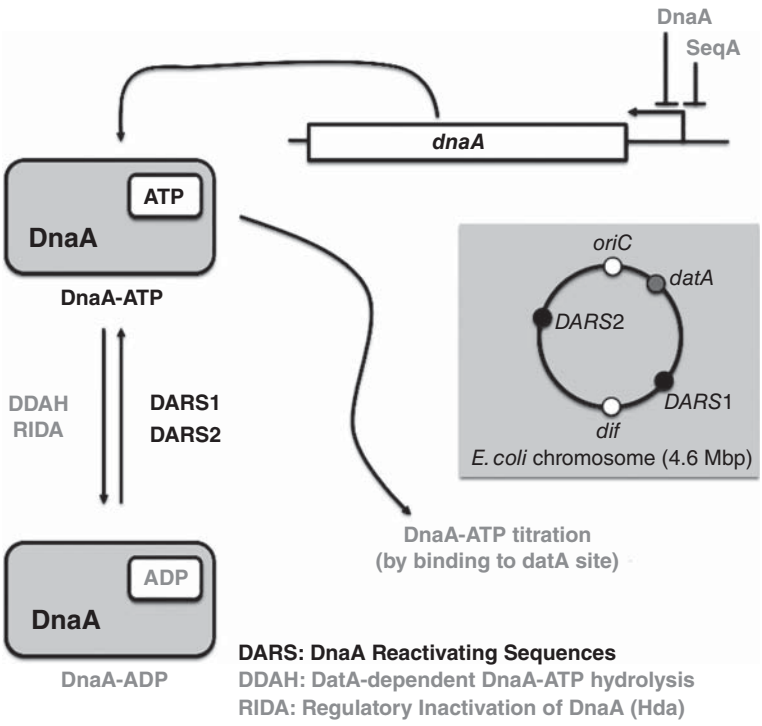

Figure 1.5 The control of DnaA production and activity. The SeqA and DnaA proteins regulate expression of the *dnaA* gene negatively. DnaA-ATP is generated at the DnaA Reactivating Sequences DARS1 and DARS2, and is converted to DnaA-ADP by ATP hydrolysis (i) at the *datA* site stimulated by binding of IHF in a process called *datA*-dependent DnaA-ATP Hydrolysis (DDAH) and (ii) by Regulatory Inactivation of DnaA (RIDA) in which the DnaA inhibitor protein Hda catalyses the hydrolysis of DnaA-bound ATP to ADP, yielding DnaA-ADP. Hda activation in RIDA follows interaction with the DNA polymerase clamp on newly synthesised DNA. The relative locations of *datA* (4.39 Mb), *DARS1* (0.81 Mb) and *DARS2* (2.97 Mb) with respect to the *oriC* and *dif* sites on the 4.6 Mb *E. coli* chromosome are shown (inset). Black lettering: generation of DnaA-ATP, grey lettering: conversion of DnaA-ATP to DnaA-ADP.

(Chiaramello and Zyskind 1989). The biological function of MioC is not firmly established, although it has been reported to be involved in biotin metabolism (Birch et al. 2001). The GidA protein contributes to tRNA modification, working in association with MnmE (GidA is also known as MnmG) (Bregeon et al. 2001). Neither protein is thought to have DNA-binding activity. Transcription of *mioC* is repressed by DnaA acting at a DnaA box in the promoter. The initiation of chromosome replication displaces DnaA and de-represses *mioC*, with the return of DnaA being delayed as the protein is recruited by the new DnaA binding sites generated by replication (Bogan and Helmstetter 1996). Transcription of *gidA* is repressed by SeqA when this protein binds to the 5′-GATC-3′ sites at the promoter that become hemimethylated following DNA replication (Bogan and Helmstetter 1997). The process of transcribing *gidA* and *mioC* is important for the initiation of chromosome replication at *oriC* (Bramhill and Kornberg 1988b; Theisen et al. 1993), at least under some circumstances (Asai et al. 1998; Bates et al. 1997; Lies et al. 2015).

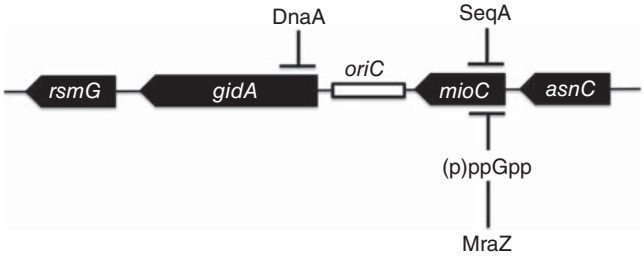

Figure 1.6 The genetic neighbourhood of *oriC* in *E. coli*. Filled arrows represent the genes and an open rectangle indicates the position of *oriC*. DnaA represses the *gidA* gene transcriptionally through DnaA boxes that overlap the *gidA* promoter. The *mioC* gene is repressed by SeqA binding to hemimethylated versions of 5′-GATC-3′ sites at the promoter that are generated by DNA replication. The *mioC* promoter is also subject to stringent control via the (p)ppGpp alarmone and it is repressed by MraZ, a protein that has been linked to the control of cell division. The *rsmG* gene encodes a methyltransferase for the modification of 16S rRNA (see Benítez-Páez et al. 2012). The *asnC* gene encodes a HTH-motif-containing transcription regulator that is related to LRP and controls genes involved in asparagine metabolism (see Kölling and Lother 1985; Willins et al. 1991). Termination of transcription extending from *asnC* to *mioC* is dependent on a DnaA-DNA complex at the *asnC* terminator, as described by Schaefer and Messer (1988).

1.4 Chromosome Replication: Elongation

Once replication has been initiated, the replisome is responsible for progressive DNA synthesis during the elongation phase of chromosome replication. This large complex is composed of a pentameric clamp loader, the DNA polymerase clamp (DnaN), the three-subunit DNA primase (DnaG), and the hexameric helicase DnaB (Bailey et al. 2007; Reyes-Lamothe et al. 2010) (Figure 1.4). The helicase uses ATP hydrolysis to unwind the DNA duplex, moving along the lagging strand of the DNA as it does so. Single-stranded DNA-binding protein (SSB) coats the separated ssDNA strands, thus preventing reformation of the duplex by religation and attack by nucleases (Beattie and Reyes-Lamothe 2015).

The primase, DnaG, possesses a central RNA polymerase domain where the RNA primers used in DNA synthesis are manufactured (Corn et al. 2008). The primer emerges from the DnaG-DnaB complex and is transferred to DNA polymerase and SSB (Corn et al. 2008). DNA Polymerase III works with the beta-clamp protein (DnaN) to extend the primer, creating a new DNA strand at a rate of 1000 bases per second (Beattie and Reyes-Lamothe 2015). It is advantageous to have DnaN as a component of the replisome because a beta-clamp must be reloaded for the synthesis of each lagging strand Okazaki fragment (Beattie and Reyes-Lamothe 2015). If the replication fork stalls or breaks, replication can be restarted through a DnaA-independent mechanism. Here, the PriA helicase, in association with accessory proteins such as PriB, PriC, and DnaT, binds to the gapped replication fork and loads DnaBC. In some cases, the restart may be associated with a strong transcription promoter that generates an R-loop where PriA can introduce DnaBC on the displaced DNA strand (Heller and Marians 2006; Kogoma 1997). Of the approximately 300 copies of DNA gyrase that are bound to the *E. coli* chromosome at any one time, about 12 accompany each moving replication fork to manage the DNA topological disturbance that is associated with fork migration (Stracy et al. 2019).

1.5 Chromosome Replication: Termination

Termination of DNA synthesis occurs within Ter, located at a point that is diametrically opposite *oriC* on the chromosome (Hill et al. 1987) (Figure 1.7). The Ter region has five copies of a 23-bp DNA element on each flank and the 36-kDa Tus protein binds to these sequences (Neylon et al. 2005). The Tus binding sites are asymmetric and have a permissive and a non-permissive orientation (Figure 1.7). Replication forks can pass the Tus-Ter nucleoprotein complexes when the DNA sequences are in the permissive orientation, but fork movement becomes arrested when the sequences are oriented in the non-permissive direction. The mechanism of replication fork passage at Ter sites that are in the permissive orientation involves displacement of Tus by the DnaB helicase; when in

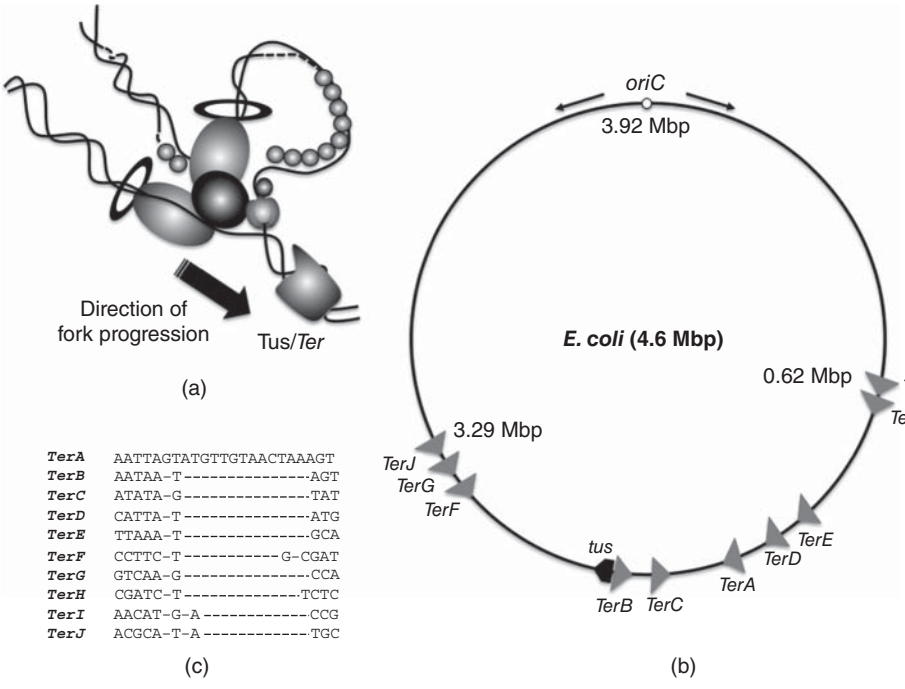

Figure 1.7 Termination of chromosome replication in *E. coli*. (a) The moving replisome encounters an appropriately oriented Tus/*Ter* nucleoprotein complex and the interaction between Tus and the DnaB helicase halts replisome movement, leading to the termination of chromosome replication. (b) The 4.6 Mb chromosome of *E. coli* is shown, indicating the relative positions and orientations of the *Ter* sequences (grey arrowheads) with respect to one another and *oriC* and the *tus* gene. The black arrows on either side of *oriC* indicate the bidirectional nature of *E. coli* chromosome replication. *Ter* sites aligned with the direction of replication are in the permissive orientation and will allow the replication fork to pass; those oriented against the direction of fork movement are in the non-permissive configuration and will halt fork movement if bound by Tus. The promoter of the *tus* gene overlaps the *TerB* sequence, resulting in negative autoregulation of *tus* transcription by the Tus protein. (c) An alignment of the DNA sequences of the *Ter* elements, showing the high degree of sequence conservation among the sites and the lack of dyad symmetry within each site. The latter feature ensures that the sites operate to stop forks moving in one direction only.

the non-permissive orientation, Tus prevents DnaB, and the replication fork, from translocating past that point (Bastia et al. 2008; Berghuis et al. 2015; Mulcair et al. 2006). Single-molecule experiments performed *in vitro* have shown that the DNA also plays a critical role: in the non-permissive orientation, the unwinding of the DNA by the approaching replication fork creates a powerful lock at the Tus-Ter site that is an effective roadblock to further translocation by the fork; in the permissive orientation the lock does not operate and the fork can proceed (Berghuis et al. 2015).

The newly synthesised DNA strand is unmethylated and forms one part of a hemimethylated duplex. For this reason, the products of chromosome replication are chemically distinct from the template duplex until a full methylation of the newly synthesised strand has taken place. DNA adenine methyltransferase, Dam, methylates DNA at 5′-GATC-3′ sites and there are 11 of these sites in *oriC* (Figure 1.2). The SeqA protein binds to these sites while they are still in their hemimethylated form, sequestering the origin and excluding DnaA (Han et al. 2003; Slater et al. 1995; von Freiesleben et al. 1994). The sequestered state persists for about one third of the cell cycle when it is relieved by dissociation of SeqA and methylation of the 5′-GATC-3′ sites by Dam (Kang, S. et al. 1999; Lu et al. 1994). SeqA also interferes with expression of the *dnaA* gene, reducing the levels of the DnaA protein available for binding to *oriC* (Campbell and Kleckner 1990). In addition, SeqA contributes to processes that ensure proper segregation of the chromosome copies at cell division (Helgesen et al. 2015; Waldminghaus and Skarstad 2009). It is interesting to note that both hemimethylated *oriC* and SeqA have been shown to associate with the cell envelope (Ogden et al. 1988; Slater et al. 1995), perhaps indicating a role for the complex in the positioning of *oriC* in the cell.

1.6 Replication Produces Physically Connected Products

The converging replication forks moving along the chromosome will create a topological problem as they approach one another in the Ter region. As chromosome replication comes to an end, the products that it generates will emerge as intertwined DNA duplexes. This physical linkage must be resolved if it is not to impede chromosome segregation. Furthermore, if homologous recombination occurs between the sister chromosomes it may create a chromosome dimer. This process is made more likely by oxidative damage to DNA, as occurs in mutants defective in the Fur iron regulatory protein (Steiner and Kuempel 1998). The dimers arise from RecBCD- and RecFOR-mediated events with roughly equal frequency (Barre et al. 2001). Once formed, this dimer must be resolved at or before cell division or an anucleate cell will be created (see Section 1.8).

1.7 Decatenating the Sister Chromosomes

Bidirectional replication produces two interlinked copies of the chromosome and these must be decatenated before they can be segregated at cell division. Decatenation of fully intact duplexes is an intermolecular event that is catalysed by type II topoisomerases. Topoisomerase IV is the most efficient decatenase in such cases (Adams et al. 1992; Espéli et al. 2003; Zechiedrich and Cozzarelli 1995), although DNA gyrase can provide this function too

(Steck and Drlica 1984). Type I topoisomerases can also supply a decatenase function, but in this case one of the DNA strands in at least one of the DNA duplexes must be nicked, with the topoisomerase providing the break in the intact strand of the same duplex to permit passage of the intact duplex through the gap (DiGate and Marians 1988).

1.8 Resolving Chromosome Dimers

The creation of chromosome dimers by homologous recombination between sister chromosomes threatens to interfere with chromosome segregation at cell division. The dimers are resolved by site-specific recombination at the *dif* sites within the terminus regions of the cointegrated chromosomes. These sites are arranged as directly repeated sequences in the dimer, albeit 4.6 million base pairs apart, and *dif* synapsis, strand exchange, and resolution separate the chromosome copies as monomeric molecules (Figure 1.8). The XerC and XerD tyrosine integrases act sequentially at *dif* to catalyse the reaction (Lesterlin et al. 2004; Sherratt et al. 1995); XerC creates the Holliday junction but this will collapse to substrate unless XerD completes the reaction (Barre et al. 2000; Hallet et al. 1999; Recchia and Sherratt 1999). The FtsK cell division protein triggers XerC/D-mediated dimer resolution at *dif* (Steiner et al. 1999). FtsK is located at the cell division septum at the mid-cell where it uses ATP hydrolysis to activate chromosome dimer resolution. Its location at the septum is

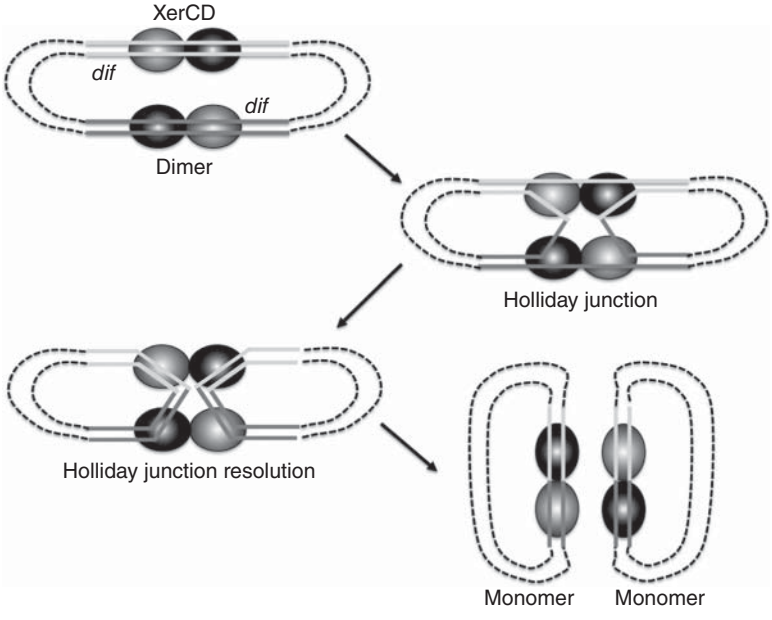

Figure 1.8 Resolution of a chromosome dimer by XerCD-mediated recombination at *dif*. Chromosome dimerisation can occur as a result of recombination-mediated DNA repair during replication. The dimers are resolved by site-specific recombination at two directly repeated *dif* sites located in the Ter region. The reaction is catalysed by the XerCD tyrosine integrases, generating monomeric chromosomes that can be segregated into the two daughter cells at cell division.

dependent on its interaction with the FtsZ septum ring protein (Dorazi and Dewar 2000a; Yu et al. 1998; Wang and Lutkenhaus 1998). FtsK moves DNA within the cell, assists sister chromosome synapsis and reduces DNA entanglement/catenation of sister chromosomes (Sherratt et al. 2004). FtsK Orienting Polar Sequences (KOPS) located near the *dif* site in the Ter region of the chromosome contribute to the directional loading of the FtsK translocase, allowing it to segregate the daughter chromosome by driving a copy into each daughter cell (Bigot et al. 2006, 2007; Sivanathan et al. 2009; Stouf et al. 2013). The *dif* site and the wider Ter region of the chromosome remain in close association with the septum/nascent septum throughout the cell cycle (Niki et al. 2000).

It is not in the interest of the bacterium to have chromosome dimers arising frequently because this poses a risk that either anucleate cells will arise or that the chromosome may be damaged by the division septum as the cell attempts to segregate a dimeric chromosome. RecA-dependent homologous recombination events, whether they arise via the RecBCD or RecFOR pathways (Section 1.6), generate Holliday junctions that must be resolved by the Ruv resolvasome-based system. There is a bias in this process in favour of Ruv-mediated resolution that does not involve crossover, and therefore the creation of chromosome dimers (Cromie and Leach 2000; van Gool et al. 1999). This bias reduces the frequency at which chromosome dimers, and the associated threat to the wellbeing of the daughter cell genomes, arise.

The XerCD system is versatile and is used in site-specific recombination reactions with sequences related, but not identical, to *dif*, and accompanied by co-factor proteins, to resolve dimers in autonomously replicating plasmids (Clerget 1991; Colloms 2013; Colloms et al. 1990, 1998; Summers 1989). In the human pathogen *V. cholerae*, the CTXϕ bacteriophage that carries the cholera toxin *ctxAB* operon integrates into chromosome I at its *dif* site using the XerC recombinase from the *V. cholerae* XerCD recombinases to catalyse the reaction (it can enter the corresponding location on chromosome II at a lower frequency) (Das et al. 2013; Huber and Waldor 2002; McLeod and Waldor 2004). CTXϕ is a filamentous phage and only the plus strand of its genome integrates. The plus strand of the CTXϕ genome folds to create a double-stranded region that encompasses the XerC and XerD binding sites of the phage flanking a mismatched and bulging phage *dif* site. Only a single stranded exchange occurs, mediated by XerC alone, and this creates a Holliday junction that is resolved by DNA replication (Val et al. 2005). The minus strand of the CTXϕ, phage fails to generate a *dif* site with enough homology to recombine with the chromosomal counterpart, so this strand of the phage genome does not integrate. The integrated phage does not excise because its *dif* sites also lack sufficient homology with the chromosomal site to promote site-specific recombination (Val et al. 2005).

1.9 Segregating the Products of Chromosome Replication

The daughter chromosomes have to be moved to locations in the mother cell that correspond to the emerging daughter cells. At the end of the movement period, it should be possible to close the cell division septum between the nascent daughter cells without damaging the chromosome copies by guillotining them. The chromosome segregation

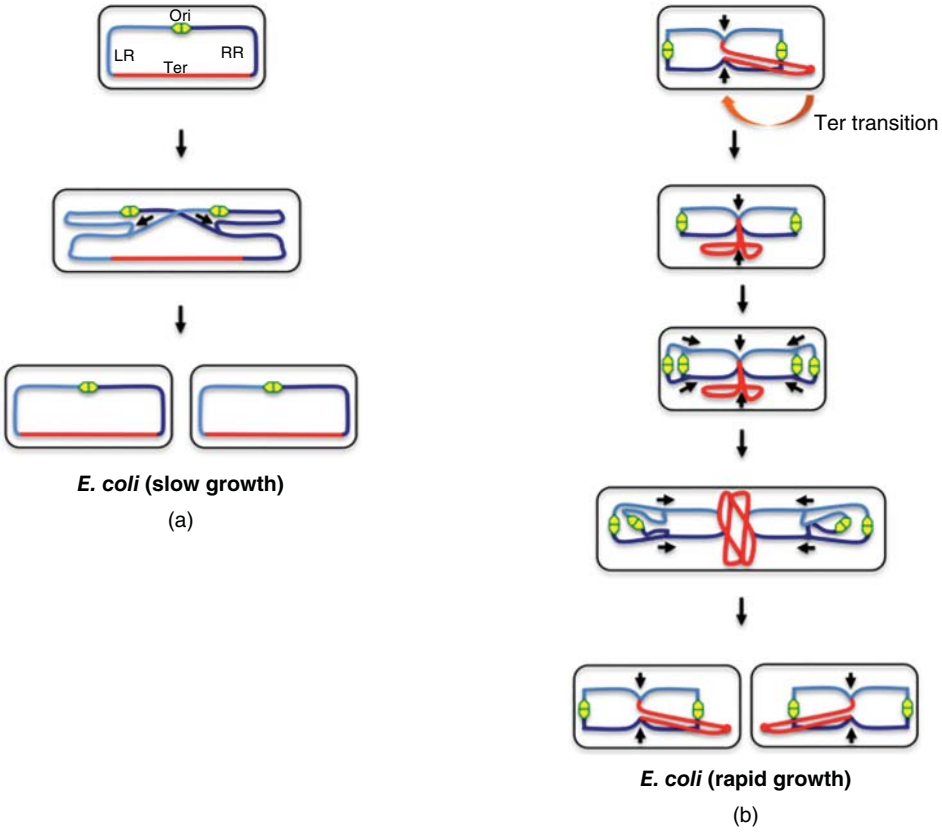

Figure 1.9 The choreography of chromosome movement in *E. coli* during the cell cycle. (a) In slow-growing *E. coli* cells, chromosome replication is initiated at the origin (Ori, green/yellow lozenge) located at mid-cell, and proceeds bidirectionally, copying the left (LR, light blue) and right (RR, dark blue) replichores and ending in the terminus (red), where the products of replication are decatenated prior to segregation into the daughter cells. Arrows drawn alongside the chromosome are used to indicate the direction of replication fork movement. (b) Rapidly growing *E. coli* cells have multiple rounds of chromosome replication underway simultaneously. Instead of the mid-cell position seen in slow-growing *E. coli* cells, the Ori is at the cell pole in the rapidly growing bacteria. The newly born cell has its Ter region displaced towards one pole of the cell and this undergoes a transition to the mid-cell. A second round of chromosome replication starts before the previous one is complete and multiple replication forks can be observed. The final separation of the daughter chromosomes is thought to exert a force at the terminus that moves this part of the chromosome to an eccentric position that is maintained in the daughter cell immediately after its birth (Youngren et al. 2014). (*See colour plate section for colour representation of this figure*)

process follows the Origin-to-Terminus axis, just as the replication process does (Bouet et al. 2014) (Figure 1.9).

The immediate products of replication behind the moving fork and replisome are catenated, positively supercoiled, interwound, double-stranded DNA molecules. Topo IV will attempt to decatenate these (Deibler et al. 2001; Espéli et al. 2003; Khodursky et al. 2000; Lopez et al. 2012). However, in a 300- to 400-kb sliding window immediately in the wake of

fork passage, the DNA is only hemimethylated. Any hemimethylated SeqA sites can bind the SeqA protein, which has the potential both to bridge DNA molecules and to exclude Topo IV, preventing decatenation (Joshi et al. 2013). The result is cohesion of the chromosome copies (Joshi et al. 2011; Nielsen et al. 2006a).

Two factors are thought to contribute to cohesion: exclusion of Topo IV by SeqA and bridging of daughter chromosomes by SeqA bound to hemimethylated copies of its binding sites. In support of the model, a positive correlation has been described between the time delay in ending cohesion and the density of SeqA-binding sites at *oriC* and at two so-called 'snap' loci (in the Right Replichore in *E. coli*) at which particularly strong physical tethers seem to fail suddenly as the replication-and-segregation process proceeds (Joshi et al. 2011). Chromosome cohesion must be overcome if segregation is to proceed to completion and this involves efficient decatenation of interwound chromosome copies and the breaking of any inter-chromosome bridges. The resulting segregation process appears to include a series of successive jerks as each tether breaks in turn (Fisher et al. 2013). Dam-mediated methylation of the newly synthesised DNA strands seems to be a key step in preventing SeqA binding and using its capacity to bridge chromosome copies and to interfere with Topo IV access to catenated DNA substrates behind the fork (Joshi et al. 2011). Cohesion does not require the Structural Maintenance of Chromosome (SMC) proteins, such as MukBEF in *E. coli*, despite their potential to encircle DNA duplexes (Adachi et al. 2008; Danilova et al. 2007; Joshi et al. 2013) (Figure 1.10).

The presence or absence of a ParAB-*parS* system is a major determinant of the pattern of chromosome segregation seen in a bacterium. *E. coli* does not possess such a system and forces of mutual repulsion acting on the chromosome copies as they emerge in the confined space of the rod-shaped cell may drive them to segregate, perhaps aided by the imprinted structural and super-structural features of the chromosomes (Jun and Mulder 2006; Jun and Wright 2010; Junier et al. 2014; Pelletier et al. 2012; Wiggins et al. 2010).

ParAB-*parS* systems may be useful rather than essential in bacteria that have just one chromosome, unless the bacterium is sporulating (Ireton et al. 1994) or going through a growth phase transition (Godfrin-Estevenon et al. 2002). If the microbe has more than one chromosome, then the partitioning system is essential if the segregation of its different chromosomes is to be properly coordinated, as, for example, in the case of *V. cholerae* (Yamaichi et al. 2007) or members of the Burkholderias (Passot et al. 2012). The roles of chromosome-encoded ParAB-*parS* systems as functioning partitioning machines was confirmed in early work where it was demonstrated that they could replace the native plasmid *par* systems on single copy episomes (Godfrin-Estevenon et al. 2002; Lin and Grossman 1998; Yamaichi and Niki 2000).

The *cis*-acting *parS* centromere-like sequences, and the genes that encode the ParA and ParB proteins, typically are found close to the *oriC* region of those bacterial chromosomes that harbour these systems (Livny et al. 2007; Reyes-Lamothe et al. 2012). This centromere positioning ensures that the first part of the chromosome to be duplicated is going to be delivered to the appropriate cellular site for the orientation of the segregation of the rest of the chromosome. Positioning varies and can be at mid-cell or quarter-cell (Fogel and Waldor 2005; Webb et al. 1997); in the case of chromosomes with *oriC* tethering at the poles of rod-shaped cells, it will be at the cell pole (Bowman et al. 2008; Fogel and Waldor 2006; Harms et al. 2013) (Figure 1.11). Dimeric ParB is in excess compared with its multiple 16-bp

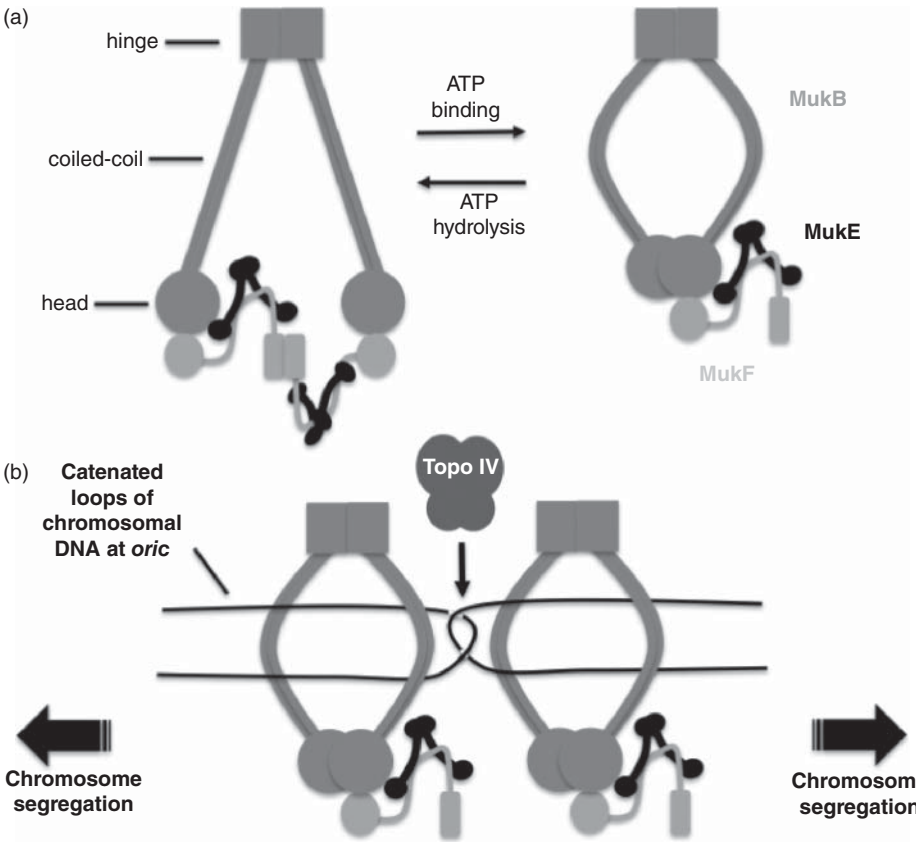

Figure 1.10 MukBEF structure and function. (a) The Dimeric MukB protein (intermediate grey) consists of a head domain with ATPase activity that is attached to a hinge domain by a coiled-coil. A 'cap' region in the head domain interacts with carboxyl terminus of MukF (light grey) while the central segment of MukF interacts with a MukE dimer (black). When ATP is absent, the complex has two copies of MukB, MukF and four of MukE. One MukF protein is displaced following ATP binding by MukB. This form of the MukBEF complex may in turn dimerise (not shown) (Nolivos and Sherratt 2014). (b) Clusters of MukBEF complexes, here represented by just two, have the potential to organise and segregate the *ori* regions of sister chromosomes following replication. The catenated *ori* regions are decatenated by Topo IV (dark grey), working in combination with the DNA segregation activity of the MukBEF motor.

parS binding sites and it seems that ParB can spread beyond *parS,* perhaps by a bridging mechanism that has the effect of folding the centromere region into a tightly organised complex (Graham et al. 2014; Sanchez et al. 2013).

The ParA protein drives bidirectional segregation of the *parS*-ParB complexes in an ATP-dependent manner. It can form filaments, and these have been proposed to be a factor in chromosome segregation (Bouet et al. 2007; Hui et al. 2010; Ptacin et al. 2010). However, it is also possible that ParA-driven chromosome segregation works by a diffusion-ratchet-type mechanism that has been described for its plasmid-encoded counterparts (Hwang et al. 2013; Vecchiarelli et al. 2014) or a *trans*-nucleoid relay mechanism (Lim et al. 2014).

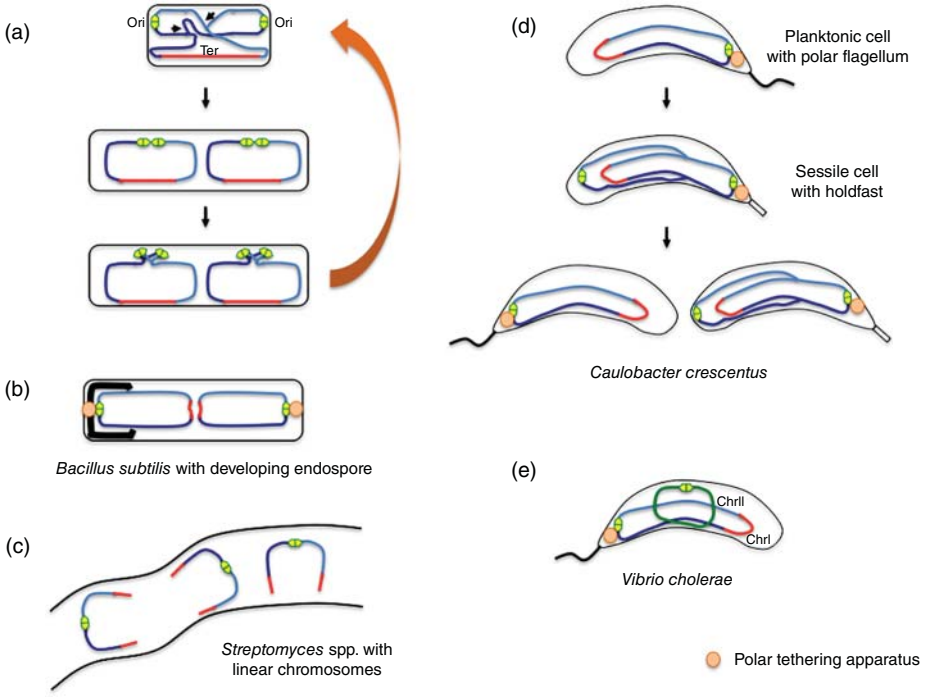

Figure 1.11 Spatiotemporal dispositions of chromosomes in model bacteria other than *E. coli*. (a) In vegetative cells of *Bacillus subtilis*, the origins of chromosome replication are at mid cell in newborn cells before moving to the poles. The origins are at quarter cell positions at the onset of cell division. (b) In sporulating cells, the origins are tethered at the poles as one chromosome copy (left) is segregated into the developing fore-spore. (c) *Streptomyces* species have linear chromosomes with the origin of replication at the midsection. (d) *Caulobacter crescentus* has two cell types: planktonic cells with a polar flagellum and sessile cells that adhere to the substratum via a polar holdfast. The origin of chromosome replication is tethered to the pole where the flagellum/holdfast is located. (e) *Vibrio cholerae* has two chromosomes. ChrI is the primary chromosome and its origin of replication is tethered to the cell pole where the single flagellum is located. The second chromosome, or chromid (ChrII), is plasmid-like and synchronises its replication with that of ChrI. (*See colour plate section for colour representation of this figure*)

Ter and associated genetic loci replicate at mid-cell and cohere for an extended period as a result of the *matS*-MatP nucleoprotein complex (Dupaigne et al. 2012; Reyes-Lamothe et al. 2008; Mercier et al. 2008; Stouf et al. 2013; Wu et al. 2019). The C-terminal domain of MatP interacts with the ZapB protein in the cell division apparatus and this interaction contributes to the extended cohesion of the Ter domain copies (Dupaigne et al. 2012; Espéli et al. 2012). MatP is displaced from *matS* by the action of the FtsK motor (or SpoIIIE in *B. subtilis*) as it drives the Ter domain copies into the nascent daughter cells (Deghorain et al. 2011; Graham et al. 2010; Marquis et al. 2008; Massey et al. 2006; Sherratt et al. 2010). FtsK uses its *dif*-oriented KOPS repeats to bind and to guide this process; the XerCD recombination *dif* site is the final component of the chromosome to be segregated (Stouf et al. 2013). The formation of FtsK hexamers, triggered by the onset of cell division, is a critical step in FtsK's own activation (Bisicchia et al. 2013) and its activation of the XerCD recombination

apparatus (Zawadzki et al. 2013), illustrating the exquisite integration of the Ter segregation and the chromosome dimer resolution systems.

1.10 Polar Tethering of Chromosome Origins

Anchoring the origin of replication to one pole of the cell is likely to assist in reinforcement of the *ori-Ter* orientation of the chromosome seen along the long axis of rod shaped cells and in ensuring that daughter cells receive an entire chromosomal copy at cell division (Badrinarayanan et al. 2015) (Figure 1.11). The PopZ protein fulfils this role in *C. crescentus* by forming a matrix at the pole and interacting with the ParB-*parS* complex at *oriC* (Bowman et al. 2008; Ebersbach et al. 2008). Displacement of *parS* to a different chromosome site interferes with this arrangement: while *parS* continues to be located at the pole *oriC*, from which *parS* is now disconnected, lies elsewhere in the cell (Umbarger et al. 2011).

The cytoplasmic protein HubP connects the origin of replication of ChrI to the cell pole in *V. cholerae*. The connection is made between HubP and the ParAI-ParBI-*parS* complex. In addition to its membrane location, the HubP protein is connected to the cell wall through a peptidoglycan-binding LysM motif, a feature that is required for its polar localisation (Yamaichi et al. 2012).

Polar attachment of the chromosome occurs in *B. subtilis* at the onset of sporulation. The RacA protein interacts with the DivIVA membrane protein that is located at the cell pole (Ben-Yehuda et al. 2003; Lenarcic et al. 2009; Oliva et al. 2010; Ramamurthi and Losick 2009; Wu and Errington 2003). RacA also binds to *ram* (RacA binding motifs) that are found in 25 copies at *oriC* (Ben-Yehuda et al. 2005). In the absence of RacA or DivIVA, sporulating bacteria fail to position the chromosome correctly and have the *oriC* at mid-cell. This misplacement leads to the production of prespore compartments without chromosomes (Ben-Yehuda et al. 2003). *B. subtilis* cells do not have their chromosomes attached to the cell pole during vegetative growth, although their origins occupy positions that alternate between pole-proximal and at quarter-cell, arrangements that require the cytoplasmic SMC complex (Wang, X., et al. 2014), just as the MukBEF equivalent in *E. coli* is required for that organism's chromosome to exhibit its customary *ori*-Ter orientation during rapid growth (Danilova et al. 2007).

1.11 Some Bacterial Chromosomes Are Linear

Most of the literature on bacterial chromosomes describes work with covalently closed, circular molecules. On the face of it, chromosome circularity is not essential for survival: work with *E. coli* has shown that linearisation of its circular chromosome through a phage-mediated process that leaves the ends closed by DNA hairpins does not interfere significantly with the life of the bacterium (Cui et al. 2007). Going in the other direction, the linear chromosome of *Streptomyces lividans* can be circularised without killing the microbe, although its genetic instability increases (Volff et al. 1997).

Some organisms have linear chromosomes naturally. For example, *Borrelia burgdorferi*, the spirochete and causative agent of Lyme disease, has a complex genome consisting of

a linear chromosome and 23 plasmids, some of which are circular while others are linear (Chaconas and Kobryn 2010). Essential metabolic functions are encoded by the plasmids, so these are parts of the core genome and not simply ancillary components. The ends of the linear DNA molecules are closed covalently by hairpin telomere-like structures (Barbour and Garon 1987). Such structures are not found widely in bacteria; other examples have been reported in the plant pathogen *Agrobacterium tumefaciens* and in some phage (Chaconas and Kobryn 2010; Slater et al. 2013). Telomere resolvases, enzymes that are related to the integrase family of tyrosine site-specific recombinases, promote fusions between the linear replicons at their telomeres, driving genome evolution (Huang et al. 2017). Replication of linear replicons in *Borrelia* spp. is thought to occur bidirectionally from a central origin, producing a double-stranded dimeric circle that is resolved by the telomere resolvase (ResT in *B. burgdorferi*) to produce two linear molecules with closed telomeres at their ends. Positive DNA supercoiling, probably arising from the local overwinding of the DNA during replication, assists telomere resolution (Bankhead et al. 2006). Although the role of DNA supercoiling, positive or negative, in linear replicons has not been studied comprehensively, there is some evidence that it is a factor in setting the level of transcription of promoters found on linear plasmids when those replicons are artificially circularised. This has led to the proposal that linear replicons may avoid instability caused by topological changes in circular molecules (Beaurepaire and Chaconas 2007). *Streptomyces* spp. also have linear chromosomes and linear plasmids, and intra-replicon interactions mediated by 'terminal proteins' that are covalently bound to the telomeres allow the creation of negatively supercoiled DNA circles from the linear replicons (Tsai et al. 2011). These negative supercoils are relaxed by DNA topoisomerase I, which is a component of the telomere complex in *Streptomyces*. It has been proposed that negative supercoiling is likely to be important for both DNA replication and for transcription, especially of genes located close to the telomeres (Tsai et al. 2011).

1.12 Some Bacteria Have More than One Chromosome

Among bacteria that have more than one chromosome are the well-studied organisms *A. tumefaciens* (Allardet-Servent et al. 1993), *Brucella* spp. (Jumas-Bilak et al. 1998), *Rhodobacter sphaeroides* spp. (Choudhary et al. 2007; Suwanto and Kaplan 1989), and *Vibrio* spp. (Val et al. 2014). Of the organisms listed here, *A. tumefaciens*, has one circular and one linear chromosome; the others have two circular chromosomes. *Paracoccus denitrificans* is a bacterium that has three chromosomes (Winterstein and Ludwig 1998).

Are all of the chromosomes in a multi-chromosome genome 'equal'? It appears that one chromosome is usually the primary replicon, with the other being relegated to secondary chromosome status. For example, the two chromosomes of the pathogen *V. cholerae* are designated chromosomes, ChrI and ChrII, with ChrI having the majority of the metabolically important and virulence-associated genes. ChrII does harbour essential genes, so its designation as a second chromosome is justified, despite its having plasmid-like features. For example, the origin of replication of ChrII shows structural features that are similar to those of plasmids, which is consistent with the secondary chromosome having evolved from an ancestral plasmid (Orlova et al. 2017). The plasmid-like nature of ChrII is also emphasised

by its encoding plasmid RK2-like toxin systems that ensure post-segregation killing of those *V. cholerae* cells that lose ChrII (Yuan et al. 2011). The replication initiation system of ChrI resembles that of *E. coli*: initiation of replication of each chromosome is independent of, but coordinated with, that of the other (Duigou et al. 2006). ChrII begins replicating later in the cell cycle than the larger ChrI, but both finish together. This has been interpreted as a mechanism that compensates for the differences in size of the two molecules and the need to end replication simultaneously so that the chromosomes can be segregated together and that any dimers can be resolved simultaneously (Rasmussen et al. 2007). Further investigation has revealed the generality of coordinated termination of replication in members of the *Vibrionaceae* with two chromosomes (Kemter et al. 2018). ChrI and ChrII each possess their own ParAB-*parS* systems and use these for efficient segregation of chromosome copies at cell division (Fogel and Waldor 2005; Yamaichi et al. 2007, 2011).

1.13 Plasmids

In many bacteria, autonomously replicating and segregating genetic elements called plasmids accompany the chromosome in the cell. Like most bacterial chromosomes, plasmids are usually covalently closed, circular DNA molecules, but this is not always the case: some are linear. Certain plasmids are categorised as additional chromosomes (or 'chromids') due to their size, their carriage of genes normally found on bona fide chromosomes, their unitary copy number, and/or the coordination of their replication and segregation with the main chromosome (Barloy-Hubler and Jebbar 2008; Fournes et al. 2018). Other very big plasmids are called 'mega-plasmids' and can encode functions required for symbiosis or virulence (Schwartz 2008). In general, plasmids carry genes that are useful rather than essential, so their loss is not usually fatal to the cell; in contrast, loss of the chromosome is fatal.

Plasmids came to attention due to their involvement in bacterial sex (the Fertility, or F factor) and when it was discovered that they carried genes for resistance to antimicrobial agents, including antibiotics (R factors). Investigations of these phenomena led to the discovery of plasmid conjugation and the existence of other mobile genetic elements such as transposons and integrons. Plasmid studies revealed a wealth of information about plasmid replication processes, segregation systems, and copy number control mechanisms. This field also provided cloning vectors to support the emergence of the recombinant DNA technology that, in part, underpins biotechnology. Plasmid research has provided important insights into gene regulation mechanisms, including the provision of early examples of the regulatory roles of small RNA molecules.

1.14 Plasmid Replication

The term plasmid was introduced in the mid-twentieth century to describe self-replicating extrachromosomal DNA elements (Lederberg 1952). Self-transmissible plasmids are important contributors to HGT; indeed, some plasmids can even participate in DNA transfer between different domains of life (Suzuki et al. 2008; Zambryski et al. 1989). Limits to the

host range of a plasmid include barriers to mating bridge establishment, to successful DNA transfer, and to successful plasmid replication (Jain and Srivastava 2013). Plasmid size is a poor predictor of host range: while many broad-host-range plasmids are large, many others are quite small. For example, pBC1 can function in both Gram-negative and Gram-positive bacteria, yet it is only 1.6 kb in size (De Rossi et al. 1992). At the other end of the scale, the intensively studied RK2/RP4 plasmid group is in the 60-kb-size range but is confined to Gram-negative hosts, albeit a wide selection of these (Thomas et al. 1982). It is an advantage to have several origins of plasmid replication as this improves the chances of being able to replicate in a given host. However, the presence of multiple origins is not in itself a reliable indicator of broad host range: the F plasmid has a narrow range yet it has three origins of replication. Instead, it is the structure of the origin(s) that seems to be important in determining host range. Plasmids with a minimum dependence on host-encoded factors for replication are likely to have a broad host range; for example, RSF1010 from incompatibility group Q (IncQ) uses a strand-displacement mode of replication that relies on no host-encoded factors for the initiation of DNA synthesis (Loftie-Eaton and Rawlings 2012).

The RK2 plasmid (IncP) has a broad host range and can replicate in *E. coli* or *Pseudomonas aeruginosa* (Shah et al. 1995). It can replicate in *E. coli* using just a minimal origin, *oriV* ('vegetative' origin), containing five iterons (directly repeated sequences) and four binding sites for DnaA (Doran et al. 1999) (Figure 1.12). In *P. aeruginosa*, RK2 needs an additional three iterons but can dispense with the DnaA boxes (Schmidhauser et al. 1983). RK2 is also capable of replicating without DnaA when in *C. crescentus* (Wegrzyn et al. 2013).

Iterons are the binding sites for plasmid-encoded Rep (replication) proteins. The Rep proteins are needed to initiate plasmid DNA replication, but they can also inhibit this process. In addition, Rep proteins have roles as transcription regulators, acting as auto-repressors. They are also subject to turnover by host-encoded proteases (Konieczny et al. 2014). The positions, numbers, orientations, lengths, DNA helical phasing, and spacer lengths of iterons vary from plasmid to plasmid and making alterations to any of these details within a particular plasmid typically has a negative effect on replication (Konieczny et al. 2014). In RK2, the Rep protein (called TrfA) and DnaA both target the origin, which has an A+T-rich DUE adjacent to the iteron arrays. Some iteron plasmids (but not RK2, which requires HU) have a requirement for IHF binding to the origin for efficient initiation of replication (Shah et al. 1995). Sites for Dam methylation (5′-GATC-3′) and SeqA binding are also features of some iteron-dependent origins (Brendler et al. 1995). SeqA binding blocks replication initiation by excluding the replication proteins. A GC-rich sequence motif is located adjacent to the DUE in RK2, but its significance is unclear (Figure 1.12). Other iteron origins have requirements for the FIS NAP and the IciA protein, an inhibitor of DNA unwinding at the DUE.

Many of the *cis* or *trans*-acting components of iteron origins, and their architectures, are reminiscent of *oriC* on the chromosome. The TrfA replication protein of RK2 interacts with, and recruits, the DnaB helicase. The ability of a plasmid replication protein to recruit a host helicase may be a determining factor limiting plasmid host range (Zhong et al. 2005). TrfA also acts with Hda, the inhibitor of DnaA activity, to prevent over-initiation of RK2 replication (Kim et al. 2003). It has been suggested that TrfA has a motif that is suitable for interaction with the β-clamp of *E. coli* DNA Pol III (Kongsuwan et al. 2006). In

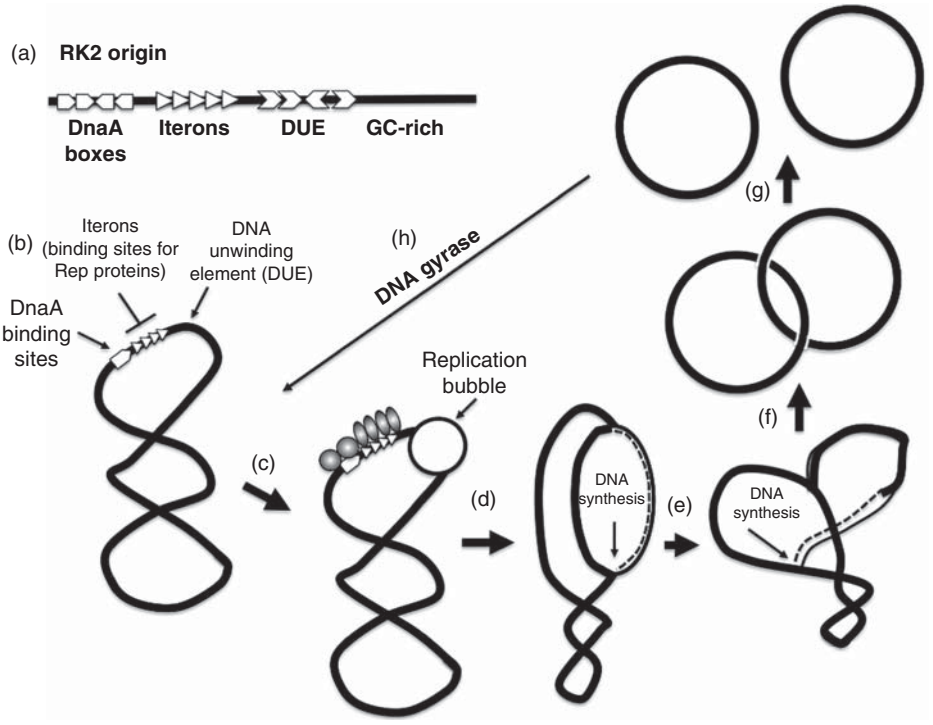

Figure 1.12 Theta model of plasmid replication. (a) The structure of the origin of replication in the broad-host-range, single-copy, IncP plasmid RK2, showing the relative positions and numbers of the DnaA boxes, the iterons (binding sites for the replication protein, TrfA), the AT-rich DNA unwinding element (DUE), and the adjacent GC-rich sequence. (b) A replication cycle is shown for an idealised plasmid using theta replication. Replication begins with the binding of the replication protein to the iterons and the recruitment of the host-encoded DnaA to the DnaA boxes. (c) The DNA in the DUE becomes single-stranded, creating a replication bubble to which host-encoded replication proteins are recruited. (d, e) Depending on the plasmid, DNA synthesis proceeds either uni- (e.g. ColE1) or bidirectionally (e.g. RK2). (f) The products of plasmid replication are catenated, double-stranded circles and these are unlinked by Topo IV. (g, h) The unlinked plasmids are negatively supercoiled by DNA gyrase, recreating the substrate for another round of replication.

addition to DnaB, iteron-based initiation also requires DnaC (in *E. coli*), the DnaG primase, DNA gyrase, the Pol III holoenzyme, and the SSB, as is also seen at *oriC* (Section 1.3). Unlike initiation of chromosome replication at *oriC,* initiation of plasmid DNA replication at iteron origins is ATP-independent; there is no requirement for the DnaA-ATP form of DnaA (Konieczny et al. 2014).

An important mechanism of copy number control in iteron plasmids is 'handcuffing', where the monomeric Rep proteins bound to iterons on two plasmids dimerise, bridging the two replicons (Das and Chattoraj 2004). Handcuffing may be counteracted by the DnaJ-DnaK-GrpE protein chaperone triad, which can convert the Rep dimers to monomers (Toukdarian and Helinski 1998). The Rep proteins downregulate replication initiation through the auto-repression of their own genes (Kelley and Bastia 1985). The level of active Rep proteins in the cell is controlled by proteolysis and protein chaperones: monomers

are active and dimers are inactive in promoting replication (Konieczny et al. 2014). Active Rep proteins are a proxy for the number of plasmid copies in the cell and allow feedback through dimer formation to downregulate Rep activity, and hence to inhibit the synthesis of new plasmid copies. Antisense RNAs also play important roles in modulating plasmid replication through their ability to interfere with the expression of plasmid-encoded replication factors (Brantl 2014).

RK2 and its plasmid relatives use a theta model of DNA replication (Figure 1.12). Plasmid ColE1, the backbone for many cloning vectors used in recombinant DNA technology, also uses theta replication, but differs from RK2-like plasmids in relying on host factors to open the double-stranded origin and to prime synthesis (Lilly and Camps 2015; Wang et al. 2004). DNA duplex unwinding is driven by transcription of a stable RNA pre-primer that forms an R-loop in the *ori* region of ColE1. This process is driven by negative supercoiling of the plasmid DNA. RNase H then processes the bound RNA to generate the primer RNA that is then extended by Pol I. This marks the beginning of leading strand DNA synthesis. As the newly synthesised DNA strand makes progress through the plasmid DNA duplex, it base pairs with the template to create a D-loop that recruits PriA. Pol III takes over leading strand synthesis and initiates the synthesis of the lagging strand; the converging replisomes continue moving until they are at or near the termination site *terH* (Nakasu and Tomizawa 1992). Gaps between the strands are then filled in by Pol I (Troll et al. 2014).

Rolling circle replication allows plasmids to replicate independently of chromosomal DNA (Khan 2005). The process relies on a nick made by a plasmid-encoded initiator protein in one plasmid DNA strand, providing a primer for leading strand initiation and a lagging strand origin (Figure 1.13). No RNA primer is required. Rolling circle replication is chiefly found in plasmids from Gram-positive bacteria, although it does occur in replicons from Gram-negatives and archaea (del Solar et al. 1993; Ruiz-Masó et al. 2015). The process replicates the leading strand and the lagging strand in two separate steps. Leading strand replication begins with the nicking of the double-strand origin (*dso*) by a plasmid-encoded replication protein, Rep. This is a member of the HuH superfamily of DNA endonucleases (Chandler et al. 2013) and it has a binding site located adjacent to the *dso* that positions it appropriately to cut the DNA. The DNA to be cleaved is presented to Rep in a single-stranded form within a stem-loop structure that extrudes from the negatively supercoiled plasmid. This extrusion event is Rep-binding-dependent (Ruiz-Masó et al. 2007). Rep forms a covalent bond with the cleaved DNA through an active site tyrosine (Noirot-Gros et al. 1994; Thomas et al. 1990). Host DNA polymerases use the intact template strand to guide DNA synthesis while simultaneously displacing the non-template strand. The displaced strand is coated with SSB and is ejected as a covalently closed, single-stranded circle at the end of leading strand synthesis. This single-stranded circle is then used as the template for lagging strand synthesis, a process that involves only host-encoded proteins (especially RNA polymerase and DNA polymerase I) and initiates at a structured region in the circle known as the single-strand origin, *sso* (del Solar et al. 1987; Gruss et al. 1987; Kramer et al. 1997). Control of rolling circle replication is achieved principally through the control of Rep protein production. For this reason, the expression of the *rep* gene is strictly regulated, typically via mechanisms that employ an antisense RNA or an antisense RNA working with a DNA-binding regulatory protein. The first type operates through transcriptional attenuation while the second involves protein-mediated transcriptional repression

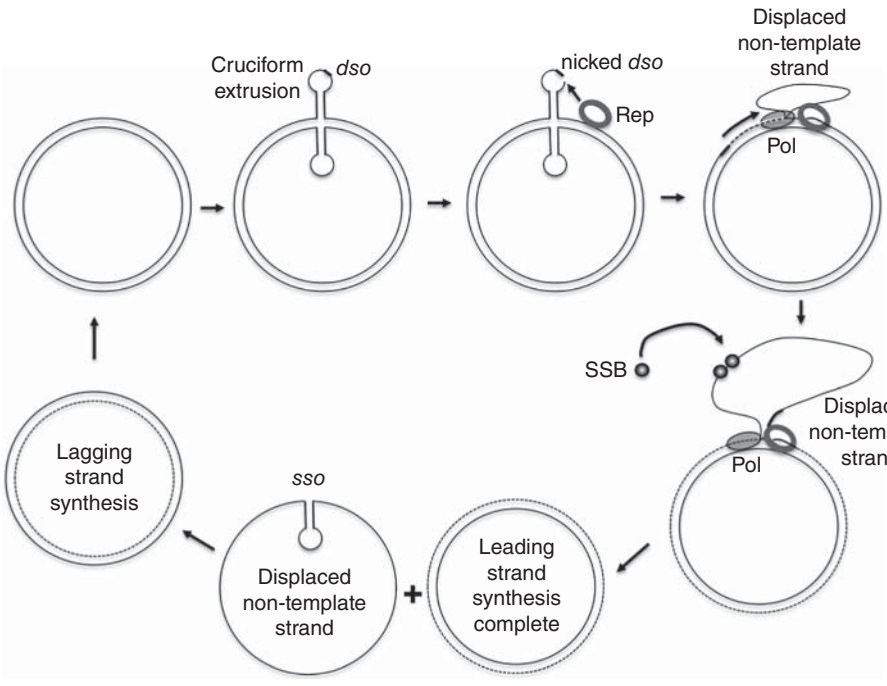

Figure 1.13 Rolling circle plasmid replication. A circular plasmid using the rolling circle mechanism of replication is shown at top left. The double-stranded circle is shown in a topologically relaxed state, but it would be negatively supercoiled in the bacterium, a state that encourages extrusion of a cruciform that contains the double-strand origin (*dso*), the site of replication initiation. The *dso* is represented by a slightly thicker line in the drawing. Extrusion of the cruciform presents the *dso* in single-stranded form to the plasmid-encoded replication protein, Rep. The Rep protein is positioned appropriately by binding to a recognition site on the plasmid adjacent to the *dso*. The bacterial DNA polymerase use the 3′-OH at the nick to prime DNA synthesis; no RNA primer is required. A dotted line represents the newly synthesised DNA and an arrow next to this line shows the direction of DNA synthesis. The plasmid duplex unwinds as DNA synthesis proceeds, displacing the non-template DNA strand, which is then coated by the single-stranded DNA-binding protein, SSB. A full round of replication displaces the non-template strand completely, producing a double-stranded plasmid (with one newly synthesised strand) and a single-stranded circle. This circle is used as the template for the synthesis of the lagging strand. Host proteins exclusively conduct lagging strand synthesis (especially RNA polymerase and DNA polymerase I), a process that begins with priming by RNA polymerase via RNA synthesis at the single-strand origin, *sso*.

backed up by translational inhibition using a *trans*-acting RNA (Brantl 2014; del Solar and Espinosa 2000; Novick et al. 1989).

1.15 Plasmid Segregation

Plasmids employ two strategies to ensure segregation of their copies at cell division: active partitioning mechanisms (low copy number plasmids) and reliance on dispersal through the cytosol of the mother cell to ensure that some copies end up in each daughter

cell (high copy number plasmids). A third strategy acts post-segregationally. It is based on toxin–antitoxin systems and eliminates those bacterial cells that do not acquire a plasmid copy at cell division (Hayes 2003) (see also Sections 2.30 and 2.35). The plasmid encodes both a stable toxin and an unstable antitoxin in an operon known as an addiction module: maintenance of the supply of the antitoxin requires the continued presence of the plasmid and the antitoxin-encoding gene. Bacteria that become liberated from the burden of plasmid-carriage may outgrow their plasmid-carrying counterparts. Eliminating the plasmidless bacteria helps to prevent extinction of the plasmid carriers by the fitter, plasmid-free, segregants. The CcdA/B antitoxin/toxin pair produced by the F plasmid provides an example of this post-segregational killing strategy. CcdB inhibits DNA gyrase by trapping it in a cleavage complex with DNA, and CcdB activity is neutralised when it binds the unstable CcdA antidote. A bacterium that loses the F plasmid will retain the toxic CcdB molecule after the unstable CcdA molecule has been broken down by the ATP-dependent Lon protease (van Melderen et al. 1994). The resulting poisoning of DNA gyrase by CcdB kills the plasmidless cell.

High copy number plasmids, i.e. those with 10 or more copies per cell, lack genes that are capable of encoding active partitioning machinery (Million-Weaver and Camps 2014). Random distribution of the multicopy plasmid through the cytosol seems to account for the faithful inheritance of plasmids such as ColE1 (Durkacz and Sherratt 1983). The formation of plasmid multimers poses a risk because this process reduces the copy number of independently segregating units, but multimer resolution systems such as *cer*/XerCD in ColE1 provide a potent antidote (Summers and Sherratt 1984). This resolution mechanism is a close relative of the chromosomal *dif*/XerCD system, albeit with additional co-factors (Section 1.8). Plasmid distribution in the cytosol is likely to be influenced by the presence of other molecules and structures, not least the nucleoid, and nucleoid exclusion does seem to be a factor in confining plasmids to the space just inside the cytoplasmic membrane (Reyes-Lamothe et al. 2014; Wang et al. 2016; Yao et al. 2007). The plasmids occur in clusters and frequently these clusters are seen at the poles of the cell; clusters are dynamic, they can divide, with some sub-clusters relocating to the mid-cell (Yao et al. 2007). The introduction of another type of plasmid produces an even more complex clustering pattern (Diaz et al. 2015; Yao et al. 2007). It appears that multicopy plasmids move between existing in clusters and being alone, and that these forms diffuse randomly within the confines imposed by the nucleoid and other cell structures (Wang 2017).

Low-copy number plasmids cannot rely on strategies based on random spatial distribution to ensure their segregation to the daughter cells at division. These plasmids have active partitioning systems, systems that have counterparts in the chromosomes of many bacteria (but not *E. coli*). These partitioning (Par) systems consist of two proteins, ParA and ParB, and a centromere-like DNA site called *parS* (Baxter and Funnell 2014; Gerdes et al. 2010). The ParB protein binds to *parS* and ParA interacts with ParB, hydrolysing ATP or GTP to provide the energy needed to drive the partitioning process.

Plasmid Par systems, such as those in the single-copy F plasmid or the P1 prophage plasmid, whose ParA protein has a Walker-type ATPase motif, use the surface of the nucleoid as a scaffold over which plasmids are actively moved. The mechanism is termed a diffusion-ratchet, with ParA diffusing over the nucleoid and ParB binding to the *parS* sequence on the plasmid to form the partition complex (Vecchiarelli et al. 2013, 2014).

ParA-ParB interaction triggers ATP hydrolysis by ParA, denuding the nucleoid surface in the vicinity of the plasmid *parS*-ParB complex of active ParA. This depletion effect creates a ParA gradient across the nucleoid surface, moving the *parS*-ParB complex (and the plasmid) along the gradient. With two daughter plasmids in play, the effect of ParA depletion and the associated gradients is to move the two plasmids away from each other, segregating them into the two daughter cells. This diffusion ratchet mechanism has replaced earlier hypothetical models of ParA-ParB-*parS* segregation systems that were based on ParA assembly into cytoskeletal filaments (Brooks and Hwang 2017).

The R1 drug-resistance single-copy plasmid has a ParMRC partitioning system that consists of a centromere-like *parC* site, an adaptor protein ParR that binds to *parC* and an actin-like ATPase, ParM. ParM forms filaments that grow bidirectionally, with a ParR-*parC* complex one either end. As the filament grows in length, the plasmid copies are separated. ParM searches the cell for ParR-*parC* complexes, the complexes stabilise ParM filaments whose dynamic instability requires ATP hydrolysis; the stabilised filaments grow, pushing *parC*-containing plasmids to opposite ends of the cell (Garner et al. 2004, 2007). The TubZ-TubR-*tubZ* partitioning system found in many plasmids in *Bacillus* spp. (e.g. *B. thuringiensis*) differs from ParMRC in that the TubZ filament grows unidirectionally by recycling TubZ subunits from the leading edge to extend the trailing edge ('treadmilling') and uses GTP hydrolysis to form the filament (Fink and Löwe 2015; Larsen et al. 2007).

1.16 The Nucleoid

A defining characteristic of prokaryotes is that they do not possess a membrane-bound nucleus. Instead, prokaryotes have a nucleoid, a body within the cytoplasm that contains the genetic material but lacks a surrounding membrane (Piekarski 1937). The nucleoid is composed of the chromosome and associated molecules including RNA polymerase, DNA polymerase, DNA-binding proteins, and RNA molecules (Dorman 2014b; Macavin and Adhya 2012). In electron micrographs of thinly sectioned bacteria, the nucleoid can be seen as an amoeboid shape surrounded by the electron-dense ribosomes within the cytoplasm (Kellenberger 1952; Robinow and Kellenberger 1994). Staining of the DNA with 4′, 6-diamidino-2-phenylindole (DAPI) has confirmed the presence of a zone around the nucleoid in *E. coli* and *B. subtilis* where translation can take place (Mascarenhas et al. 2001). While even more sophisticated imaging has improved our knowledge of the structure of the nucleoid, it has taken a multi-pronged approach using a variety of techniques over several decades to bring about our current (but still incomplete) understanding of the bacterial nucleoid.

The chromosome is packaged within the bacterial cell in a conformation that permits gene expression and DNA replication to proceed. The 4.6 Mb circular chromosome of *E. coli* strain MG1655 has a circumference of 1.5 mm and, if opened out fully, a diameter of approximately 0.5 mm. In contrast, the bacterial cell is approximately 2 μm in length, 1 μm in diameter and has a volume of 1 fl, or 1×10^{-15} l (Dorman 2013; Kubitschek and Friske 1986). Understanding how the need to package the DNA efficiently is reconciled with the requirements of DNA replication, gene transcription, DNA recombination, and DNA repair is a major goal of research into the structure of the nucleoid.

1.17 The Chromosome Has Looped Domains

Examination of electron micrographs of thinly sectioned *E. coli* cells gives few clues as to the fine detail of chromosome organisation in the nucleoid. The images of chromosomes extruded from gently lysed *E. coli* obtained by Cairns (1963a,b) using autoradiography hint at a subdivision of the chromosome into looped, supercoiled domains. The nature of the domain boundaries was obscure but seemed to be associated with RNA (Kavenoff and Bowen 1976). Analysis with electron microscopy suggested that the chromosome was subdivided into between 12 and 80 supercoiled loops (Delius and Worcel 1974).

Sinden and Pettijohn (1981) used photobinding of trimethylpsoralen to intracellular DNA to estimate the number of independently looped domains. This agent binds to duplex DNA at a rate that is proportional to the superhelical tension in the DNA (Sinden et al. 1980). By estimating the number of gamma-radiation-induced nicks that were required to release most of the superhelical tension, an estimate of the number of topologically independent domains was obtained. For *E. coli* growing with a generation time of 30 minutes, it was estimated that the chromosome is divided into between 33 and 53 independent domains (or between 90 and 150 domains per nucleoid) (Sinden and Pettijohn 1981). The existence of independent domains is an important concept in nucleoid architecture: genes in one domain may be isolated from DNA topological changes taking place in other domains, making gene location along the chromosome significant for reasons other than differences in copy number arising as a result of gene distance from *oriC*.

More refined measurements of domain number in the *E. coli* chromosome or that of its close relative, *Salmonella enterica* serovar Typhimurium, have been made by exploiting site-specific recombination reactions that can take place within but not between domains, by counting the number of looped domains in multiple images of extruded chromosomes, and by releasing superhelical tension from domains using restriction enzymes and observing the distance over which an effect on the transcription of a supercoiling-sensitive gene can be exerted (Postow et al. 2004; Stein et al. 2005). The results from these experimental approaches indicate that the chromosome is subdivided into about 400 independent domains in *E. coli* cells during exponential growth, with each being approximately 12–14 kb in length. The boundaries between the domains do not seem to be fixed and fewer of them are found in bacteria that have entered stationary phase (Staczek and Higgins 1998).

1.18 The Macrodomain Structure of the Chromosome

The bacteriophage lambda integrase-mediated site-specific recombination system has been exploited in studies of nucleoid organisation in *E. coli* and *Salmonella* (Garcia-Russell et al. 2004; Valens et al. 2004). Recombination between copies of the lambda attachment site requires physical contact between the sites and these can be created by random collision (Crisona et al. 1999; Dorman and Bogue 2016). Sites placed at different distances from one another around the chromosome can be assessed for interaction frequency, providing an estimate of the frequency of contact between different parts of the chromosome. At the same time, regions of the chromosome that rarely interact have also been discovered. This analysis led to the proposal that the chromosome is divided into a small number of

large territories called macrodomains (Valens et al. 2004) (Figure 1.1). *E. coli* and its close relatives have four macrodomains (Ori, Left, Ter, and Right) and two non-structured (NS) regions: NS-Left and NS-Right (Cameron et al. 2017; Jiang et al. 2015; Thiel et al. 2012). The NS domains are determined by their proximity to the Ori macrodomain: any region that is placed next to Ori acquires the features of an NS domain (Duigou and Boccard 2017).

1.19 The Chromosome Displays Spatial Arrangement Within the Cell

The bacterial chromosome is oriented in the cytoplasm with reference to the poles and the midpoint of the cell (Figure 1.9). This positioning is important for the successful segregation of the daughter chromosomes at cell division and it assigns domains to specific regions of the cytoplasm. Experiments using fluorescence *in situ* hybridisation (FISH) in *E. coli* show that in newborn cells, Ori and Ter are located at the cell poles and that genetic loci in between follow approximately the gene order along the chromosome (Niki et al. 2000). Before the initiation of chromosome replication, Ori and Ter move to the mid-cell position. Chromosomes with gross rearrangements of the macrodomains misplace Ori and Ter, leading to the appearance of anucleate daughter cells at cell division (Niki et al. 2000).

FISH experiments involve the permeabilisation and fixing of the bacterial cells; these treatments may produce artefacts, making less intrusive approaches more desirable. Experiments in which arrays of binding sites for fluorescently labelled DNA-binding proteins are placed at specific chromosomal locations have allowed chromosome dynamics to be studied in live bacteria (Robinett et al. 1996). This approach has revealed that in slow-growing *E. coli* cells, the origin and terminus are found at mid-cell and the replichores are located in each cell half (Nielson et al. 2006b; Wang et al. 2006) (Figure 1.9). In fast-growing *E. coli* cells, the Ori copies are found at the cell poles while the Ter region is recovered from the pole to the mid-cell position as the replication forks close in (Youngren et al. 2014) (Figure 1.9). This situation is reminiscent of that described in *B. subtilis* for the Ori and Ter regions during chromosome replication (Teleman et al. 1998; Webb et al. 1997). *C. crescentus* has also served as an important model organism in nucleoid structure studies and its chromosome has been observed as adopting an arrangement in which Ori is located at one cell pole and Ter at the other (Viollier et al. 2004) (Figure 1.11). Analysis of *C. crescentus* using 'carbon copy chromosome conformation capture' (5C) has shown that the replichores lying in between the cell-pole-located Ori and Ter regions are intertwined (Umbarger et al. 2011).

Methods based on different forms of chromosome conformation capture offer the possibility of generating high-resolution data of the co-location of different parts of the chromosome at different points in time. This offers the possibility of assembling a four-dimensional map of the bacterial genome. In contrast, investigations that rely on arrays of binding sites for fluorescently labelled DNA-binding proteins are limited to information about just those few positions where the arrays have been placed. There may also be technical difficulties that arise if the binding site copies in the array recombine; shortening array length and so reducing the strength of the fluorescent signal as fewer labelled proteins bind. The nucleoprotein complexes at the arrays may impede replication fork passage or interfere with local

transcription or the diffusion of DNA supercoils (Le and Laub 2014). It is also possible that the bound proteins may interact with one another, bridging together sister chromosomes prior to chromosome segregation (Mirkin et al. 2014).

The movement of the Ori and Ter domains within the *E. coli* cytosol is thought to reflect the separation of sister chromosomes that are connected along their length (Bates and Kleckner 2005). The molecular nature of the inter-sister connections is unknown (Kleckner et al. 2014). The final phase of the replication process copies the Ter region, setting the stage for chromosome segregation (Figure 1.9). In fast-growing bacteria, another round of chromosome replication will have started by the time this stage is reached (Youngren et al. 2014). The *E. coli* pattern of chromosome domain positioning with Ori and Ter at mid-cell is not universal: *C. crescentus* has its Ori and Ter regions in opposite poles of the cell while *B. subtilis* oscillates between the two modes (Figure 1.11). The differences are thought to reflect different chromosome segregation mechanisms (Wang et al. 2014).

Decatenation of the interlinked chromosome copies by topoisomerase IV and the resolution of any chromosome multimers by XerCD are needed prior to sister chromosome segregation (Hiraga 1993). In the absence of Topo IV, XerCD-*dif*-FtsK can achieve the same outcome by a process of local reconnection involving multiple rounds of site-specific recombination (Grainge et al. 2007). Finally, the segregation process will move one chromosome, together with any associated live replication forks, into one of the daughter cells. In *E. coli* and its close relatives, this occurs without the aid of a dedicated protein-based active partitioning system equivalent to the ParAB proteins and the *parS cis*-acting partitioning DNA sequence that are found in most other bacteria (Badrinarayanan et al. 2015; Bignell and Thomas 2001). Radial confinement of the two sister chromosomes has been proposed as playing a role in segregation in the case of *E. coli*. Here the two chromosome polymers repel one another through an entropic exclusion mechanism that drives the copies into separate compartments before the closure of the cell division septum (Jun and Wright 2010; Junier et al. 2014).

The specificity of chromosome orientation within the cytoplasm has led to the interesting proposal that the chromosome provides the prokaryotic cell with an internal frame of reference, something that has been lost in eukaryotes because the genetic material there is in a membrane-enclosed nucleus (Theriot 2013). This reference frame is useful in providing each molecule in the cell with a set of spatial coordinates. Developing the point further, Theriot has proposed that eukaryotes rely on their cytoskeleton to provide a reference frame (Theriot 2013). We will return to the issue of spatial and temporal positioning of molecules (Chapter 8).

1.20 SeqA and Nucleoid Organisation

DNA-binding proteins play important roles in organising the structure of the folded chromosome, with some of these proteins having a chromosome-domain-specific binding pattern (Dame et al. 2011). The SeqA protein was introduced during the description of factors involved in the control of the initiation of chromosome replication (Section 1.3). SeqA accompanies the moving replication fork (Brendler et al. 2000; Onogi et al. 1999), resulting in a dynamic pattern of binding around the chromosome (Sánchez-Romero et al.

2010; Waldminghaus and Skarstad 2010). This protein seems to be excluded from binding within the Ter macrodomain, possibly reflecting the absence from Ter of matches to the consensus sequence for SeqA high-affinity binding sites (Sánchez-Romero et al. 2010; Waldminghaus and Skarstad 2010). The SeqA protein can interact with the cell envelope as well as hemimethylated DNA (D'Alençon et al. 1999; Mika et al. 2015; Shakibai et al. 1998; Slater et al. 1995) so may it play a role in the positioning of Ori during cell division (Dame et al. 2011).

1.21 MukB, a Condensin-Like Protein

The bacterial chromosome is maintained in an orderly superstructure to facilitate replication, transcription, and other DNA-based transactions. The family of SMC proteins play an important role in achieving this organisation (Uhlmann 2016). These large polypeptides have a DNA-binding head domain and long coiled-coil domains that bring the head-domain-DNA complexes together in a condensed nucleoprotein complex (Figure 1.10). The head domains have ATPase activity and a DNA-binding hinge region in the coiled-coil domain promotes dimer formation (Chen, N., et al. 2008; Kumar et al. 2017a). SMC activity is found in eukaryotes and in prokaryotes, with one of the best-studied examples of an SMC-like protein in bacteria being the MukB protein from *E. coli* (Niki et al. 1991; Rybenkov et al. 2014).

MukB forms topologically stable loops in the chromosomal DNA and protects the supercoils in these protected loops (Kumar et al. 2017a). It forms a complex with the MukE and MukF proteins, with these seeming to play a role in the turnover of the MukB complex on DNA in combination with the ATPase activity of MukB itself (Kumar et al. 2017a). MukF performs a bridging role between the ATPase heads of the two MukB in the complex (Figure 1.10). Proteins performing this task in SMC complexes are called kleisins. The equivalent system in *B. subtilis* and *C. crescentus* consists of the proteins Smc (MukB), ScpA (MukF, kleisin), and ScpB (MukE): the 'Scp' designation indicates that the protein is a 'segregation and condensation protein' (Britton et al. 1998; Burmann et al. 2013; Jensen and Shapiro 1999, 2003; Mascarenhas et al. 2005).

The MukBEF complex has important architectural and segregational roles in the nucleoid, operating mainly outside the Ter macrodomain of the chromosome. Its principal site of action seems to be at *ori* and MukB requires MukE, MukF, and ATP hydrolysis to gain and maintain this association; MukBEF/SMC complexes do not seem to track moving replisomes (Badrinarayanan et al. 2012a,b; Danilova et al. 2007; Gruber and Errington 2009; Sullivan et al. 2009). MukBEF is responsible for guiding the newly replicated *ori* regions into the two halves of the cell, driving bipolar segregation; if MukBEF is removed, the *ori* shifts from mid-cell to the pole, disturbing normal chromosome orientation and segregation patterns (Danilova et al. 2007). Inside the Ter macrodomain, the MatP protein prevents MukBEF from playing a structural role by displacing it and so making it available for *ori* binding (Lioy et al. 2018; Nolivos and Sherratt 2014; Nolivos et al. 2016). The MukBEF complex is not required for sister chromosome cohesion because *muk* mutants have a higher degree of cohesion of sister chromosomes than wild-type cells (Danilova et al. 2007).

Despite being able to stimulate Topo IV activity by direct interaction (Hayama and Marians 2010; Hayama et al. 2013; Li et al. 2010; Vos et al. 2013), MukBEF forms a complex with Topo IV that seems to stabilise MukBEF on the DNA and drive chromosome condensation independently of the catalytic activities of the topoisomerase (Kumar et al. 2017b). This observation is indicative of an architectural role for the MukBEF-Topo-IV complex. Data from chromosome conformation capture experiments show that, together with the HU NAP, MukBEF promotes long-range DNA contacts in the megabase range (Lioy et al. 2018), adding further detail to our picture of the nucleoid-structuring contributions of MukBEF. However, the MukBEF-Topo-IV complex also has functions in chromosome segregation that do depend on the catalytic properties of Topo IV. The complex is responsible for the timely decatenation of newly replicated *ori* copies and their segregation (Figure 1.10), while also contributing to the management of DNA supercoiling set points (Zawadzki et al. 2015). The primary site of action of the MukBEF-Topo-IV complex has been regarded as *ori*, with MukBEF recruiting the topoisomerase to that region of the chromosome (Nicolas et al. 2014). It is becoming clear that MukBEF-Topo-IV has a much more dynamic relationship with the chromosome, both spatially and temporally, and that its relationship with the MatP protein in *ter* has an important management role in its choreography. The final stages of chromosome replication and the associated need to effect decatenation in a timely manner are consistent with a need to grant MukBEF-Topo-IV access to *ter* to perform the necessary cohesion/decatenation steps, with MatP then evicting the complex, making it available for re-association with *ori* (Nolivos et al. 2016).

1.22 MatP, the *matS* Site and Ter Organisation

The 17-kDa MatP protein binds to 23 high-affinity *matS* sites found exclusively within the Ter macrodomain of the *E. coli* chromosome (Mercier et al. 2008; Thiel et al. 2012). The Ter domains of both chromosome I and chromosome II of *V. cholerae* also have *matS* sites that are bound by MatP and these affect the spatiotemporal coordination of the replication and segregation of both chromosomes (Demarre et al. 2014). Counterparts of *matS* are present in the Ter regions of the chromosomes of *Erwinia carotovora*, *S. enterica* serovar Typhimurium LT2, and *Yersinia pestis* (Mercier et al. 2008). MatP forms bridged tetramers that link distant *matS* sites to condense the Ter DNA (Dupaigne et al. 2012). MatP interacts with ZapB, a cell division-associated protein, to position the Ter macrodomain at the cell midpoint and to ensure its segregation (Espéli et al. 2012). With proteins ZapA, C, and D, ZapB functions to organise the Z ring that acts as a scaffold for the assembly of the complexes responsible for cell division (Buss et al. 2013). ZapB is dependent on ZapA for interaction with the tubulin-like FtsZ protein (Galli and Gerdes 2010). MatP also interacts with the MukBEF-Topo-IV complex at the Ter macrodomain, displacing MukBEF from Ter and facilitating its interaction with the Ori macrodomain. This is part of a step-wise process in which MukBEF manoeuvres the chromosome such that Ori and Ter adopt their assigned positions at the pole and mid-cell, respectively, by the time cell division takes place (Lioy et al. 2018; Nolivos et al. 2016). Together with MukBEF and SeqA, MatP is part of a group of proteins found exclusively in bacteria that express the Dam methyltransferase (Brezellec et al. 2006; Mercier et al. 2008).

1.23 MaoP and the *maoS* Site

The MaoP protein is important for the organisation of the Ori macrodomain (Duigou and Boccard 2017; Valens et al. 2016). It binds to a 17-bp sequence that lies adjacent to the *maoP* gene. Both MaoP and *maoS* are required for accurate positioning of Ori within the cell during the division cycle and for restricting the ability of the Ori macrodomain to interact with other territories of the chromosome, especially the Right Macrodomain and the NS-Right region (Valens et al. 2016). MaoP has been described as belonging to a class of DNA-binding proteins that has co-evolved with Dam, including MatP (see below), MukBEF, and SeqA: all of these proteins are involved in chromosome replication/segregation (Brezellec et al. 2006; Valens et al. 2016).

1.24 SlmA and Nucleoid Occlusion

The nucleoid is protected from bisection by the Z ring through a process known as nucleoid occlusion, or NO (Woldringh et al. 1991). If NO fails to occur the result is guillotining of the chromosome, fragmentation of the DNA, and cell death. In *E. coli,* the DNA-binding SlmA protein plays a key role in NO through an interaction with FtsZ (Bernhardt and de Boer 2005). SlmA works by interfering with the polymerisation activity of FtsZ, inhibiting Z-ring formation (Cho, H., et al. 2011). SlmA binds to DNA and to FtsZ simultaneously. Its DNA-binding sites are distributed around the chromosome, but it does not bind in the Ter macrodomain. The inhibitory activity of SlmA on FtsZ polymerisation outside of Ter is thought to delay septum formation until after the Ter macrodomain has been replicated (Tonthat et al. 2011).

1.25 The Min System and Z Ring Localisation

The cell division septum must be placed centrally if rod-shaped bacteria like *E. coli* are to divide into daughter cells of equal size. This placement is achieved through a gradient of FtsZ-inhibitor Min proteins. The gradients extend from regions of maximum Min density at the cell poles to a region of minimum density (and therefore minimum FtsZ inhibition) at mid-cell (Bramkamp and van Baarle 2009; Monahan and Harry 2012; Rowlett and Margolin 2015). While nucleoid occlusion operates to prevent guillotining of the nucleoid by the closing division septum, the Min system works independently of the nucleoid and is concerned with the correct localisation of the septum. Indeed, bacteria that are rendered chromosomeless still tend to form the cell division septum at the mid cell (Sun et al. 1998).

The term 'Min' is derived from 'minicell', a phenotype in *E. coli min* mutants (and other rod-shaped bacteria) where eccentric placement of the division septum produces two daughter cells of uneven length, one of which is too small to accommodate a chromosome (Adler et al. 1967; Reeve et al. 1973), although it can house plasmids (Roozen et al. 1971).

E. coli uses the MinC protein to inhibit FtsZ polymerisation (Hu et al. 1999; Hu and Lutkenhaus 2000) but, unlike *B. subtilis,* it lacks a cell-pole-anchoring protein that can be used to recruit MinC and other Min complex components to that part of the envelope. It relies instead on a MinC protein gradient extending from each pole to the midcell, with MinC forming a complex with the membrane-binding MinD ParA-like ATPase protein (de Boer et al. 1989, 1991; Hu and Lutkenhaus 2003). A third protein, MinE, is used to target the MinCD complex to the cell poles, with MinE (and phospholipid) stimulating the ATPase activity of MinD (Hu and Lutkenhaus 2001). MinE binds to the membrane at the pole, targeting MinCD complexes, displacing both MinC and MinD and stimulating ATP hydrolysis by MinD (Loose et al. 2011; Park et al. 2011). MinE and MinD set up a high-speed oscillating system in which MinC is trafficked from pole to pole, on average spending a minimum of time at mid-cell and most of the time at the poles (Raskin and de Boer 1997; Hu and Lutkenhaus 1999; Hu et al. 2002). It is the relative paucity of MinC at mid-cell that diminishes the inhibitory influence on FtsZ polymerisation and Z-ring formation (Hu and Lutkenhaus 1999; Raskin and de Boer 1999a,b). In addition to inhibiting FtsZ polymerisation by protein-protein interaction, the oscillation of MinC populations from pole to pole has an impact on the distribution of other FtsZ-interactors. Together with FtsZ itself, the ZapA, ZapB, and ZipA proteins oscillate oppositely to MinC and with a similar dynamic pattern. ZapB does not bind FtsZ directly but through ZapA, which does bind FtsZ. ZipA, with FtsA, connects FtsZ to the cytoplasmic membrane (Pichoff and Lutkenhaus 2005) while ZapA-ZapB stimulates Z-ring formation and stabilises it (Buss et al. 2013; Galli and Gerdes 2010; Gueiros-Filho and Losick 2002). Therefore, the oscillatory movements of MinC proteins probably trigger periodic assembly and disassembly of the Z ring complexes (Bisicchia et al. 2013; Thanedar and Margolin 2004).

B. subtilis possesses the cell-pole-targeting protein DivIVA, which is involved both in chromosome attachment at the pole in sporulating cells (Section 1.10) and in directing the cellular localisation of MinC (Cha and Stewart 1997; Edwards and Errington 1997). The utility of DivIVA as a general pole-targeting protein arises from its ability to sense cell membrane curvature, which is maximal at the poles (Edwards et al. 2000; Lenarcic et al. 2009). The MinC protein is bound by MinD and an adaptor protein, MinJ, connects this complex to DivIVA (Bramkamp et al. 2008; Patrick and Kearns 2008). As the division septum develops, invagination of the membrane, and the associated membrane curvature, recruit DivIVA from the pole to the mid-cell (which is the soon-to-be pole of the new daughter cell) (Gregory et al. 2008; Rodriguez and Harry 2012; van Baarle and Bramkamp 2010).

Genetic elimination of the Min system and of nucleoid occlusion is deleterious for cell growth in rich medium, but the mutants can grow and divide in minimal medium (Bernhardt and de Boer 2005; Yu and Margolin 1999). This suggests that additional systems exist to ensure chromosome segregation and cell division (Bailey et al. 2014; Cambridge et al. 2014). A link between the Ter-*matS*-binding MatP protein and ZapB connects the Ter macrodomain of the chromosome to the divisome's ZapB-ZapA-FtsZ complex (Espéli et al. 2012) and this may afford the nucleoid itself a role in determining Z ring placement (Rowlett and Margolin 2015; Yu and Margolin 1999).

The Min system operates on Z ring placement through an inhibitory mechanism. This strategy is not used universally among bacteria. For example, positive placement is used

to direct Z ring positioning in *Streptomyces* spp. Here, the SsgA protein takes up position at mid-cell and recruits a partner, SsgB, which is thought to promote FtsZ polymerisation and to link the Z rings to the membrane. These rings form the basis of sporulation septa in these actinomycetes (Traag and van Wezel 2008; Willemse et al. 2011). Sporulation takes place in the aerial hyphae where up to one hundred division septa are laid down, dividing the hyphae into compartments that each contain one genome copy (Zhang, L. et al. 2016). The SepG membrane protein recruits SsgB to future septum sites; it is also required for nucleoid compaction indicating that plays a role in coordinating chromosome organisation and placement of the division septum (Zhang et al. 2016). The PomZ protein in *Myxococcus* spp. is also a positive regulator of Z-ring positioning; PomZ shares with MinD the property of being a ParA-like ATPase (Treuner-Lange et al. 2013). It forms a complex with PomX and PomY that moves over the surface of the nucleoid in a biased, randomised motion that becomes constrained at the mid-cell. Once at the mid-cell location, PomXYZ recruits FtsZ (Schumacher et al. 2017).

Macrodomains play important roles in determining the choreography of the daughter chromosomes, as these segregate prior to cell division (Espéli et al. 2008). They also correlate with global gene expression patterns, suggesting that the overall gene expression programme of the cell is written into the architecture of the nucleoid (Cameron et al. 2017; Sobetzko 2016; Sobetzko et al. 2012). To appreciate the significance of the connections between nucleoid structure and gene expression, it will be necessary to consider the contributions made to both by variable DNA structure and NAPs.

1.26 DNA in the Bacterial Nucleoid

DNA in bacterial cells is maintained in an underwound state and this affects the shape that the DNA duplex adopts as it seeks to adopt a minimal energy conformation. The underwinding arises due to a deficit in helical turns, i.e. the number of times the two DNA strands twist around the duplex axis. The twist deficiency imparts torsional stress to the duplex, which is relieved by allowing the duplex to adopt a writhed confirmation in which the helical axis coils around itself. This coiling of the already coiled DNA duplex creates supercoiling and has the effect of making the DNA molecule more compact. In the context of the nucleoid, such compaction assists with solving the problem of packaging the genetic material within the cell. The most supercoiled parts of the chromosome form branches, facilitating further compaction.

1.27 DNA Topology

The topological state of a DNA molecule is described by three parameters: the linking number (the number of times one DNA strand crosses the other in the duplex); the twist (the number of complete turns made by the strands around one another along the length of the duplex); and the writhe (the number of writhing turns made by the duplex axis around itself) (Bauer et al. 1980; Boles et al. 1990; Vinograd et al. 1965). Changes to the linking number can be made by breaking one or both strands of the duplex, twisting the DNA with

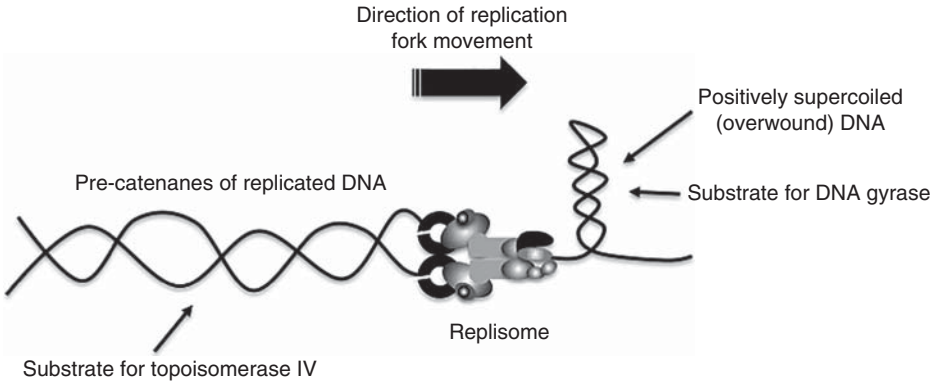

Figure 1.14 The topological consequences of replisome activity. The replication fork, moving from left to right, over winds the DNA ahead to create a domain of positive supercoiling. Unless this is relaxed, the replisome will be unable to advance further. Relaxation of the positively supercoiled domain is carried out by DNA gyrase: it neutralises the positive supercoils by introducing negative ones. In *C. crescentus*, the GapR protein binds to positively supercoiled DNA and stimulates its relaxation by type II topoisomerases (see Guo et al. 2018). The newly synthesised DNA behind the replisome becomes catenated. These interwound, double-stranded DNA molecules are decatenated by DNA topoisomerase IV, allowing the daughter chromosomes to be segregated at cell division. For further information, see Lopez et al. (2012) and Postow et al. (2001).

or against the sense of the double helix, and then resealing the strand breaks (Higgins and Vologodskii 2015). Twisting the DNA in keeping with the sense of the double helix tightens the duplex and imparts positive writhe, resulting in positive supercoiling. Twisting the DNA against the sense of the double helix underwinds the molecule, imposing negative writhe and therefore negative supercoiling (Sinden 1994). In nature, negative supercoiling is the norm although positive supercoils do arise naturally, especially during the movement of the DNA replication fork (Figure 1.14) and during transcription (Liu and Wang 1987) (Figure 1.15). In contrast to mesophilic bacteria like *E. coli*, hyperthermophilic archaea that live in environments that are characterised by very high temperatures possess a reverse gyrase activity and maintain their DNA in a positively supercoiled state (Couturier et al. 2014; Lipscomb et al. 2017). Reverse gyrase, first described in *Sulfolobus acidocaldarius* in 1984, uses ATP to introduce positive supercoils into DNA (Kikuchi and Asai 1984; Ogawa et al. 2015).

A covalently closed, circular duplex DNA molecule that is neither overwound nor underwound is said to be topologically relaxed. If this circular duplex undergoes an increase in its linking number (overwinding) or a decrease (underwinding) it retains an identical nucleotide sequence compared with the relaxed form but differs from this form topologically (Sinden 1994). For this reason, the relaxed, overwound, and underwound isomeric forms of the circular duplex are referred to as topoisomers of the same DNA molecule. Enzymes that produce topological changes in DNA by altering the linking number are called topoisomerases and *E. coli* has four: topoisomerase I (topo I), DNA gyrase (a topo II family member), topoisomerase III (topo III), and topoisomerase IV (topo IV) (Bates and Maxwell 2005; Wang 2002) (Table 1.1).

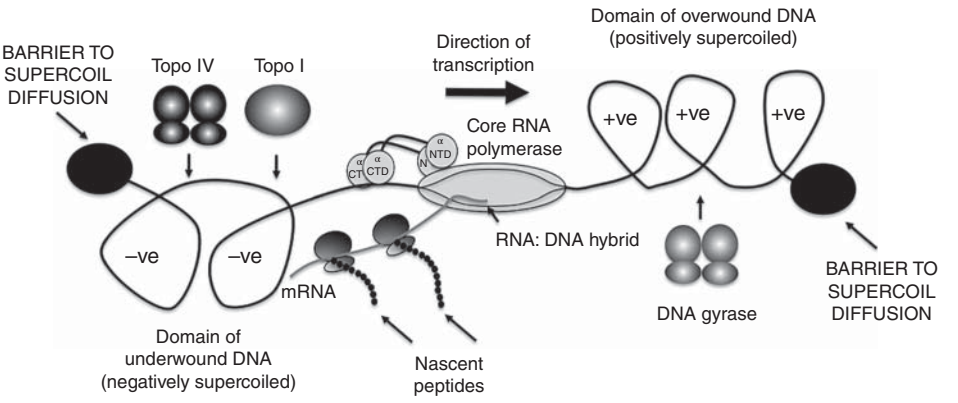

Figure 1.15 The topological consequences of transcription. This is the twin supercoil domain model, as proposed by Liu and Wang (1987) and supported by numerous independent experiments. Core RNA polymerase is engaged in transcript elongation and the mRNA is simultaneously being translated by polyribosomes to produce nascent peptides. As the coupled transcription-translation complex moves from left to right, the DNA template ahead becomes overwound, or positively supercoiled, while the DNA behind becomes underwound, or negatively supercoiled. This situation will bring transcription to a halt as the machinery jams. This is because: (a) the domains of supercoiled DNA cannot be resolved by supercoil lateral diffusion because the transcribed region is bounded by topological barriers (solid black discs) and (b) the bulky transcription-translation complex cannot rotate around the DNA to relieve the torsional tension in the DNA duplex. The solution to the problem is provided by the DNA-relaxing activities of topoisomerases: DNA gyrase removes the positive supercoils by introducing negative supercoils, while the negatively supercoiled domain is relaxed by DNA topoisomerase I and IV. Interference with these relaxation processes can result in undesirable outcomes, such as R-loop formation (Figure 1.16). Topological barriers can be created by head-to-head transcription collisions and by collisions between converging replisomes and RNA polymerases; they can also arise from the presence of nucleoprotein complexes and distortions (e.g. sharp bends) in the DNA duplex.

1.28 DNA Topoisomerases: DNA Gyrase

Topoisomerases are classed as type I if they change the linking number of DNA in steps of one, and as type II if the linking number changes in steps of two (Champoux 2001; Wang 2002) (Table 1.1). DNA gyrase is a type II enzyme and it has the property, unique to prokaryotes, of being able to introduce negative supercoils into DNA (Gellert et al. 1976a; Higgins et al. 1978; Nöllmann et al. 2007). This negative supercoiling activity is ATP dependent and there is an ATP-binding site in the B subunit of gyrase (Gellert et al. 1979; Mizuuchi et al. 1978). Gyrase is an A_2B_2 hetero-tetramer and it is essential; knockout mutations in either of the genes that encode its A (*gyrA*) or B (*gyrB*) subunits are lethal (Bates and Maxwell 2005). Its essentiality has made gyrase a very attractive target for antimicrobial therapy and a number of drugs are available that target its subunits (Collin et al. 2011; Maxwell 1999). The coumarin class of antimicrobials have been particularly useful as research tools because they compete with ATP for access to the B subunit ATPase and inhibit gyrase activity without inducing the SOS response (DeMarini and Lawrence 1992; Gellert et al. 1976b; Gormley et al. 1996; Pugsley 1981; Sugino and Cozzarelli 1980; Sugino et al. 1978).

Table 1.1 The topoisomerases of *E. coli*.

Enzyme name (type[a])	Molecular mass (kDa)	Gene(s)	Comments
(Type I)			
Topoisomerase I	97	*topA*	DNA swivelase that makes a transient cut in one strand of the DNA duplex, binds to the cut site via a 5′-phosphotyrosine bond; relaxes negatively supercoiled DNA; requires Mg^{2+}
Topoisomerase III	73.2	*topB*	Relaxes negatively supercoiled DNA; decatenase; has catenase activity in association with RecQ; requires Mg^{2+}
(Type II)			
DNA gyrase (Topoisomerase II)	105 (A subunit) 95 (B subunit)	*gyrA* (A subunit) *gyrB* (B subunit)	ATP-dependent negative supercoiling activity; relaxes negative supercoils in an ATP-independent manner; relaxes positively supercoiled DNA; binds DNA transiently via a 5′-phosphotyrosine bind; requires Mg^{2+}
Topoisomerase IV	75 (ParC) 70 (ParE)	*parC* (ParC; GyrA-like) *parE* (ParE; GyrB-like)	Decatenase activity; interacts with MukBEF; relaxes negative supercoils; requires ATP, Mg^{2+}

a) Type I enzymes change the linking number of the duplex DNA substrate in steps of 1 ($\Delta Lk = 1$) while type II enzymes change the linking number in steps of 2 ($\Delta Lk = 2$).

In contrast, those drugs (e.g. quinolones) that inhibit the A subunit during DNA cleavage and religation cause DNA damage that results in induction of the SOS response (Gellert et al. 1977), something that can complicate experimental design and data interpretation. Gyrase also has an ATP-independent DNA relaxing activity that is unmasked only in the absence of ATP (Gellert et al. 1977; Higgins et al. 1978; Williams and Maxwell 1999). The ATP-dependent mechanism by which gyrase introduces negative supercoils into DNA is also capable of relaxing positive supercoils (Ashley et al. 2017). This activity is especially important when gyrase processes the positively supercoiled DNA that is a by-product of transcription and DNA replication (Koster et al. 2010) (Figures 1.14 and 1.15). DNA gyrase in living cells responds to the [ATP]/[ADP] ratio, linking the management of DNA topology to the metabolic activity of the bacterium (Hsieh et al. 1991a,b; Snoep et al. 2002; van Workum et al. 1996). Gyrase activity is also tuned in living bacteria by stresses such as the acidification of the bacterial cytosol that accompanies adaptation to acid stress (Colgan et al. 2018).

1.29 DNA Topoisomerases: DNA Topoisomerase IV

Topo IV was discovered in *E. coli* 14 years after gyrase, its type II topoisomerase companion (Kato et al. 1990) (Table 1.1). It is encoded by two genes, *parC* and *parE*, whose names hint at a defect in chromosome partitioning that is associated with mutants with a topo IV deficiency (Kato et al. 1990). Topo IV is an ATP-dependent topoisomerase but, unlike gyrase, which it closely resembles in amino acid sequence and subunit structure, it cannot introduce negative supercoils into DNA. Instead, Topo IV relaxes both positively and negatively supercoiled DNA and is an important DNA decatenase (Bates and Maxwell 2007; Crisona and Cozzarelli 2006; Kato et al. 1992; Peng and Marians 1993; Zawadzki et al. 2015). Its relationship with the MukBEF SMC-like complex is emerging as one of Topo IV's most physiologically significant functions, one that is important for the correct spatiotemporal management of chromosome replication, architecture, and segregation during the cell cycle (Section 1.21).

1.30 DNA Topoisomerases: DNA Topoisomerase I

The principal source of relaxation activity for negatively supercoiled DNA is Topo I, a monomeric, ATP-independent enzyme that is encoded by the *topA* gene in the Ter macrodomain (Margolin et al. 1985) (Table 1.1). This enzyme also has a catenase/decatenase activity on double-stranded circular DNA with single-stranded regions (Terekhova et al. 2012; Tse and Wang 1980) and prevents over-replication of the chromosome originating at *oriC* (Usongo and Drolet 2014). Topo I is a type I topoisomerase that has a 'swivelase' activity. It cuts one of the two DNA strands in a negatively supercoiled molecule, forming a covalent link to the cut strand, and allows the torsional strain in the DNA to drive the rotation of the intact strand through the gap (Kirkegaard and Wang 1978). The result is an increase of 1 in the linking number of the DNA (Bates and Maxwell 2005). The *topA* gene is not essential, although knockout mutants grow slowly (Margolin et al. 1985; Sternglanz et al. 1981). Bacteria that lose Topo I through *topA* knockout mutations can compensate in different ways, restoring a growth rate that is close to that of wild-type strains (Raji et al. 1985; Richardson et al. 1984). One option is to acquire non-lethal mutations in *gyrA* or *gyrB* that result in the expression of a gyrase with a reduced negative supercoiling activity (DiNardo et al. 1982; Pruss and Drlica 1985; Pruss et al. 1982, 1986; Richardson et al. 1984, 1988). Another possibility exploits the amplification of the copy number of the *parC* and *parE* genes, resulting in increased expression of Topo IV. In these strains the elevated expression of Topo IV with its DNA-relaxing activity can compensate for the missing Topo I, restoring the growth rate of the mutant to one that is similar to wild type (Dorman et al. 1989; Free and Dorman 1994; McNairn et al. 1995). Bacteria that are not exposed to stressful growth conditions such as elevated temperature, low pH, or raised osmotic stress or lack of oxygen do not require compensatory mutations in order to display normal rates of growth (Ní Bhriain and Dorman 1993). This suggests that a link exists between environmental stress and the management of DNA supercoiling in bacteria (Dorman and Dorman 2016).

1.31 DNA Topoisomerases: DNA Topoisomerase III

Topo III is a second type I topoisomerase that, like Topo I, is an ATP-independent monomeric enzyme (Table 1.1). It is encoded by the *topB* gene and is not essential for the survival of the bacterium (Usongo et al. 2013). However, mutants deficient in both Topo I and in Topo III do not survive (Stupina and Wang 2005). Topo III has weak DNA-relaxing activity and functions principally as a decatenase (Nurse et al. 2003; Perez-Cheeks et al. 2012). Its apparent weakness as a DNA-relaxing enzyme compared with Topo I arises from a difference in the mechanisms used by the two topoisomerases: Topo I operates in a processive manner with short pauses between processive runs, whereas Topo III takes long pauses, leading to a relaxing process with an overall rate that seems slower (Terekhova et al. 2012). While Topo I plays an important role in controlling the frequency of chromosome replication initiation at *oriC*, Topo III contributes to the management of replication fork collision in the Ter macrodomain (Suski and Marians 2008). In fact, all four topoisomerases are important components of the chromosome replication machinery and display both a division of labour and an interesting degree of redundancy that allows the cell to continue to function even if one of the enzymes experiences interference.

1.32 DNA Replication and Transcription Alter Local DNA Topology

The linking number of DNA is changed at a local level by the processes of transcription and DNA replication. In 1987, Liu and Wang proposed, in a landmark theoretical paper, that the process of transcription would induce overwinding of the DNA template ahead of RNA polymerase and underwinding behind (Liu and Wang 1987) (Figure 1.15). Experimental studies provided support for the proposal, leading to the realisation that topoisomerases play important roles in transcription by relieving the torsional stress that the process creates (Ahmed et al. 2017; Chong et al. 2014; Higgins 2014; Rahmouni and Wells 1992; Rani and Nagaraja 2019; Wu et al. 1988). The role of local DNA supercoiling in the modulation of transcription and in gene-to-gene communication will be addressed in Section 8.2. Here we will consider the impact of transcription on nucleoid architecture and on DNA replication.

1.33 Transcription and Nucleoid Structure

Several investigations have made links between patterns of transcription and the superstructure of the bacterial nucleoid. At a practical level, replication fork movement must be reconciled with the needs of transcription (initiation, elongation, and termination), so aligning replisome movement with the direction of gene transcription avoids significant conflicts between DNA and RNA polymerases. Collisions between the replisome and RNA polymerase are known to cause severe inhibition of replisome progression (Mirkin and Mirkin 2005). Transcriptional promoters that oppose the direction of replisome movement serve to pause the replication fork, while transcription terminators that are aligned with the direction of replisome movement also act as replication fork pause sites (Mirkin et al.

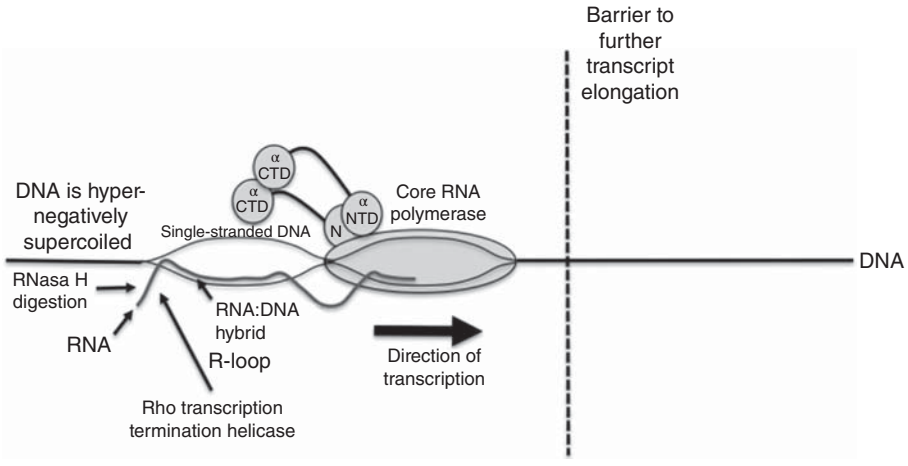

Figure 1.16 R-loop formation. When RNA polymerase reads a G+C-rich template, stalls and backtracks, it leaves a domain of hypernegatively supercoiled in its wake and the associated transcription stalling may allow the RNA transcript to base pair with its DNA template strand, leaving the non-transcribed strand as a single-stranded bubble. Other impediments to RNA polymerase progression include head-on collisions with other transcription units or with replisomes (the barrier is represented by the vertical gapped line). Loss of topoisomerase I activity is known to promote R-loop formation because it encourages the accumulation of hyper negative superhelicity in DNA that is being transcribed (or replicated). Failure to process and remove RNA loops can lead to DNA damage, including double-stranded breaks and hyper-recombination. The Rho transcription-terminating helicase (Figure 3.4) helps to suppress R-loop formation by preventing backtracking by RNA polymerase while RNase H eliminates R-loops by removing the RNA component of the RNA:DNA hybrid in R-loops.

2006). Conflicts between the replisome and RNA polymerase can generate R-loops, stalling replication, and transcription in the affected region until RNase H removes the R-loop (Kuziminov 2018). Unresolved R-loops also result in hyper-recombination and genome instability, so avoiding replication–transcription conflicts is very desirable (Figure 1.16).

A correlation has also been reported between gene *essentiality* and alignment with the direction of replication fork movement (Rocha and Danchin 2003). Overall, one finds more genes on the leading strand than on the lagging strand of the chromosome and this may reflect the outcome of evolutionary pressure to minimise collisions (Rocha 2008). The question of replication and transcription alignment/collision also has a DNA topological dimension, in that converging polymerases will create, and trap between them, a domain of positively supercoiled DNA that must be resolved by type II topoisomerases: gyrase or topo IV (Crisona et al. 2000; Kato et al. 1992). The mechanisms of action of these enzymes bring an increased risk of double-stranded breaks occurring in the chromosome with potentially lethal consequences for the cell (Hiasa et al. 1996; Lockshon and Morris 1983). These factors may impose limits on the options for gene orientation on the chromosome, influencing its evolution.

Experiments using chromosome conformation capture (3C) methods (Dekker et al. 2002) have suggested a link between DNA replication, transcription, and the organisation of the bacterial nucleoid (Cagliero et al. 2013; Le et al. 2013; Le and Laub 2016; Lioy et al.

2018; Meyer et al. 2018). Work that exploited site-specific recombination as a measure of interaction frequency within the bacterial genome suggested that transcription activity and the associated changes in local DNA topology have a structural function in the nucleoid (Booker et al. 2010; Higgins 2014). Furthermore, the global level of DNA supercoiling in the nucleoid can be tuned by treating the bacterium with rifampicin, an inhibitor of RNA polymerase (Rovinskiy et al. 2012). Experiments with trimethylpsoralen crosslinking have provided evidence that a gradient of DNA supercoiling exists extending from the origin of chromosome replication to the Ter macrodomain (Lal et al. 2016). Bioinformatic and experimental studies show that the distribution of binding sites around the chromosome for DNA gyrase is non-uniform, with more sites being detected close to the origin of replication (Jeong et al. 2004; Sobetzko et al. 2012; Sutormin et al. 2019). This could indicate that the Ori-proximal part of the chromosome is the most underwound, contradicting the data from the psoralen-binding-and-crosslinking studies (Lal et al. 2016). On the other hand, it may indicate a need to compensate for the paucity of negative supercoiling in the Ori-proximal part of the chromosome that is predicted by those studies. Further evidence that the chromosome is not uniformly supercoiled has come from investigations in which supercoiling-sensitive genes have been placed at different locations in the genome (Bryant et al. 2014). A comprehensive survey of the effects of gene position on transcription in *E. coli* showed that the propensity for transcription varies with chromosomal location: horizontally acquired genetic elements are associated with quiescent regions while ribosomal and other metabolic genes are in highly active zones (Scholz et al. 2019). It should be noted, however, that several studies which have explored the influence of gene position (including the possible contribution of differences in local DNA supercoiling) did not detect clear changes in the level of expression of the test gene(s) at different sites around the chromosome (Block et al. 2012; Brambilla and Sclavi 2015; Chandler and Pritchard 1975; Miller and Simons 1993; Pavitt and Higgins 1993; Schmid and Roth 1987; Sousa et al. 1997; Thompson and Gasson 2001; Ying et al. 2014).

1.34 Nucleoid-associated Proteins (NAPs) and Nucleoid Structure

Sequence-dependent DNA-binding proteins such as MaoP, MatP, SeqA, and SlmA play important roles in the organisation of the chromosome in the nucleoid. Yet the term ‘nucleoid-associated protein’, or NAP, is usually reserved for another group of DNA-binding proteins, not all of which are sequence-dependent for DNA binding. Most NAPs were discovered in roles other than organising the nucleoid. Several were first encountered as contributors to the efficient operation of site-specific recombination systems in bacteria or their phage (reviewed in Dorman and Bogue 2016). Later, their more general contributions to cell physiology became appreciated. We have already encountered two, FIS and IHF, as components of the systems that govern the initiation of chromosome replication (Section 1.3). Originally, FIS was identified as an enhancer-binding protein that improved the efficiency of the DNA inversion events responsible for phase-variable expression of tail fibre genes in bacteriophage Mu, and for flagellar phase variation in *Salmonella* (Koch and Kahmann 1986; Johnson et al. 1986). IHF was detected as an

essential factor for the site-specific entry of the bacteriophage lambda genome into the *attλ* site on the chromosome of *E. coli* (Nash and Robertson 1981) (Section 1.35). These and other NAPs have emerged as important regulators of a multitude of bacterial genes (Dillon and Dorman 2010). They have mechanisms of action that typically involve the making of adjustments to local DNA architecture. In many cases, these adjustments affect gene expression at the level of transcription (or beyond) and include direct or indirect effects on nucleoid structure. A summary of the key features of some of the most important (i.e. best-studied) NAPs is given in the following sections.

1.35 DNA Bending Protein Integration Host Factor (IHF)

IHF is essential both for the integration and the excision of bacteriophage lambda into/from the *E. coli* chromosome (Bushman et al. 1984; Seah et al. 2014) (Figure 1.17). These site-specific recombination reactions are catalysed by the tyrosine integrase Int (Craig and Nash 1983; Han et al. 1994; Hoess et al. 1980; Kikuchi and Nash 1979; Tong et al. 2014). IHF is usually expressed as a heterodimer composed of an alpha and a beta subunit encoded by the *ihfA* and *ihfB* genes, respectively, which are located at different places on the chromosome (Haluzi et al. 1991; Mendelson et al. 1991; Miller and Friedman 1980; Nash et al. 1987). IHF has strict DNA sequence requirements for binding and its binding sites typically are located in A+T-rich DNA (Miller and Friedman 1980). The protein inserts a looped beta strand with a proline amino acid at its apex into the minor groove of the DNA at the target site, producing a distortion that causes the DNA duplex to bend (Engelhorn and Geiselmann 1998; Engelhorn et al. 1995; Rice et al. 1996; Sun et al. 1996; Vivas et al. 2012) (Figure 1.18). The combined effects of the bends introduced by each subunit is to create a turn of up to 180° in the pathway taken by the DNA helix (Rice et al. 1996; Sugimura and Crothers 2006). This allows IHF to play important roles in nucleoid architecture, chromosome replication, site-specific recombination, transposition, plasmid maintenance, and transcription regulation (Biek and Cohen 1989; Crellin et al. 2004; Dorman and Bogue 2016; Prieto et al. 2012; Ryan et al. 2004; Saha et al. 2013; Swinger and Rice 2004). In terms of amino acid sequence, protein structure, and subunit composition, IHF is a close relative of the HU NAP. Both proteins have an αβ heterodimeric structure and all four proteins have similar amino acid sequences (Dey et al. 2017). IHF appears to be a specialist member of the HU superfamily of DNA-binding proteins, a group that includes relatives encoded by the human genome that contribute to chromosome partitioning (Burroughs et al. 2017). Although IHF is usually considered as acting in an αβ heterodimeric form, transcriptomic studies carried out in *Salmonella* indicate that bacteria expressing just the α, just the β, and both α and β subunits, are altered in expression of distinct-yet-overlapping groups of genes, implying that IHF can form homodimers that are biologically active (Mangan et al. 2006). Indeed, previous investigations have found that IHF homodimers have DNA-binding activity (Hiszczynska-Sawicka and Kur 1997; Werner et al. 1994; Zablewska and Kur 1995; Zulianello et al. 1994). When reading the older IHF literature, the reader should be aware that in 1996 the names of the genes were changed from *himA* and *himD* (where *'him'* referred to H.I. Miller) to *ihfA* and *ihfB*, respectively (Weisberg et al. 1996).

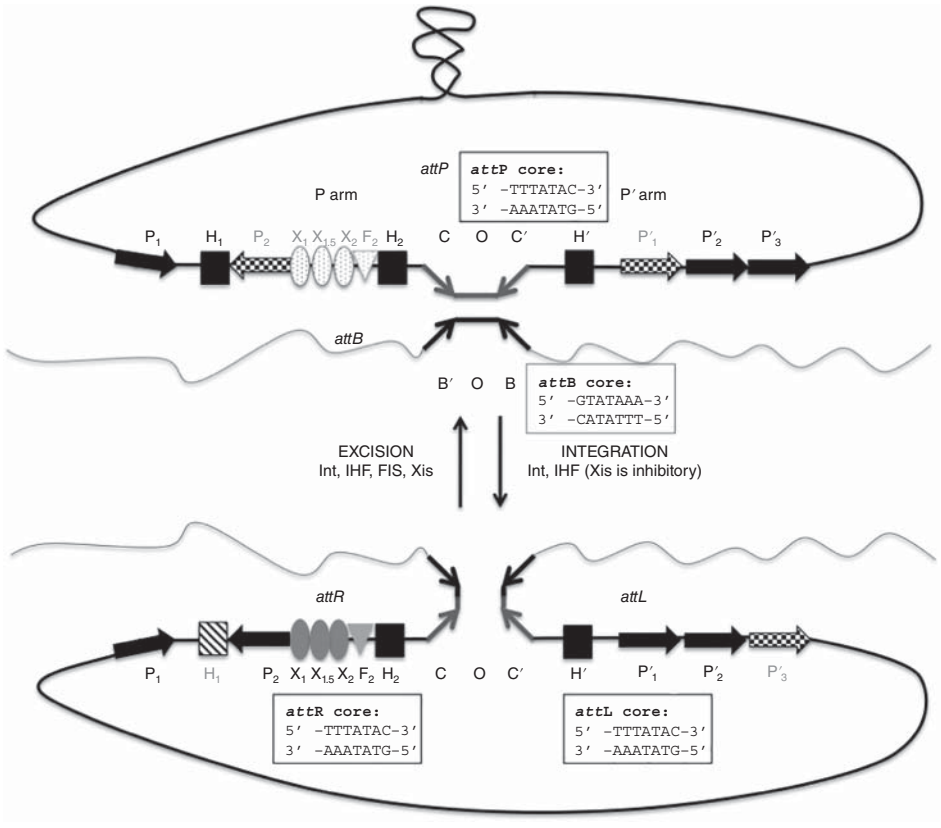

Figure 1.17 Integration of bacteriophage lambda at the lambda attachment site on the *E. coli* chromosome and excision of the prophage. Integration and excision are catalysed by the phage-encoded Int tyrosine integrase. In the integration step, the recombining sites at *attP* (on the phage) and *attB* (on the bacterial chromosome) undergo intermolecular site-specific recombination to generate the lambda prophage. Excision recreates *attB* and *attP* from *attL* and *attR,* the direct repeats that form the boundaries between the prophage and chromosomal DNA. The nucleotide sequences of each of these four elements are shown in the figure. In addition to the Int recombinase, the integration step requires the architectural protein, integration host factor, IHF. Excision also requires IHF together with the phage-encoded directionality factor, Xis, and the host-encoded FIS protein. Xis is inhibitory to integration and FIS has a stimulatory effect on excision (see Ball and Johnson 1991; Seah et al. 2014). Binding sites for these proteins are represented by labelled arrows (P, Int), squares (H, IHF), ovals (X, Xis), and a triangle for a FIS (F) binding site. Binding sites in the P′ arm of the free circular phage genome have primes added to their designations and these are retained in the *attL* segment of the prophage. Not every protein-binding site is occupied in each reaction. Occupied sites have a solid filling while unoccupied sites have a speckled or hatched filling. During integration, Int-binding sites P_1, P'_2, and P'_3 are occupied while P_2 and P'_1 are not; all three IHF-binding sites are occupied but the XIS and FIS sites are vacant. During excision, Int sites P_1, P_2, P'_1, and P'_2 are occupied, as are IHF sites H_2 and H′ and all of the Xis and FIS sites; IHF site H_1 and Int site P_3 are vacant.

1.36 HU, a NAP with General DNA-binding Activity

The HU protein from *E. coli* is the founder member of a superfamily of related NAPs found throughout the prokaryotic world and beyond (Burroughs et al. 2017). HU interacts with DNA in the minor groove and this encourages the bound DNA to follow a looped path (Figure 1.18). This property helps HU to overcome the resistance of the DNA to loop formation by overcoming DNA's intrinsic stiffness (Johnson et al. 1986). HU-assisted loop formation contributes to the formation of nucleoprotein complexes involved in the control of transcription and site-specific recombination (Haykinson and Johnson 1993; Semsey et al. 2004). It also has RNA-binding activity, enabling it to influence translation (Balandina et al. 2001).

Each HU subunit inserts a beta sheet with an apical proline amino acid into the minor groove of the DNA at the binding site, inducing the DNA to bend (Figure 1.18). The bend angle is typically in the range of 105°–140° and bends are not coplanar, having a dihedral angle that is consistent with the path taken in negatively supercoiled DNA (Swinger et al. 2003). The flexibility in the bend angle, coupled with the absence of a strict nucleotide sequence for DNA binding, may allow HU to participate as an architectural component in a wide variety of DNA-based transactions.

The α and β subunits of HU are encoded, respectively, by the *hupA* and *hupB* genes, located at distinct positions on the chromosome: *hupA hupB* double mutants that fail to express the HU protein display a filamentous cell phenotype because of disruption of the

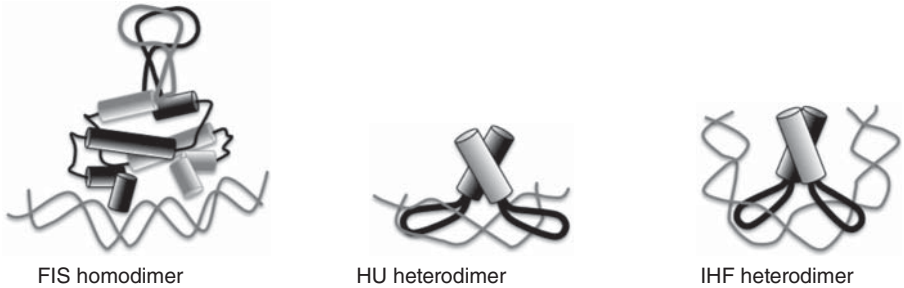

Figure 1.18 The interactions of the nucleoid-associated proteins FIS, HU, and IHF with DNA. The FIS protein is homodimeric, while HU and IHF are heterodimeric. HU and IHF are closely related at the level of amino acid sequence and the alpha and beta subunits of each protein are similar in sequence and secondary structure. The tertiary structures of HU and IHF are also quite similar, as their modes of binding to DNA. Each inserts a beta sheet from each subunit into the minor groove of its DNA target. IHF differs from HU in having a strict nucleotide sequence requirement for binding and in making more contacts with the DNA at its binding site. IHF also induces a much greater bend angle in the DNA. The bends on either side of the HU-DNA complex are non-coplanar. FIS binds in the major groove of DNA using an alpha helix, one of four alpha helices found in each FIS monomer. The protein uses an induced fit binding mechanism that compresses the minor groove lying between the two sites of insertion of the alpha helices in the major groove. FIS binds to a variety of sites with differing binding affinities; a Logo has been assembled that summarises the chief sequence characteristics of the highest affinity sites (see Stella et al. 2010).

cell cycle due to the arrest of DNA replication (Dri et al. 1991). HU interacts with the DnaA protein at *oriC* where it stimulates formation of the initiation complex in chromosome replication. Like IHF, HU is usually heterodimeric and is composed of an alpha and a beta subunit. The alpha subunit seems to have the primary responsibility for interacting with DnaA. This preference for the alpha subunit may facilitate enhanced HU–DnaA interaction at early stages of growth when an HU α_2 homodimer predominates rather than the αβ heterodimer (Chodavarapu et al. 2008). HU can also influence chromosome replication initiation indirectly by repressing the expression of the gene that encodes SeqA (Lee, H., et al. 2001), the protein that sequesters *oriC* and excludes DnaA (Han et al. 2003; Slater et al. 1995; von Freiesleben et al. 1994).

The HU protein can form nucleosome-like structures in *E. coli* that are dependent on the local HU-to-DNA ratio (Sagi et al. 2004). It has been described as insulating transcription units on the chromosome by preventing changes in DNA supercoiling caused by transcription in one unit from influencing an adjacent one (Berger et al. 2016). HU may be particularly important for the maintenance of DNA supercoiling levels in the Ter macrodomain as the bacterium enters the stationary phase of the growth cycle (Lal et al. 2016). It has been reported to induce, together with FIS, weak and transient domain boundaries around the *E. coli* chromosome (Wu et al. 2019).

In laboratory-grown cultures, the subunit composition of the HU protein changes as a function of growth phase: In lag phase, as the bacterium adapts to its new environment, the α_2 form of HU occurs; in exponential growth the αβ form predominates and the β_2 form is detected as the culture enters stationary phase (Claret and Rouvière -Yaniv 1997). The changing subunit composition of HU and the different DNA interaction properties of the distinct HU forms may contribute to processes that differentially compact the chromosome in the nucleoid and affect gene expression patterns (Hammel et al. 2016). Transcriptomic studies in *Salmonella* have shown that each form of the HU protein seems to govern a distinct group of genes, with overlaps between the three sub-regulons (Mangan et al. 2011).

1.37 The Very Versatile FIS Protein

FIS is the Factor for Inversion Stimulation, so called because it was discovered originally as an important architectural element in the DNA inversion mechanisms responsible for the phase-variable expression of flagella in *Salmonella* (Johnson et al. 1986) and of tail fibre proteins in bacteriophage Mu (Koch and Kahmann 1986). FIS is now known to contribute to a wide range of molecular events in bacteria, including DNA replication (Cassler et al. 1995; Filutowisz et al. 1992; Gille et al. 1991), site-specific recombination (Dhar et al. 2009; McLean et al. 2013), transposition (Weinreich and Reznikoff 1992), transcription regulation (Grainger et al. 2008; Hirvonen et al. 2001; Kelly et al. 2004; Pemberton et al. 2002), bacteriophage life cycles (Betermier et al. 1993; van Drunen et al. 1993; Papagiannis et al. 2007; Seah et al. 2014), illegitimate recombination (Shanado et al. 1997), and chromosome domain boundary formation (Hardy and Cozzarelli 2005; Wu et al. 2019).

1.38 FIS and the Early Exponential Phase of Growth

FIS is a homodimeric NAP that is encoded by the second gene in the *dusB-fis* operon and shows strong homology to the DNA-binding domain of the NtrC transcription factor (Bishop et al. 2002; Morett and Bork 1998). Transcription of the *fis* gene is maximal in the early stages of exponential growth and FIS plays an important role in boosting the expression of genes that encode components of the translational machinery of the cell (Appleman et al. 1998; Ball and Johnson 1991; Hirvonen et al. 2001; Osuna et al. 1995). FIS binds to the major groove of the DNA using a helix-turn-helix (HTH) motif that interacts with A+T-rich sites that match a weak consensus sequence (Hancock et al. 2016). The protein uses an induced fit binding mechanism that compresses the minor groove between those parts of the major groove that accommodate the HTH motifs of the two subunits (Figure 1.18) (Hancock et al. 2016; Stella et al. 2010). This creates a bend in the DNA of 65° according to FIS-DNA co-crystal structure data (Stella et al. 2010) with bends of up to 90° also being reported (Kostrewa et al. 1992; Pan et al. 1996).

Transcription factors that introduce bends into DNA can facilitate additional contacts between DNA (including proteins bound to that DNA) located upstream of the promoter and bound RNA polymerase, increasing the efficiency of transcription initiation (Huo et al. 2006; Rivetti et al. 1999; Verbeek et al. 1991). FIS acts as a 'conventional' transcription factor at some promoters, making protein–protein contacts with RNA polymerase (Bokal et al. 1997) and its DNA-bending activity has the potential to enhance the efficiency of the early stages of the transcription process. FIS can also influence promoter function without the need to contact RNA polymerase. The *leuV* operon consists of three genes that encode three of the four $tRNA_1^{Leu}$ isoreceptors and its promoter is under the positive control of FIS. The single binding site for the FIS protein upstream of the *leuV* promoter is located in a DNA segment that is prone to becoming single-stranded under the torsional stress imposed by negative supercoiling. This phenomenon is known as supercoiling-induced DNA duplex destabilisation, SIDD (Benham 1992, 1993). Binding of the FIS protein to its site within the SIDD element displaces the tendency towards duplex destabilisation to the nearest susceptible site, in this case, the *leuV* promoter – assisting in the formation there of an open transcription complex (Opel et al. 2004). This mechanism is not peculiar to FIS and has been demonstrated for the IHF NAP too (Sheridan et al. 1998). It is likely to be used at many other promoters and represents an under-researched aspect of the link between NAP binding, DNA topology, and promoter activation.

FIS has also been shown to create a nucleoprotein complex at promoters with a series of FIS-binding sites that stabilise the topological state of the DNA in ways that favour transcription initiation (Rochman et al. 2004). Many of these promoters express genes that encode components of the translational apparatus, such as ribosomal proteins, tRNA, and rRNA (Champagne and Lapointe 1998; Newlands et al. 1992; Nilsson et al. 1990). Increased translation capacity is necessary to support rapid bacterial growth, so the stimulatory role of FIS during the lag-to-log phase of the growth cycle is important. Consistent with this is the observation that while mutants that lack the FIS protein remain viable, they display reduced competitive fitness when grown in co-culture with their otherwise isogenic wild-type parent (Schneider et al. 1997).

1.39 FIS and the Stringent Response

Stable RNA (tRNA and rRNA) genes that are stimulated by FIS are subject to control by the stringent response (Condon et al. 1995b; Potrykus and Cashel 2008). Here, an intracellular signal known as an 'alarmone' interferes with the ability of RNA polymerase to transcribe a subset of genes, including the stable RNA genes. The alarmone is guanosine tetraphosphate (ppGpp) or pentaphosphate (pppGpp) and it is synthesised in response to a build-up of uncharged tRNA molecules and the interaction of the RelA protein with stalled ribosomes (Brown et al. 2016; Hauryliuk et al. 2015; Richter 1976) (see Section 6.18 for a more complete description of the stringent response). This accumulation results from a shortage of amino acids to charge the tRNAs and is an indication that the translational capacity of the cell exceeds demand. Hence the feedback loop that shuts down the transcription of genes involved in the production of ribosomes and other parts of the translational apparatus. The stringent response also affects DNA synthesis and mRNA translation both negatively and directly, while stimulating the transcription of genes outside the stringently regulated group (Ferullo and Lovett 2008; Haugen et al. 2006; Paul et al. 2005). What distinguishes the members of the two groups? One important factor is the possession by stringently regulated promoters of a discriminator sequence consisting of a G+C-rich DNA between its −10 and +1 elements (Figure 1.19) (Lamond and Travers 1985; Mizushima-Sugano and Kaziro 1985; Travers 1980; Travers et al. 1986; Zacharias et al. 1989). The discriminator is an effective barrier to open complex formation, possibly due to the extra hydrogen bonding between DNA strands consisting of G+C-rich sequences. Negative supercoiling of the DNA has a stimulatory effect on the promoters of stable RNA genes and this may assist with the melting of the recalcitrant discriminators when negative supercoiling is available (Schneider et al. 2000). However, in bacteria experiencing low metabolic flux (e.g. those in lag phase or stationary phase) this stimulatory influence is absent and the resulting relaxation of the DNA template, combined with the negative influences of the (p)ppGpp alarmone and the DksA protein, cooperate to repress transcription of stable RNA genes (Potrykus and Cashel 2008; Schneider et al. 2000). Genes subject to stimulation by (p)ppGpp and DksA also possess a discriminator, but in these cases this element is an A+T-rich DNA sequence (Figure 1.19) (Gummesson et al. 2013).

1.40 FIS and DNA Topology

The involvement of FIS in SIDD-based regulatory mechanisms has already been described. The promoter of the *dusB-fis* operon is subject to transcriptional stimulation by DNA negative supercoiling (Schneider et al. 2000) in addition to being auto-repressed by FIS and controlled negatively by the stringent response (Ninnemann et al. 1992). At a global level, the FIS protein is intimately associated with the general management of DNA topology in the bacterial cell. It represses the transcription of the *gyrA* and *gyrB* genes in *E. coli* (Schneider et al. 1999) and *Salmonella* (Keane and Dorman 2003) and has a complicated relationship with the promoters of the *topA* gene, where its influence is conditional on factors such as oxidative stress (Weinstein-Fischer and Altuvia 2007). Although *E. coli* and *Salmonella* have distinct DNA supercoiling set points, with *Salmonella* DNA being more

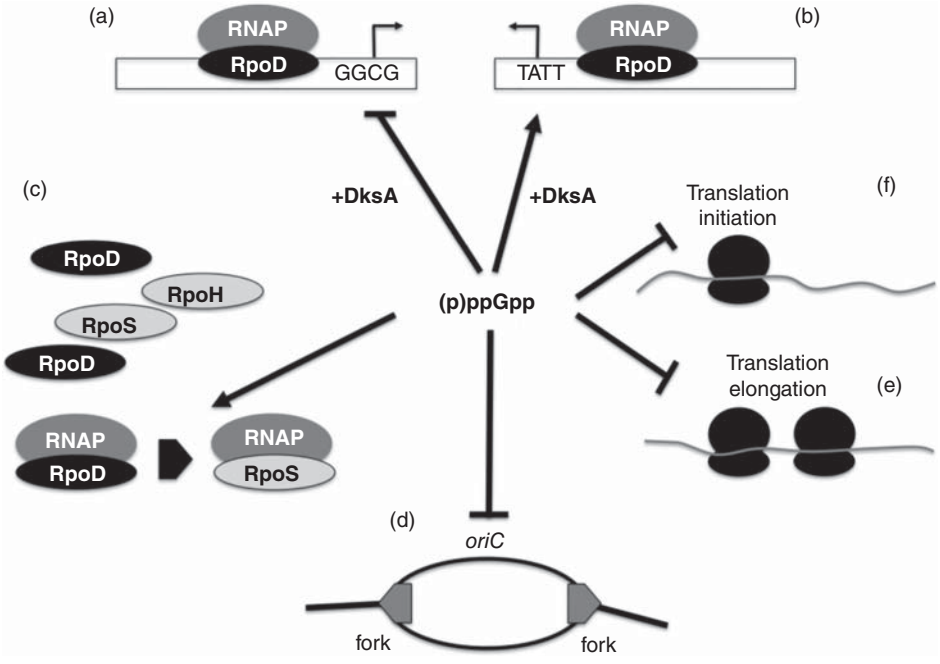

Figure 1.19 The multifaceted stringent response. A summary is shown of the processes that are inhibited or enabled by the alarmone (p)ppGpp. (a) (p)ppGpp and DksA affect a stringently regulated promoter that contains a G+C-rich discriminator sequence negatively. Genes encoding rRNA or tRNA are in this category. (b) In contrast, (p)ppGpp and DksA affect a promoter with an A+T-rich discriminator positively. Genes involved in amino acids biosynthesis are in this category. (c) The (p)ppGpp alarmone biases the selection of sigma factors by RNA polymerase away the RpoD housekeeping sigma factor and towards sigma factors that are required for various stress responses. (d) The initiation of chromosome replication is inhibited by (p)ppGpp. (e) Translation initiation and translation elongation are affected negatively because (p)ppGpp has an inhibitory influence on Initiation Factor 2 (IF2) and on the translation elongation factor, EF, respectively.

relaxed than in *E. coli* (Champion and Higgins 2007), this distinction is dependent on the presence of FIS (Cameron et al. 2011). Thus, the pattern of expression of the topoisomerases responsible for negative supercoiling (DNA gyrase) and relaxation (Topo I) of DNA is modulated by FIS. The activities of these topoisomerases is also affected by FIS because the protein influences their access to DNA by binding to it: since FIS prefers to bind to DNA with intermediate levels of negative supercoiling it acts to preserve this topological form (Schneider et al. 1997; Cameron and Dorman 2012).

In order to exert its influence on DNA topology, FIS must be present in the cell. This restricts its influence to the early stages of exponential growth when it is most abundant (Schneider et al. 1997). An exception has been discovered in bacteria growing under micro-aerobic conditions: here FIS levels are sustained into the stationary phase of growth (Cameron et al. 2013; O Cróinín and Dorman 2007). This may be of special significance in environments such as the mammalian gut epithelial surface where FIS-dependent gene expression is required for colonisation and invasion (Falconi et al. 2001; Kelly et al. 2004; Prosseda et al. 2004; Rossiter et al. 2015).

1.41 Ferritin-Like Dps and the Curved-DNA-binding Protein CbpA

While FIS is associated with the early stages of rapid exponential growth, the Dps (DNA-binding protein from starved cells) and CbpA proteins exhibit the polar opposite expression pattern and are seen predominantly in stationary phase (Ali Azam and Ishihama 1999). Both are NAPs and Dps has been studied in the most detail. CbpA expression is prevented during exponential growth by the FIS protein. FIS, which is abundant in this period of the growth cycle, binds and represses the activity of an RpoD-dependent promoter that is located in a gene (*yccE*) adjacent to *cbpA* that is partly responsible for *cbpA* transcription in stationary phase. A second promoter immediately upstream of *cbpA* depends on RpoS, a sigma factor that is only available in stationary phase or in stressed cells (Figure 1.20) (Chintakayala et al. 2013). CbpA forms dimers in solution and aggregates following binding to DNA, forming nucleoprotein complexes similar to those produced by the Dps NAP (Cosgriff et al. 2010). The preferred DNA targets of CbpA are A+T-rich and intrinsically curved; this protein has a marked preference for binding within the Ter macrodomain of the chromosome, a zone of high DNA curvature and with a high A+T content (Chintakayala et al. 2013). CbpA binds at the minor groove of DNA and *cbpA* mutants display aberrant DNA topology, observations that are consistent with a role in organising the DNA in the Ter macrodomain during stationary phase (Chintakayala et al. 2013, 2015).

Dps is dodecameric in *E. coli* and has ferritin-like properties (Grant et al. 1998). This protein accumulates in stationary phase bacteria and was found initially to protect the genomic DNA from oxidative damage (Almirón et al. 1992; Martinez and Kolter 1997). It does not impede transcription, despite being an abundant DNA-binding protein (Janissen et al. 2018). Dps was subsequently discovered also to afford protection against gamma radiation, ultraviolet light, copper and iron toxicity, heat stress, and pH shock (Algu et al. 2007; Jeong et al. 2008; Nair and Finkel 2004). It also protects DNA from cleavage by restriction enzymes (Janissen et al. 2018).

Although Dps is usually grouped with the NAPs (Ali Azam and Ishihama 1999), its relationship with DNA has been difficult to determine with precision. This protein can form a co-crystal with DNA, perhaps accounting for its ability to protect the chromosome from damage in stressed cells (Wolf et al. 1999). Through the application of SELEX (systematic evolution of ligands by exponential enrichment) to *E. coli*, a DNA sequence has been identified that seems to contain the elements of a Dps-binding site (Ishihama et al. 2016). A closely related motif has been detected in *E. coli* by chromatin immunoprecipitation on chip (Antipov et al. 2017). These Dps-binding sites overlap with those of other NAPs, leading to speculation that Dps supplies the genome architectural functions of those proteins (such as FIS) that are no longer expressed as the bacterium enters stationary phase (Antipov et al. 2017). Alternatively, Dps binding and the binding of other NAPs, such as IHF, may alternate depending on the environmental conditions that accompany entry of the bacterium into stationary phase (Lee et al. 2015).

The FIS protein plays a key role in controlling the expression of the *dps* gene during the growth cycle (Grainger et al. 2008). This gene is transcribed from a single promoter that is recognised by both the RpoD- and the RpoS-containing forms of RNA polymerase (Altuvia et al. 1994). Three NAPs are involved in the regulation of *dps* transcription: FIS, H-NS,

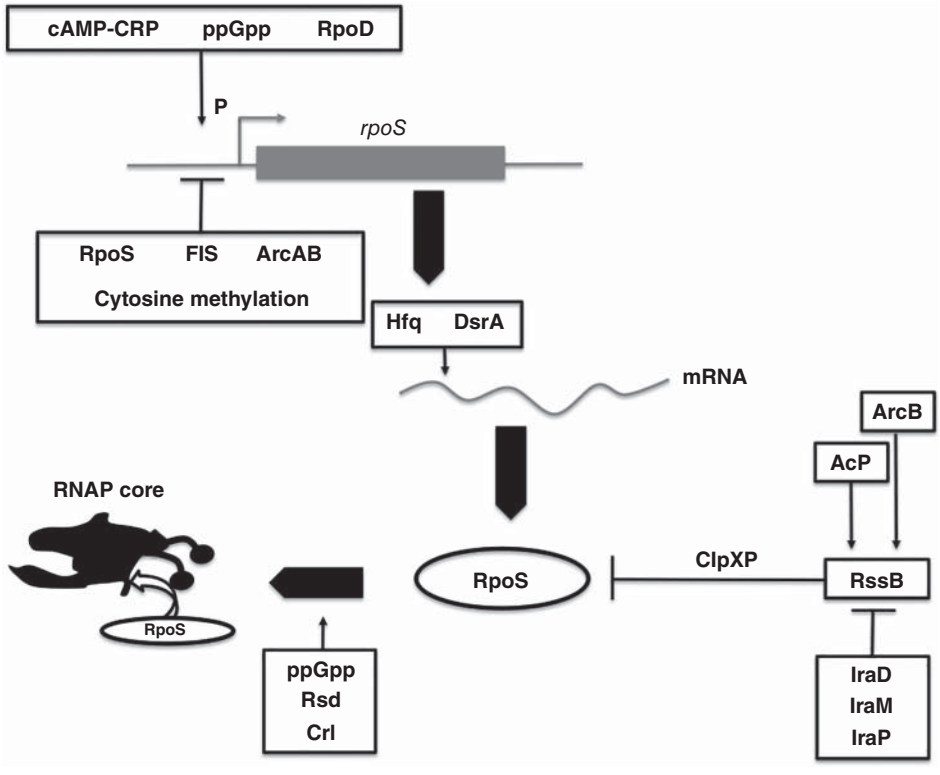

Figure 1.20 The stress and stationary phase sigma factor, RpoS. The *rpoS* gene is influenced at the transcriptional level by several factors. The glucose-sensitive cAMP-CRP complex and the stringent-response signal, ppGpp, act at the RpoD-dependent promoter to enhance *rpoS* transcription; production of RpoS is enhanced in mutants deficient in cytosine methylation (see Kahramanoglou et al. 2012). It is important to note that *rpoS* is expressed under all growth conditions and that the principal regulatory effects are imposed at the level of RpoS protein stability; rapidly growing bacteria have few copies of RpoS and non-growing bacteria have many. The expansion of the population of RpoS proteins occurs in bacteria when growth is slowed or stopped due to stress. The stress can be physical or chemical in nature. Once transcribed, RpoS mRNA is translated poorly due to the formation of secondary structures that sequester the translation initiation signals. These stem-loops are eliminated by the DksA sRNA that binds to the 5′ end of the mRNA in the presence of the Hfq RNA chaperone protein. DksA also controls the translation of the *hns* transcript, albeit negatively due to sequestration of the translation initiation signals. The RpoS protein is degraded by the ClpXP protease. Proteolytic cleavage of RpoS is enhanced by the adaptor protein, RssB. RssB activity is in turn modulated negatively by the sRNAs IraD, IraM, and IraP in response to stresses that impede the growth of the bacterium. In *Salmonella,* IraM is called RssC. RssB activity is controlled in response to changes to oxygen supply (ArcB) and carbon levels (Acp). The sigma factor must compete with RpoD and other sigma factors for access to the core RNA polymerase and it is assisted in doing so by ppGpp, Crl, and the anti-sigma factor, Rsd.

and IHF. IHF and the OxyR transcription factor activate the *dps* promoter in association with RpoD in bacteria experiencing oxidative stress; in stationary phase the same promoter is utilised by RNA polymerase containing RpoS (Altuvia et al. 1994). During exponential growth, FIS holds RNA polymerase containing RpoD at the promoter while H-NS excludes the RpoD form of the RNA polymerase holoenzyme from the same promoter. The transcriptionally inert FIS-RpoD-RNA-polymerase complex prevents entry of the RpoS-containing RNA polymerase holoenzyme. Since FIS levels decline to negligible values as the bacterium enters stationary phase, this negative control is no longer exerted, and as H-NS is unable to impede the activity of the RpoS-containing form of RNA polymerase, *dps* transcription can begin (Grainger et al. 2008).

1.42 The H-NS Protein: A Silencer of Transcription

Throughout the 1980s, the gene that encodes the H-NS NAP was discovered and rediscovered by investigators working independently of each other because this protein is involved in controlling the expression of so many different components of the bacterium. One of the consequences of the broad influence of H-NS is that the gene that encodes it has been given many names, such as *bglY*, *osmZ*, *pilG*, *virR*, and *dxdR*, among others. In each case, the name linked a mutation in the gene, now referred to universally as *hns*, to a specific H-NS-dependent system in the cell such as beta-glucoside uptake and utilisation (*bglY*), pilus expression (*pilG*), the osmotic stress response (*osmZ*), expression of a virulent phenotype in the pathogen *Shigella* (*virR*), or thermo-regulated adhesin expression in *E. coli* (*drdX*) (Defez and de Felice 1981; Higgins et al. 1988; Göransson et al. 1990; Maurelli and Sansonetti 1988; Spears et al. 1986). The *hns* gene is located in the Ter macrodomain of the chromosome, close to the *topA* gene, and early experiments revealed a connection between some *hns* alleles and alterations in the DNA topology of reporter plasmids in those strains (Dorman et al. 1990; Higgins et al. 1988). The finding that H-NS binding to DNA impedes access to that DNA by DNA gyrase may explain these observations (Sutormin et al. 2019). H-NS can influence transposition and site-specific recombination as well as transcription (Corcoran and Dorman 2009; Liu et al. 2011; Whitfield et al. 2009) and it has effects at the level of mRNA translation too (Park et al. 2010).

The H-NS protein is a small, abundant NAP that is produced at all stages of the growth cycle (Ali Azam and Ishihama 1999; Dorman 2013; Free and Dorman 1995). It binds to A+T-rich DNA and has been described as having a preference for DNA with intrinsic curvature (Yamada et al. 1991). These features are commonly associated with transcriptional promoters, allowing H-NS to target large numbers of genes for repression, even if the functions of the gene products are not related in any obvious way. The protein is almost always a repressor, allowing it to silence transcription of a large subset of the genes in the genome (Figure 1.21).

The very low base sequence requirement, as opposed to base content requirement, of H-NS for its binding sites allows this protein to interact with DNA on the basis of DNA shape rather than nucleotide sequence. Its reliance on indirect readout for DNA-binding-site recognition underlies the contribution made by H-NS to silencing the transcription of genes that have been acquired by HGT in Gram-negative organisms (Dorman 2007, 2014a). This xenogeneic silencing is hypothesised to allow potentially

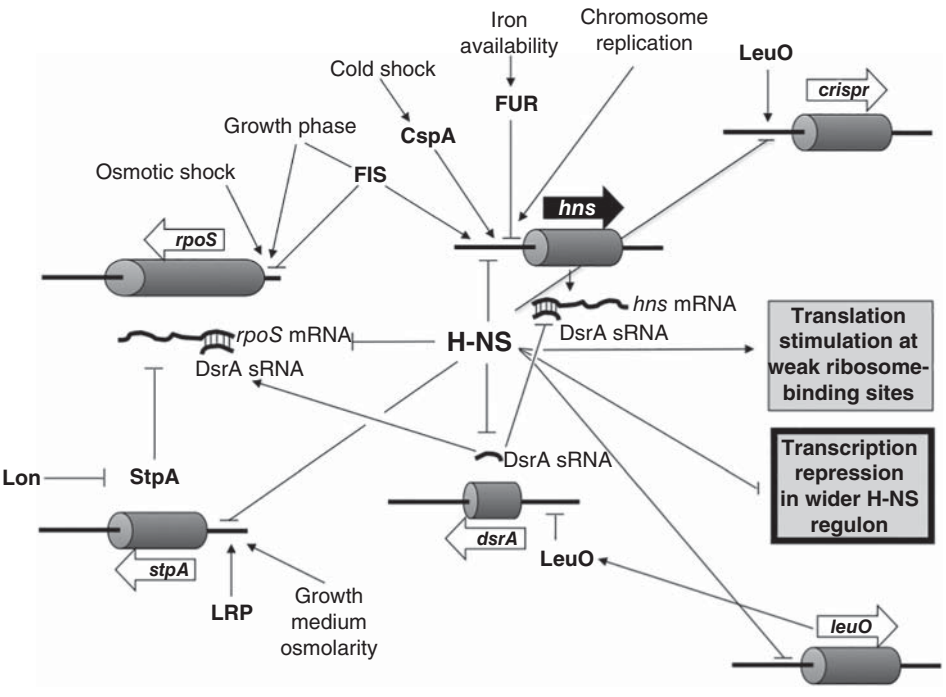

Figure 1.21 The vast H-NS regulon. The H-NS protein controls the expression of hundreds of genes in the pan genome, the accessory genome, and the core genome. Its own gene is also subject to complex control at the transcriptional and posttranscriptional levels. Among the factors influencing *hns* transcription positively are chromosome replication, the growth-phase-dependent FIS protein, and the cold-shock regulatory protein CspA; it is auto-repressed by its own gene product and repressed by the iron-binding Fur protein, and translation of H-NS's own mRNA is inhibited by the DsrA sRNA in an Hfq-dependent manner. H-NS, in turn, inhibits DsrA expression, resulting in a positive effect throughout the DsrA regulon, including production of the RpoS sigma factor. H-NS silences *crispr* transcription, an effect that is antagonised by the LeuO transcription factor, with the *leuO* gene in turn being H-NS-repressed. Like StpA (whose gene H-NS represses) the H-NS protein can act at the level of RNA, for example improving the translation of the maltose regulatory gene by repositioning ribosomes so that translation can proceed. The H-NS paralogue, StpA, is a substrate for Lon-mediated proteolysis but dimerisation with H-NS protects StpA from this fate (H-NS is not degraded by Lon). StpA can act as a backup for H-NS and its expression is governed by an independent set of cues, such as the LRP protein and bacterial growth phase (H-NS is present at all stages of growth). The H-NS regulon includes genes involved in bacterial virulence and whose expression is controlled by pH, osmotic stress, temperature, and other environmental influences. Its (usually negative) influence is overcome by an impressive array of mechanisms that link the expression of the H-NS target genes to information relevant to the infection process (see Stoebel et al. 2008b). Arrows represent positive regulatory inputs and negative ones are indicated by 'T' symbols.

harmful genes to be imported into the genome without compromising the competitive fitness of the bacterium (Dorman 2004, 2007; Lucchini et al. 2006; Navarre et al. 2006; Oshima et al. 2006). It should be pointed out that although H-NS displays low nucleotide sequence specificity at its binding sites, a preferred sequence has been identified at the *proU* operon (Bouffartigues et al. 2007; Lang et al. 2007). This operon encodes a transport

system for glycine-betaine, a modified amino acid that protects macromolecules in bacteria from the deleterious effects of water loss following osmotic shock (Cairney et al. 1985). The *proU* promoter is induced by osmotic up-shock in the presence of potassium ions and is subject to repression by the H-NS protein (Gowrishankar and Manna 1996; Higgins et al. 1988; Sutherland et al. 1986). It contains two matches to an A+T-rich consensus sequence that contains the very flexible TpA di-nucleotide step, possibly important in specifying local DNA curvature (Bouffartigues et al. 2007; Lang et al. 2007). This motif may represent an evolutionary step towards a more specific binding site for the H-NS protein.

H-NS forms dimers and these can link together to form higher-order oligomers (Arold et al. 2010; Shahul Hameed et al. 2018) (Figure 1.22). The monomer consists of an amino-terminal dimerisation domain that is connected by a linker region to a carboxyl-terminal nucleic-acid-binding domain (Dorman et al. 1999). The dimer can bridge different parts of the same DNA molecule or link separate DNA molecules together, activities that have been confirmed *in vitro* using single molecule methods and atomic force microscopy (Dame et al. 2000, 2005, 2006). The protein displays two DNA-binding modes, one involving bridging of two DNA duplexes (or two sections of the same DNA duplex) and the other involving polymerisation of H-NS along a single DNA duplex (Figure 1.23) (Corcoran and Dorman 2009; O'Gara and Dorman 2000; Lim et al. 2012). Polymerisation, with or without bridging, occurs following initial binding of H-NS to a

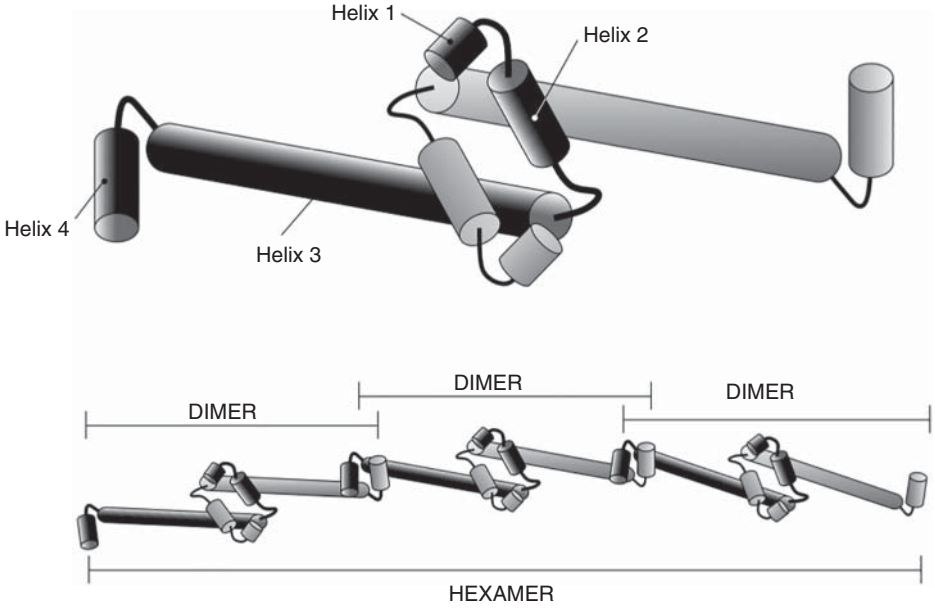

Figure 1.22 The dimerisation and oligomerisation of H-NS. The amino-terminal domain of H-NS is shown, with its four alpha helices represented by linked cylinders. The carboxyl terminal domain with its nucleotide binding activity and the linker region that connects this domain to the amino-terminus are omitted. Interlinkage of alpha helices 1 and 2 of one monomer with the corresponding parts of another allows dimer formation. Linking alpha helices 4 allows two dimers to form a tetramer, and this process can continue indefinitely to produce an H-NS polymer. For further information, see Arold et al. (2010).

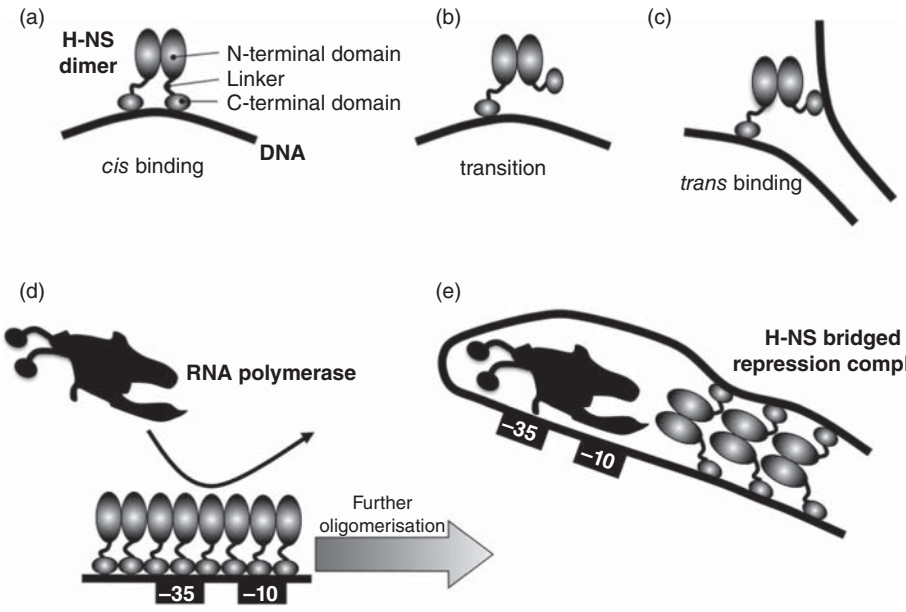

Figure 1.23 The DNA-binding modes of H-NS. The H-NS monomer is shown as two linked ovals with one oval representing the amino-terminal dimerisation/oligomerisation domain and another the carboxyl-terminal domain with the nucleic acid binding activity. The functional domains are connected by a flexible linker. (a) An individual dimer is shown in a *cis*-binding mode, with both DNA-binding modules on the same DNA molecule. (b) It then undergoes a transition to a *trans*-binding mode in which it attaches simultaneously to two DNA molecules or to two separate sections of the same DNA molecule. (c) This *trans*-binding mode is the basis for H-NS-mediated DNA bridging and the transition between the two modes is toggled by Mg^{2+} concentrations. Either mode can silence transcription. (d) In one case polymerisation of H-NS along DNA excludes RNA polymerase from a promoter. (e) In the other case, H-NS bridges DNA from upstream and downstream of the transcription start site, blockading the promoter so that RNA polymerase is either excluded or becomes trapped in the initiation stages of transcription.

preferred nucleation site (Badaut et al. 2002). *In vitro* data indicate that changes in the concentration of magnesium ions can toggle H-NS between these two binding modes (Liu et al. 2010), though the significance of this effect in the bacterial cell is unknown. In addition to the influence of divalent cations, the toggling of H-NS between these two binding modes is modulated by co-regulator proteins such as Hha, YdgT, and YmoA, which resemble the dimerisation domain of H-NS (van der Valk et al. 2017). By coating the DNA surface, H-NS can exclude other proteins or protein complexes, including RNA polymerase (Figure 1.23). However, the negative influence of H-NS on transcription can be overcome in a variety of ways (Dorman and Kane 2009; Stoebel et al. 2008b).

H-NS displacement can be assisted by properties of the DNA to which it binds. In the genetic switch that controls the expression of the major virulence genes of *Shigella flexneri*, a region of curved DNA undergoes a thermally induced reorganisation that displaces H-NS, leading to derepression of the *virF* regulatory gene (Prosseda et al. 2004). H-NS may also use an internal conformational change in response to rising temperature to disrupt its transcription-silencing complexes on DNA (Shahul Hameed et al. 2018). However, most

of the anti-H-NS mechanisms that have been described involve roles for a second protein. Also in *S. flexneri*, the VirB DNA-binding protein derepresses the promoters of the principal virulence gene operons by binding and wrapping DNA, making H-NS binding and bridging untenable (Turner and Dorman 2007). This form of antagonism can be transferred to other H-NS-repressed promoters simply by appropriate placement there of the VirB-binding site (Kane and Dorman 2011). The similarity of VirB to proteins involved in the partitioning of plasmids with protein-dependent active partitioning systems (Section 1.15) shows that DNA-binding molecules can be co-opted from other purposes to oppose the transcription silencing imposed by H-NS (Adler et al. 1989; Kane and Dorman 2012; Turner and Dorman 2007). This suggests that simple genetic switches based on transcription silencing and anti-silencing can arise quickly in the evolution of gene regulatory circuits.

The DNA-binding and bridging activity of H-NS may lend itself to nucleoid organisation as well as to transcription control (Dorman 2013; Japaridze et al. 2017). The distribution pattern of H-NS binding sites around the chromosome appears to be periodic and genetic experiments have identified the *hns* gene as being important for the formation of chromosomal domain boundaries (Hardy and Cozzarelli 2005). Experiments with super resolution imaging and chromosome conformation capture have produced some data that indicate a central role for H-NS in nucleoid architecture (Wang et al. 2011) while other 3C experiments seem to rule out a role for this protein in the organisation of long range interactions in the nucleoid (Cagliero et al. 2013). Other HiC experiments show that H-NS promotes DNA interactions in the chromosome at short ranges, but not the long-range interactions between H-NS-regulated genes and operons that were detected by Wang et al. (2011) (Lioy et al. 2018). Work with HiC in *Caulobacter* has implicated the high-frequency transcription of long genes as playing a role in boundary formation and maintenance, independent of any effect of translation (Le and Laub 2016). This work suggests that the act of transcribing a long gene inhibits interactions between the DNA regions that flank that gene, causing this long gene to act as a boundary element between domains, a process that may become modulated by H-NS-mediated transcription silencing.

1.43 StpA: A Paralogue of H-NS

The ratio of H-NS to its genomic binding sites appears to be important for the competitive fitness of the bacterial cell. Bacteria that gain extra copies of the *hns* gene through the introduction of multicopy recombinant plasmids that encode this NAP gain in their capacity to replicate themselves (C.J. Dorman, unpublished). The model organisms *E. coli* and *Salmonella typhimurium* encode the StpA protein, a second H-NS-like molecule. It shares many features with H-NS and has been described as an RNA chaperone (Doetsch et al. 2010). StpA can substitute for H-NS and it can form heterodimers with its paralogue (Johansson et al. 2001; Leonard et al. 2009; Sonden and Uhlin 1996; Zhang et al. 1996 Refs). The *stpA* gene is expressed maximally during exponential growth, perhaps to provide an auxiliary supply of H-NS-like protein at a point in the growth cycle where the number of H-NS binding sites is most numerous (Deighan et al. 2003; Free and Dorman 1997). In this context, it is interesting to note that the transcription of the *hns* gene is linked positively to chromosome replication (Free and Dorman 1995).

1.44 H-NS Orthologues Encoded by Plasmids and Phage

Copies of genes encoding H-NS-like proteins occur naturally on plasmids, including large, self-transmissible plasmids (Shintani et al. 2015). These molecules have A+T-rich DNA and may impose a competitive fitness cost on bacteria that receive them in conjugation by diverting H-NS from A+T-rich DNA sites on the chromosome, leading to disruption of the gene expression pattern in the bacterium. By providing its own supply of H-NS activity, the plasmid can avoid this regulatory disturbance and its associated impact on competitive fitness (Doyle et al. 2007). The R27 plasmid, originally detected in *Salmonella*, and more recently in *S. flexneri* 2a 2457T, encodes the H-NS orthologue Sfh (Beloin et al. 2003a; Deighan et al. 2003). The two proteins exhibit considerable overlaps in their binding sites on the bacterial chromosome. When H-NS is present, Sfh is restricted to a subset of the sites that it can occupy when H-NS is removed by inactivation of the *hns* gene (Dillon et al. 2012). These observations are consistent with Sfh acting as an auxiliary to H-NS, a role that it shares with StpA. The expression patterns of the three proteins are instructive in this regard: H-NS is present at a constant level per chromosome throughout the growth cycle, StpA is expressed when the cells are in exponential phase, and Sfh appears at the beginning of stationary phase (Deighan et al. 2003).

H-NS-like paralogues encoded by self-transmissible plasmids (Shintani et al. 2015) and bacteriophage (Skennerton et al. 2011) can downregulate the expression of CRISPR-*cas* loci, allowing the mobile genetic element to evade the host immune system (Dillon et al. 2012; Lin et al. 2016; Medina-Aparicio et al. 2011; Pul et al. 2010). The LysR-like transcription factor LeuO overcomes H-NS-mediated repression of the CRISPR-*cas* locus, but the *leuO* gene is itself silenced by H-NS, and its paralogues (Dillon et al. 2012; Medina-Aparicio et al. 2011; Pul et al. 2010). Stochastic upregulation of *leuO* transcription may provide a mechanism for overcoming silencing of the immunity function in some cells in the population that encounter plasmid or bacteriophage invaders.

1.45 H-NSB/Hfp and H-NS2: H-NS Homologues of HGT Origin

Genes encoding proteins related to H-NS are found in pathogenicity islands that have been acquired by HGT. Hfp/H-NSB is an H-NS-like protein that is expressed by a gene in the *serU* pathogenicity island in the chromosome of uropathogenic *E. coli* (Müller et al. 2010; Williams and Free 2005). It can form heterodimers with H-NS and may modulate its activity in helping UPEC to adapt to environmental conditions encountered during the infection process (Dorman 2010; Müller et al. 2010). The H-NSB/Hfp protein is also encoded by a chromosomal island in *E. carotovora* but is absent from an island in enteropathogenic *E. coli* (EPEC) that is closely related to the *serU* island of UPEC (Williams and Free 2005). This association of a gene encoding an H-NS-like protein with a former mobile genetic element is reminiscent of similar associations with elements that are currently mobile such as self-transmissible plasmids and bacteriophage (Section 1.44). The absence of the *hnsB* gene from the version of the *serU* island that is present in EPEC is intriguing, given that *hnsB* is both present and expressed in the corresponding island in UPEC. Perhaps the gene had performed its role once the EPEC island was established in the chromosome and it

was subsequently lost in the absence of a selective pressure to keep it? If so, the different environmental circumstances experienced by EPEC and UPEC seem to have selected for retention of *hnsB* by the latter organism.

Enteroaggregative *E. coli* (EAEC) strains express, in addition to H-NS and StpA, an H-NS2 protein that is closely related to H-NS. H-NS2 behaves somewhat like H-NS when the latter is in a complex with Hha: it targets A+T-rich genes that have been acquired by HGT and silences them transcriptionally (Prieto et al. 2018). The amino acid sequence of H-NS2 is similar to those of H-NSB and Hfp, but differs from them in a number of respects. It does not exhibit the sensitivity to proteolytic turnover that is a characteristic of these H-NS homologues and StpA (Prieto et al. 2018). It is possible, and plausible, that H-NS2 and other 'third homologues' could form heteromeric complexes with H-NS or StpA that have distinct activities from those of the homodimers. Certainly, H-NS heterodimers with StpA have properties that are distinct from those of the homodimers (Johansson et al. 2001; Leonard et al. 2009), so expanding the number of interacting partners may represent a way of modulating NAP function (Beloin et al. 2003a; Sonden and Uhlin 1996; Zhang et al. 1996).

1.46 A Truncated H-NS-Like Protein

The *serU* island in UPEC that encodes H-NSB/Hfp also encodes a protein that resembles a truncated H-NS. This is H-NST and its gene is tightly linked to the *hnsB* gene in the chromosomal island. H-NST consists of the first 80 amino acids of H-NS and the corresponding island in EPEC encodes a closely related protein; EAEC also encodes a relative of H-NST (Williams and Free 2005). H-NST from UPEC can form a heterodimer with H-NS and it can antagonise its activity as a transcription silencer. The corresponding protein from EPEC is much attenuated in its ability to interact with H-NS and to attenuate its biological activity: a key substitution at residue 16 of the amino acid sequence seems to be responsible for this difference between the UPEC and EPEC H-NSTs (Williams and Free 2005).

The action of H-NST recalls that of the gene 5.5 protein that is encoded by bacteriophage T7. Like H-NST, the gene 5.5 protein co-purifies with H-NS and antagonises the transcription silencing activity of H-NS, presumably to the benefit of the phage (Liu and Richardson 1993). H-NST and the gene 5.5 protein resemble one another in size and mode of action structure but not in amino acid sequence (Williams and Free 2005). The ability of H-NST from EPEC to inhibit H-NS activity has been exploited to explore the H-NS$^-$ phenotype of *Yersinia enterocolitica,* a bacterium where H-NS is essential (Baños et al. 2008). The essential nature of H-NS in *Y. enterocolitica* probably reflects the absence of a paralogous protein such as StpA that can offset the severe phenotype associated with the loss of H-NS. Expressing EPEC H-NST ectopically in *Y. enterocolitica* titrates the transcription silencing of H-NS, revealing that it has similar effects on global gene expression patterns to those seen in other Gram-negative bacteria such as *E. coli* (Baños et al. 2008).

1.47 Hha-like Proteins

H-NST's close similarity to the oligomerisation domain of H-NS and its ability to form heteromeric complexes with H-NS is superficially similar to the relationship of H-NS to

the Hha family of proteins. Found only in the *Enterobacteriaceae*, these too mimic the oligomerisation domain of H-NS in their structure but unlike H-NST, their interaction does not interfere with H-NS-mediated transcription silencing. Instead it channels the negative influence of H-NS to certain target promoters (Baños et al. 2009; Madrid et al. 2007).

Y. enterocolitica possesses just one housekeeping Hha-like protein (called Hha), in contrast to other model organisms like *E. coli* and *Salmonella* that have both Hha and a closely related paralogue, YdgT. However, pathogenic strains of *Yersinia* express YmoA (*Yersinia* modulator) from a chromosomal locus. YmoA is a founding member of the Hha protein family that regulates virulence genes negatively in *Yersinia* spp. (Cornelis et al. 1991; de la Cruz et al. 1992). It does this by forming a complex with H-NS in which H-NS provides the DNA-binding activity (Ellison and Miller 2006b). YmoA potentiates the transcription repression activity of H-NS, targeting virulence gene promoters in *Yersinia* (Ellison and Miller 2006b). It shares this property with Hha itself and with the Hha paralogue YdgT (Nieto et al. 2002; Starke and Fuchs 2014). YdgT is a paralogue of Hha and shares with Hha an ability to form heteromeric complexes with H-NS and StpA (Paytubi et al. 2004).

YmoA is structurally closely related to Hha, it mimics the oligomerisation domain of H-NS (McFeeters et al. 2007), and it is turned over by Lon- and ClpXP-mediated proteolysis (Jackson et al. 2004). YmoA and Hha each interact with an H-NS dimer, stabilising the transcription-silencing complex at target promoters (Cordeiro et al. 2015).

In the case of *Salmonella*, Hha-like proteins target H-NS to the major virulence genes in the SPI1 and SPI2 pathogenicity islands and on the *Salmonella* virulence plasmid, pSLT (Silphaduang et al. 2007; Vivero et al. 2008). They can also influence the DNA-binding mode of H-NS and whether this protein forms polymers along DNA or creates bridges between different segments of DNA (van der Valk et al. 2017). Hha and YdgT direct H-NS towards horizontally acquired genes, causing them to be silenced preferentially (Aznar et al. 2013). In *E. coli*, Hha/YdgT also targets horizontally acquired genes via H-NS/StpA binding, together with genes involved in the osmotic and carbon starvation stress responses (Ueda et al. 2013). Genes encoding Hha-like proteins also occur on self-transmissible plasmids and their products can interact with H-NS. For example, the virulence plasmid in the pathogenic *E. coli* strain O157:H7 encodes a form of Hha that directs H-NS to just a subset of the targets that are bound by H-NS when in a complex with the chromosomally encoded Hha protein (Paytubi et al. 2013). Plasmid-encoded H-NS proteins, such as the one expressed by the R27 self-transmissible plasmid in *Salmonella*, target genes of HGT origin in the chromosome, a task that requires the Hha helper protein when it is performed by the chromosomally encoded H-NS protein (Baños et al. 2009).

1.48 Other H-NS Homologues: The Ler Protein from EPEC

Ler is a paralogue of H-NS that controls virulence gene expression in EPEC (Bustamante et al. 2001) and Enterohaemorrhagic *E. coli*, EHEC (Hansen and Kaper 2009). The principal Ler-dependent virulence genes are located in the locus of enterocyte effacement, LEE, an A+T-rich pathogenicity island that has been acquired by HGT (Clarke et al. 2003; Nataro and Kaper 1998) (Figure 1.24). LEE encodes a type 3 secretion apparatus and its effector proteins and consists of five operons: *LEE1* to *LEE5,* with the *ler* gene being

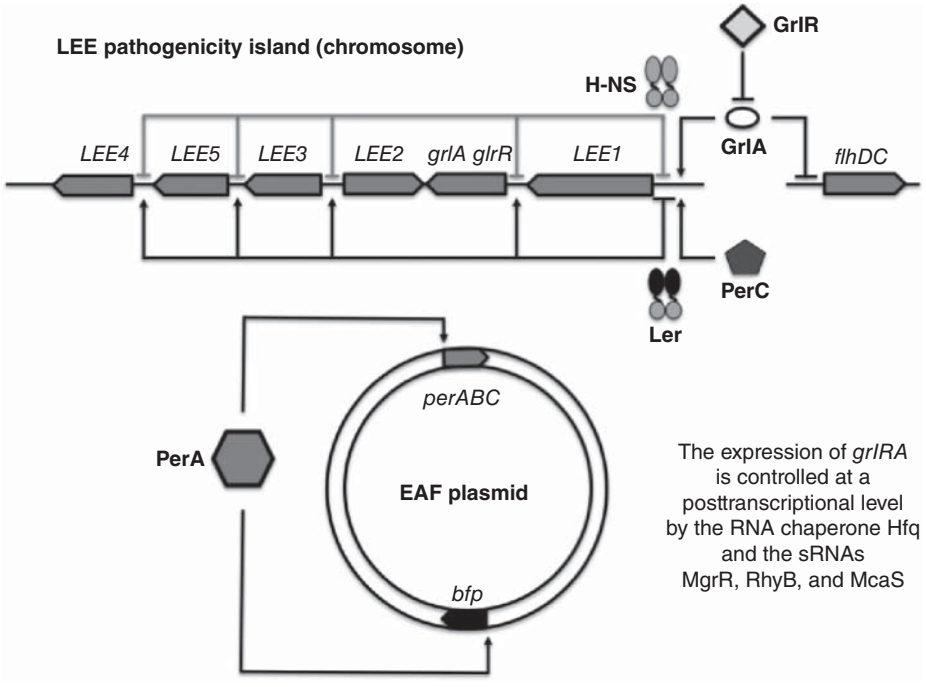

Figure 1.24 The genetic switch controlling LEE virulence gene expression in EPEC. The LEE pathogenicity island in the EPEC chromosome encodes a T3SS and its associated effector proteins for mammalian epithelial cell invasion. Transcription of the LEE operons is silenced by the H-NS protein and this is relieved by the antagonistic action of the Ler DNA-binding protein, an H-NS homologue. The *ler* gene is the first in the *LEE1* operon and its transcription is negatively autoregulated by Ler and positively controlled by GrlA (encoded by the LEE island) and the PerC protein that is expressed from the EAF virulence plasmid. Production of PerC depends on the PerA regulatory protein, which also activates the transcription of the *bfp* operon on the EAF plasmid (this operon expresses the bundle-forming pilus that attaches EPEC to its target host cells). GrlA is a negative regulator of the *flhDC* operon, the master switch for bacterial motility: when LEE is activated, the bacteria cease to be motile. GrlA activity is inhibited by the LEE-encoded GrlR protein. Ler and H-NS share a common nucleation site and a competition between these proteins determines whether or not LEE genes are expressed. The concentration of the Ler protein reflects the activities of the GrlA, PerC, Ler, and H-NS proteins at the *ler* promoter. In enterohaemorrhagic *E. coli* strains, the Hfq RNA chaperone negatively regulates *grlRA* post-transcriptionally and exerts a GrlRA-independent negative effect on LEE gene expression in stationary phase (Hansen and Kaper 2009). In EPEC, Hfq exerts its effects at *grlRA* mRNA through the sRNAs MgrR, RyhB, and McaS (see Bhatt et al. 2017).

located in *LEE1* (Elliott et al. 1998). H-NS silences transcription throughout LEE (Leh et al. 2017; Shin et al. 2012). Ler, the LEE-encoded regulator, controls LEE operon expression positively by relieving the transcriptional silencing that is imposed by the H-NS NAP in LEE (Umanski et al. 2002) and at some other loci (Bingle et al. 2014; Elliott et al. 2000).

The LEE system is under complex control that involves plasmid-encoded regulators in addition to those encoded by genes on the chromosome (Figure 1.24). The EPEC Adherence Factor (EAF) plasmid encodes Bfp type IV (bundle-forming) pili for adherence to host cells

and a *perABC* operon that is autoregulated through the PerA protein and cross-regulates the *ler* gene in *LEE1* via the PerC protein. PerC is redundant with the island-encoded GrlA regulatory protein, another positive regulator of *ler* transcription. In addition, GrlA represses expression of the flagella master regulator locus, *flhDC* (Kitagawa et al. 2011; Padavannil et al. 2013). The *grlRA* operon is the subject of posttranscriptional regulation by the Hfq RNA chaperone (Bhatt et al. 2017; Hansen and Kaper 2009). GrlR is an inhibitor of GrlA activity and it achieves this effect by direct protein–protein interaction at the GrlA HTH motif (Padavannil et al. 2013).

Upregulation of the *LEE* operons occurs in minimal medium at 37°C and transcription is repressed in EPEC cells growing in LB. Two switches operate in the system to create physiological variety. In one, the PerA protein activates the *bfp* adherence operon on the EAF plasmid directly and the *LEE* genes indirectly through PerC (Figure 1.24). Positive auto-control of *perABC* transcription by PerA is stochastic, creating sub-populations of bacteria that maintain a hypervirulent (LEE^+) phenotype even if inducing conditions are removed and a second population of non-virulent (LEE^-) cells. Allowing the culture to reach stationary phase resets the system (Ronin et al. 2017).

The second switch involves a competition between Ler and H-NS for access to a nucleation site upstream of the *LEE5* promoter (Leh et al. 2017) (Figure 1.24). Although Ler and H-NS are paralogues, they create distinct nucleoprotein complexes when they bind to DNA, with the Ler complex favouring transcription and the H-NS one causing transcriptional silencing (Leh et al. 2017). Stochastic expression of the *perABC* operon, with downstream effects via PerC on *ler* transcription, may be expected to tip the balance in the Ler/H-NS competition back and forth, leading to LEE^+ and LEE^- phenotypes among members of the EPEC population.

Ler is not a general antagonist of H-NS because it binds only to a small subset of H-NS targets in the genome, mostly those associated with the LEE pathogenicity island. The two proteins are dissimilar in amino acid sequence at their N-termini but share similar C-terminal domains, including the nucleic acid-binding domain. However, a key arginine residue, found in Ler but not H-NS, seems to underlie the more restricted range of Ler binding in DNA. Both proteins rely on an indirect readout mechanism for binding site recognition: in the case of Ler, the introduction of its arginine residue into the minor groove of DNA is permitted at only a subset of H-NS binding sites (Cordeiro et al. 2011). This represents an interesting example of specialisation within the large family of H-NS-like proteins.

1.49 H-NS Functional Homologues

Proteins performing a foreign-gene-silencing function analogous to that associated with H-NS seem to be restricted to bacteria and to fall into four classes: H-NS itself, Rok, MvaT, and Lsr2. In contrast, other types of NAP are widely distributed among prokaryotes. When a bacterium possesses one type of xenogeneic silencer, it typically will not also have an example of a different type, indicating specialisation between each protein type and its genome (Perez-Rueda and Ibarra 2015).

1.50 H-NS Functional Homologues: Rok from *Bacillus* spp.

The Rok protein was discovered in *B. subtilis* during an investigation of gene regulation in the competence system: Rok emerged as a transcription silencer of *comK*, the autoregulated master controller of competence (Hoa et al. 2002). Rok controls the expression of an extensive regulon of genes (Albano et al. 2005) and at some of its gene targets its activity is amplified by co-binding of the DnaA protein (Seid et al. 2017). Rok binds to A+T-rich DNA targets (Smits and Grossman 2010) and, like H-NS, it has been implicated in the silencing of genes that have been acquired by HGT (Duan et al. 2018). Rok exhibits a higher preference for specific DNA sequences than other xenogenic silencer proteins (e.g. H-NS) and these targets are relatively rare in the *B. subtilis* core genome, allowing Rok to focus on imported genes (Duan et al. 2018). Rok binds only in the DNA minor groove and uses a winged helix fold to do this. It avoids rigid poly-A tracts with their very narrow minor grooves (Rohs et al. 2009), preferring 5′-AACTA-3′ and 5′-TACTA-3′ (both underrepresented in the core genome) and sequences that contain the flexible TpA step (Duan et al. 2018; Travers 2005).

1.51 H-NS Functional Homologues: Lsr2 from Actinomycetes

The 12-kDa Lsr2 NAP has been described as a functional analogue of H-NS in actinomycetes, including *Mycobacterium* spp. (Datta et al. 2019a; Kriel et al. 2018). It targets genes that have high A+T content that are thought to have been acquired by HGT (Gordon et al. 2010). Like H-NS, it can form DNA–protein–DNA bridges (Chen, J.M., et al. 2008), has a similar domain structure, and it can substitute functionally for H-NS (Gordon et al. 2008). Lsr2 and H-NS also bind DNA in the minor groove through a similar mechanism: using a so-called AT-hook-like grip (Gordon et al. 2011). The similarities between Lsr2 and H-NS are primarily functional and probably arose by convergent evolution: their amino acid sequences and their DNA-binding domains have distinct tertiary structures (Gordon et al. 2010, 2011; Shindo et al. 1995). Like H-NS, Lsr2 can polymerise along DNA to form stiff nucleoprotein structures from which other DNA-binding proteins are excluded (Qu et al. 2013).

Rv3852 is a 13.8-kDa protein that is highly conserved among *Mycobacterium* spp. and has been annotated as H-NS because its N-terminus resembles histone 1 from humans (Cole et al. 1998). Rv3852 is not an essential protein and a careful study of its properties rules out a role for it in controlling the virulence phenotype of *Mycobacterium tuberculosis* or in compacting the bacterial nucleoid (Odermatt et al. 2017).

1.52 H-NS Functional Homologues: MvaT from *Pseudomonas* spp.

Identified originally as a transcription regulator of *mvaAB,* an operon involved in mevalonate metabolism in *Pseudomonas mevalonii* (Rosenthal and Rodwell 1998), MvaT is now recognised as a NAP with properties analogous to those of H-NS (Castang and Dove 2010; Tendeng et al. 2003; Winardhi et al. 2012). MvaT binds to AT-rich DNA in genes that have

been acquired by HGT but it uses a binding mechanism that is distinct from other xenogeneic silencers: MvaT prefers binding sites that contain a series of flexible TpA steps and is tolerant of GC interruptions to the target sequence (Ding et al. 2015). MvaT has a paralogue, MvaU, with which it can form heteromeric complexes (Castang et al. 2008). Like MvaT, MvaU can bridge DNA and form filaments along the DNA that exclude other DNA-binding proteins, enabling it to silence transcription (Winardhi et al. 2014). Mutants deficient in these proteins have altered phenotypes affecting prophage activation, pyocyanin expression biofilm production, and the elaboration of surface fimbriae (Li et al. 2009; Vallet et al. 2004; Vallet-Gely et al. 2005).

Genes encoding MvaT-like proteins are found on self-transmissible plasmids and these proteins influence the transcriptome of the host cell in cooperation with their chromosomally encoded counterparts (Yun et al. 2015). Some bacteria express multiple members of the MvaT family; for example, *Pseudomonas putida* KT2440 encodes five MvaT orthologues: TurA, TurB, TurC, TurD, and TurE (Renzi et al. 2010). TurC, TurD, and TurE have species-specific properties while TurA and TurB are similar to MvaT proteins found in all members of the *Pseudomonadaceae*. TurB is reported not to act generally as a repressor and to affect a smaller group of genes than TurA. These findings illustrate the versatile nature of MvaT-like proteins and their capacity to acquire new functions through evolution (Renzi et al. 2010).

1.53 The Leucine-responsive Regulatory Protein, LRP

The leucine-responsive regulatory protein (LRP) DNA-binding protein affects the expression of about 10% of the protein-encoding genes in *E. coli*, many of which are involved in determining the structure of the bacterial surface, in transport, in metabolism, and in adaptation to stationary phase (Cho, B.K., et al. 2008, 2011; Engstrom and Mobley 2016; Tani et al. 2002). More recent data, based on ChIP-seq and RNA-seq analyses, have led to a revision of the estimate of LRP's influence to up to 38% of the *E. coli* genome (Kroner et al. 2019). In many cases, LRP interacts with target promoters in a poised mode, not influencing promoter activity until it operates in combination with other regulatory proteins; it also shifts between more- and less-sequence specific DNA-binding modes in response to nutrient signals (Kroner et al. 2019).

LRP contributes to the genetic switches that govern the phase-variable expression of Pap and type 1 fimbriae in *E. coli* (and fimbriae in *Salmonella*), linking LRP to bacterial virulence and to biofilm formation (Aviv et al. 2017; Hernday et al. 2004; Kelly et al. 2009; Lahooti et al. 2005; McFarland et al. 2008). LRP is also a regulator of the *stpA* gene, encoding the H-NS paralogue StpA that is both a DNA- and an RNA-binding protein (Free and Dorman 1997; Sonden and Uhlin 1996). Together with StpA (and with H-NS and FIS) LRP controls the transcription of *rsd,* the gene encoding the Rsd anti-sigma factor that targets RpoD and, to a lesser extent, RpoS (Hofmann et al. 2011). These links confer on LRP the potential to influence transcription patterns throughout the genome.

The 18.8-kDa LRP monomer forms octamers and hexadecamers and has the ability to wrap, bend, and bridge DNA (Chen et al. 2001); the *B. subtilis* homologue, LrpC,

forms structures with DNA that are reminiscent of a eukaryotic histone core (Beloin et al. 2003b). LRP expression peaks at the transition from the exponential phase to the stationary phase of the growth cycle in rapidly growing *E. coli* (Landgraf et al. 1996). In keeping with its name, the interactions of LRP with its target genes can be potentiated, inhibited, or unaffected by leucine and other branched-chain amino acids (Calvo and Matthews 1994; Lahooti et al. 2005; Peterson and Reich 2010). Leucine can also influence the oligomeric state of LRP, perhaps helping to explain the distinct effects (including no effects) that branched chain amino acids can have on different LRP regulated systems.

The LRP protein competes with the Dam methylase for access to two 5′-GATC-3′ sites in the *pap* and *pef* fimbrial operons of uropathogenic *E. coli* and *Salmonella*, respectively. This outcome of competition decides if the Dam sites will be methylated or not and this, in turn, determines if the fimbrial structural genes will be transcribed or not. The result is a stochastic switch that is reset by the synthesis of hemimethylated DNA during chromosome (*pap*) or virulence plasmid (*pef*) replication (Hernday et al. 2002; Nicholson and Low 2000). In contrast, LRP acts as a directionality determinant at the invertible *fimS* genetic switch that governs the phase-variable expression of type 1 fimbriae in *E. coli,* an inversion event that is catalysed by two tyrosine integrases, FimB and FimE (Corcoran and Dorman 2009; Kelly et al. 2006). In the case of *pap/pef*, LRP is acting as a DNA-binding protein in competition with a DNA modifying enzyme; in the case of *fimS*, LRP's ability to shape DNA is likely to be influencing the directionality of the On/Off invertible genetic switch. It also plays a role in virulence in *M. tuberculosis* by modulating the innate immune response of macrophage (Liu and Cai 2018). These examples illustrate the versatility of the LRP protein and its ability to influence diverse systems by different molecular mechanisms.

1.54 Small, Acid-soluble Spore Proteins, SASPs

Aerobic and anaerobic spore-forming bacteria rely on small, acid-soluble spore proteins (SASPs) to protect their DNA from damage during the long (or very long) periods that may elapse between sporulation and spore germination. Most research on SASPs has been concerned with those produced by *Bacillus* spp. and *Clostridium* spp., aerobic and anaerobic organisms, respectively (Setkow 2007). The SASPs fall into two broad groups, the α/β type and the γ type. Their genes are transcribed with the G sigma factor (Nicholson et al. 1989), protect the genomic DNA in the spore, and after germination they are cannibalised as a source of amino acids by the emerging bacterial cell (Hackett and Setlow 1988; Setlow 1988). SASPs are specialists in that they are expressed specifically to accompany the genome during its period of storage in the spore and are degraded during germination; they do not have physiological roles in vegetative cells, yet they exhibit properties that are shared with NAPs. For example, they stiffen DNA, and eliminate DNA bends, they increase the persistence length of DNA and introduce supertwists into relaxed or nicked circular DNA (Griffith et al. 1994; Nicholson et al. 1990), including plasmids from spores (Nicholson and Setlow 1990). When cloned *ssp* genes expressing SASPs are

introduced to *E. coli,* they induce nucleoid condensation; however, they also induce mutagenesis and cell killing (Setlow et al. 1991, 1992). The mutations accompanied expression of the *B. subtilis* SspC SASP and required RecA and Pol V, suggesting that the effects followed the arrest of replication forks in growing *E. coli*; a derivative of SspC that was deficient in DNA binding failed to elicit these deleterious effects when expressed in *E. coli* (Setlow et al. 1992). Overall, the effect of SASP expression in *E. coli* was to cause the Gram-negative bacterium to assume some of the characteristics of a sporulating organism (Setlow et al. 1991).

2

Conservation and Evolution of the Dynamic Genome

The previous chapter described the structure of the bacterial genome in the context of the nucleoid, together with the molecules and processes that replicate it and transfer the copies faithfully to daughter cells. We saw that the genome is dynamic and that it moves around within the cell during the growth and the cell cycles. In this chapter we will consider the processes that threaten the integrity of the genome and the countervailing influences that seek to maintain it. The genetic material is kept under constant surveillance, with damage being detected and repaired rapidly. However, some changes to the genome have to be tolerated if evolution is to proceed, so an antagonism exists between the conservative and the disruptive forces that are at work in the genome. The outcome of the tension between these conservative and disruptive processes provides the genetic material upon which natural selection operates.

2.1 Disruptive Influences: Mutations

Mutation refers to any change to the genome, extending in scale from changes affecting single base pairs to large additions, subtractions, or rearrangements of the DNA. Many of the molecular events at DNA level described in this work involve mutation and each has the potential to alter the competitive fitness of the bacterium. Mutations can be caused by recombination processes: site-specific recombination, general recombination, or illegitimate recombination, or they can arise as a result of errors associated with processes such as transcription or DNA replication. They can also arise when the bacterium is exposed to a mutagenic agent, such as ionising radiation, oxidative stress, or another chemical mutagen. The bacterium has machinery to monitor genome integrity and to make repairs rapidly. If the repair machinery is damaged (e.g. by mutation) then the rate at which mutations accumulate will increase. Once a mutation tolerance threshold is exceeded, the level of damage sustained becomes lethal because a vital system has been corrupted or the repair apparatus cannot keep up, or both. Even minor genetic damage can have long-term negative consequences if it makes the organism uncompetitive: it will be overtaken by more competitive bacteria and rendered extinct. On the other hand, genetic change is a very important generator of the physiological variety that, when beneficial under selective pressure, promotes the evolution of the organism.

Structure and Function of the Bacterial Genome, First Edition. Charles J. Dorman.

DNA replication and transcription both involve making double-stranded DNA become locally single-stranded. This introduces a risk of cytosine deamination, leading to mutation (Francino and Ochman 2001; Frank and Lobry 1999). The GC skew seen in bacterial chromosomes is thought to represent a risk reduction measure. The skew refers to the enrichment of the leading strand in G and T (keto) bases and the lagging strand in C and A (amino) bases. Replication of the leading strand is continuous, that of the lagging strand is semi-discontinuous (Figure 1.3). During the replication process, the leading strand is in a single-stranded state for a longer time than the lagging strand, so minimising the C-content of the leading strand can be seen as a strategy to reduce the C deamination risk (Frank and Lobry 1999; Necsulea and Lobry 2007). Similarly, in transcription, the coding strand is single-stranded for a longer period (Beletskii and Bhagwat 1996; Klapacz and Bhagwat 2002). This may contribute to the alignment of transcription units with the direction of replication fork passage, along with other influences, such as the avoidance of collisions between RNA polymerase and the replisome and selection for a higher number of genes, including essential genes, on the leading strand (Lopez and Philippe 2001; Rocha 2008; Rocha and Danchin 2003).

Mutations in wild-type *Escherichia coli* genomes (including MG1655) arise at different rates in a geographically correlated pattern, with the neutral mutation rate differing between chromosomal regions by up to an order of magnitude. Highly expressed genes represent cold spots for mutation, as do regions subject to strong purifying selection. The authors of this study (Martincorena et al. 2012) found the findings difficult to reconcile with models in which errors associated with transcription in the coding strand arise during transcription-coupled repair of the non-coding strand (Francino et al. 1996): Martincorena et al. (2012) detected transcription-associated mutations in the non-coding strand too.

Transcription and DNA replication alter DNA topology at a local level. The underwinding of the DNA duplex that creates negative supercoils also encourages single-strandedness: about 40% of the bacterial genome consists of unconstrained supercoils (Bliska and Cozzarelli 1987). Supercoiling patterns are dynamic and shift with the growth conditions and growth stage of the organism, and most of what is known about this topic has come from studies of a few model bacteria (Lal et al. 2016). The extrusion of plectonemes, or interwound segments of writhing DNA, from the chromosome, is a response to under- or overwinding of the DNA in which the molecule seeks a minimal energy conformation. Single molecule studies have identified DNA sequence motifs that are typically found at the apices of plectonemes (Kim et al. 2018), raising the possibility that these could be predicted bioinformatically. A study of mutation patterns in a derivative of *E. coli* strain MG1655 that was deficient in mismatch repair due to a deletion of *mutL* revealed a periodic pattern of mutations in which mutation density correlated with regions of high negative superhelicity (Foster et al. 2013). Base-pair substitutions occurred symmetrically in both replichores and regions of high mutation density were detected symmetrically in the Ter region. Replisome movement is associated with DNA topological disturbance and topoisomerases are intimately involved in managing the process (Figure 1.14). This study suggests that a feature that is written into the chromosome structure makes local regions of the genome liable to accumulate base substitutions during replication fork passage and that the mismatch repair machinery normally eliminates them.

2.2 Repetitive Sequences in the Chromosome and Their Influence on Genetic Stability

DNA sequence repeats have the potential to recombine with one another, contributing to genome structural changes. At the intramolecular level, repeats can contribute to the deletion, inversion, and amplification of the DNA between them, with the outcome of the recombination being determined by the relative orientation of the repeats. Deletions can result in the excision of genetic elements that are capable of independent replication, and in many cases, this process is bidirectional. If it is not, this may be because the recombinases

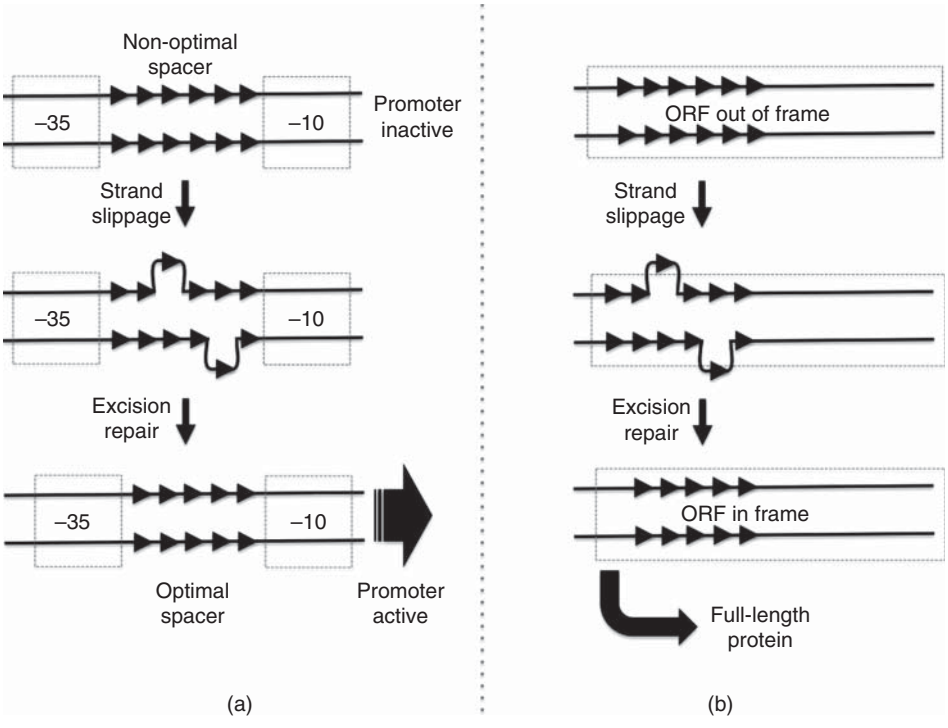

Figure 2.1 Simple sequence repeats (SSRs) and phase-variable gene expression. (a) Transcription initiation varies between an off setting (top) and an on setting (bottom) depending on the length of the spacer region between the −10 and −35 elements of the promoter. This sequence contains a number of small direct repeats (they could be as short as a single nucleotide, in which case orientation is not relevant). Slipped-strand mispairing causes a copy of one repeat to loop out from the top strand and another from the bottom strand. Excision repair eliminates the extruded repeats, adjusting the length of the promoter spacer to an optimal value for productive interaction with RNA polymerase holoenzyme. Transcription can now commence. (b) A series of repeats within the open reading frame of a constitutively transcribed protein-encoding gene interferes with the reading frame and only non-functional truncated proteins are expressed. Slipped-strand mispairing between repeat copies followed by excision repair eliminates looped out repeats, adjusting the reading frame so that a full-length, functional protein is expressed. The transcriptional phase-variation shown in A can be combined with the translation phase-variation shown in B to introduce stochasticity into gene expression at both levels.

that drove the reaction in one direction do not recognise the products or are unable to process them (e.g. bacteriophage CTXϕ integration by the XerCD recombinases at the *dif* sites of *Vibrio cholerae*; Section 1.8). Directly repeated sequences can recombine with one another or participate in replication-driven slipped strand mispairing, leading to expansion or contraction of the numbers of repeats (Figure 2.1). Genes associated with these events can be amplified or deleted along with the repeat sequences (Goldberg and Mekalanos 1986; Mekalanos 1983). At the intermolecular level, repeats can be used to integrate novel sequences from one DNA molecule into another, as in the case of bacteriophage integration by site-specific recombination.

Large catalogues of repeat sequences have been assembled (Rudd 1999; Zhou et al. 2014) and these will not be reviewed here. The ribosomal operons represent a specialist group of repeat sequences, but their functions have been well known for some time. Other repeats, despite intensive investigation that has produced a large body of published research, have functions that are only beginning to be appreciated fully. Among these are the Rhs (Hill et al. 1994), the BIME/REP/RIB/RIP (Gilson et al. 1991a,b; Higgins et al. 1982; Bachellier et al. 1994; Boccard and Prentki 1993; Oppenheim et al. 1993) and the Enterobacterial Repetitive Intergenic Consensus (ERIC) (Hulton et al. 1991; Sharples et al. 1990) repeats found in *E. coli* and related organisms. Repeats with clear biological functions include the 9-bp DNA uptake signal (5′-AAGTGCGGT-3′) required for horizontal gene transfer (HGT) by transformation in *Haemophilus influenzae*, with 1465 copies in a genome of just 1.8 mb (Fleischmann et al. 1995; Smith et al. 1995). The 8-bp Chi site (5′-GCTGGTGG-3′) occurs every 4–5 kb in the *E. coli* chromosome and plays a key role in DNA repair and recombination (Section 2.32) (Stahl 1979). Even more repeats with known functions are found as tandemly repeated sequences in contingency loci where they contribute to the generation of variety in cell composition and physiology (see Section 2.3).

2.3 Contingency Loci and the Generation of Microbial Variety

Contingency loci have been described in many bacteria, especially pathogens, and consist of tandemly repeated DNA sequences capable of undergoing rearrangement to generate diversity in gene expression. This enables the organism to survive environmental challenge and, if pathogenic, to infect and to evade host defenses (Moxon et al. 2006; Zhou et al. 2014). Contingency loci typically affect the expression of cell surface components and do so by rearranging elements of the gene control or expression machinery. However, they have also been associated with housekeeping genes (Guo and Mrazek 2008), stress response genes (Rocha et al. 2002), and with genes encoding restriction-modification systems (Adamczyk-Poplawska et al. 2011; Srikhanta et al. 2009, 2011).

Simple Sequence Repeats (SSRs) in *Helicobacter pylori* operate through slipped-strand mispairing to modify the transcription-initiating power of promoters such as the gene-encoding sialic acid-binding adhesin SabA (Aberg et al. 2014) (Figure 2.1). In the case of *sabA*, the spacing between the core promoter and the UP element is adjusted by SSR-mediated slipped strand mispairing, resulting in a retuning of the promoter. The spacer regions of overlapping and divergently arranged promoters are targets for SSR-mediated variation in the case of the *hif* genes that produce LKP fimbriae in *H. influenzae*.

Here, changes to promoter spacer length result in low, high, and zero expression levels for the genes (van Ham et al. 1993). Transcription-level diversity is generated by variations in the lengths of poly-C tracts in the promoter of the *fim* operon of *Bordetella pertussis* (Willems et al. 1990) and in the promoter of the gene that encodes the Opc surface protein in *Neisseria meningitidis* (Sarkari et al. 1994). A similar mechanism involving a poly-C tract of variable length at the promoter is responsible for transcription-level variation in the expression of *fetA*, the gene encoding the enterobactin receptor FetA in gonococci (Carson et al. 2000). The FetA protein is thought to be immunogenic, so varying its expression randomly may allow the population randomly to enjoy the benefits of access to iron-siderophores or immune evasion. It is also possible that this surface protein is targeted by a bacteriophage, in which case the same strategic benefits would apply at a population level. The promoter wiring of the gene for PorA surface protein expression in *N. meningitidis* facilitates complex sequence length variations to be generated using both a G-tract and a T-tract; an A-tract within the open-reading frame of the same gene supports phase variation at a post-transcriptional level (van der Ende et al. 2000). This two-level strategy permits variations in *porA* transcript levels to be combined with variations in protein amino acid sequence to outwit the host defenses.

SSRs can affect gene expression at the translational as well as the transcriptional level. For example, phase-variable expression of the surface-located opacity proteins of *Neisseria* spp. operates through variation in the number of 5′-CTCTT-3′ repeats in the open reading frames of the *opa* genes or by recombination between *opa* loci (Bayliss 2009; Sadarangani et al. 2011). An extreme example of physiological diversity driven by SSRs involves the conversion of the *ahpC*-encoded peroxidase into a disulphide reductase through the expansion of a TCT tract within the open reading frame (Ritz et al. 2001). Here, a completely new enzyme activity is expressed, not simply a modified version of the activity previously produced by the *ahpC* gene. An interesting variation on the post-transcriptional phase-variation theme arises when the affected protein is itself a global regulator. The PrfA transcription factor is the master regulator of virulence gene expression in the pathogen *Listeria monocytogenes*. A tandem repeat sequence in the DNA specifying the HTH motif of PrfA can mediate the deactivation and reactivation of the gene product and the virulence programme of the bacterium (Lindback et al. 2011).

2.4 Rhs: Rearrangement Hotspots

E. coli K-12 has five *rhs* or rearrangement hotspots, RhsA–E; they are up to 10.5 kb in length and contribute to chromosomal DNA duplications (Lin et al. 1984). Collectively, Rhs elements represent 1% of the DNA in the chromosome; after the ribosomal operons, they are the largest repeated sequences in the *E. coli* K-12 genome. These elements are mosaic genetic structures that seem to have been assembled from smaller units; their G+C content indicates that they were acquired horizontally from a source outside the *Enterobacteriaceae*. Indeed, Rhs elements are widespread in the eubacteria and the open reading frames they contain are part of an ancient gene family (Jackson et al. 2009). Each encodes a core protein with a conserved region that contains 28 peptide repeats. This core ORF extends into a region that shows amino acid sequence diversity, resulting in a very large protein

of up to 160 kDa in molecular mass. The repeats in the core are reminiscent of repetitive motifs seen in surface-expressed proteins such as fibronectin-binding proteins in *Staphylococcus aureus*, *Clostridioides difficile* toxin A, and repeats-in-toxins (RTX) in Gram-negative pathogens (Hill et al. 1994).

Rhs share characteristics with Contact-dependent Growth Inhibition (CDI) systems (Poole et al. 2011). CDIs use a stick-like surface protein to insert a toxin into a target cell, inhibiting its growth (Aoki et al. 2010, 2011; Hayes et al. 2010). One protein, CdiA has the toxin activity and a second, CdiB is responsible for CdiA export and presentation to the target cell. In addition, each CDI system has an immunity protein, CdiI, encoded by a gene immediately downstream of *cdiA*. CdiI protects the producer cell from the inhibitory effects of the CdiA toxin (Aoki et al. 2010). The CdiA proteins resemble the large core proteins of Rhs elements in overall size and organisation and both systems have the genetic potential to shuffle the make-up of their toxin-immunity modules, extending their biological range (Poole et al. 2011). In several Rhs-like systems, there is evidence that the Rhs proteins are expressed at the cell surface, in keeping with a role in cell-to-cell communication (Foster 1993; McNulty et al. 2006; Youderian and Hartzell 2007). Furthermore, proteins RhsA and RhsB from the plant pathogen *Dickeye dadantii* 3937 carry a nuclease activity that degrades target cell DNA. These proteins are probably exported through a type VI secretion system (T6SS, Section 5.16) and an immunity protein RhsI protects the producer cell from the toxic effects of the nuclease (Koskiniemi et al. 2013). A role in cell-to-cell communication, including contact-mediated competition, might be expected to exert a strong selective pressure for the maintenance of Rhs systems in the *E. coli* genome in some environments, even if partial duplication of the chromosome from time to time proved to be an inevitable trade-off. In other environments Rhs may not provide a selective advantage and instances have been reported of strains of *E. coli* that have no *rhs* copies (Hill et al. 1994). When an Rhs system is present, it can evolve by recombination-mediated co-option of an otherwise silent orphan toxin-encoding DNA sequence and its cognate immunity genes. As has been demonstrated in *Salmonella* Typhimurium, the bacterium with the new Rhs combination then eliminates its own ancestors (Koskiniemi et al. 2014). This shows that the Rhs system contributes to intraspecies competition and evolution.

2.5 REP Sequences

The REP sequence was identified during the DNA sequence analysis of the histidine uptake operon of *S.* Typhimurium and other bacterial transcription units (Higgins et al. 1982). Also known as Palindromic Units (PUs) (Gilson et al. 1984) REPs consist of a 38-bp imperfect inverted repeat that is capable of specifying a stem with a central 5-nucleotide loop when transcribed (Stern et al. 1984) and are also found in *E. coli*, other enterobacteria and *Pseudomonas* spp. (Gilson et al. 1984; Stern et al. 1984; Tobes and Pareja 2005). These structures can protect upstream transcripts from degradation by 3′-to-5′ exoribonucleases, influencing the ratio of proteins encoded by genes upstream and downstream of the REP in a polycistronic operon with an internal, intergenic REP (Newbury et al. 1987a,b; Stern et al. 1984). REP sequences at the ends of transcription units can form complexes with the HU nucleoid-associated protein (NAP) and DNA gyrase (Yang and Ames 1988,

1990), leading to speculation that these REPs may act as a focus for topoisomerase activity aimed at eliminating the DNA topological consequences of transcription. REPs may also participate in the higher-level organisation of the nucleoid (Gilson et al. 1990; Qian and Adhya 2017; Qian et al. 2015, 2017) (Section 4.12).

2.6 RIB/RIP, BIME-1, and BIME-2 Elements

REP sequences can occur in clusters around a binding site for Integration Host Factor (IHF). These REP derivatives have been named RIP (repetitive IHF binding palindromic) sequences (Oppenheim et al. 1993) or RIB (reiterative IHF binding) sequences (Boccard and Prentki 1993). The IHF-binding RIP elements are actually a sub-class of the BIME mosaic elements, the BIME-1 class. BIMEs are Bacterial Interspersed Mosaic Elements (Gilson et al. 1991a,b) and are defined by their REP and non-REP repeat sequence composition and the relative orientations of the components (Bachellier et al. 1993, 1994, 1999). *E. coli* has around 600 BIMEs in its genome and across Gram-negative bacteria they exhibit compositional diversity due to expansion or deletion of components, and they can be associated with novel repeat sequences (e.g. the *boxC* repeat) or mobile genetic elements (e.g. IS*621*, IS*1397*, ISKpn1) (Bachellier et al. 1997; Choi et al. 2003; Clement et al. 1999; Wilde et al. 2001, 2003). The discovery that genes encoding HuH-type transposases can be found associated with some REP and BIME repeats in *E. coli* and other bacteria has led to speculation that these elements may be (or have been) non-autonomous mobile elements (Ton-Hoang et al. 2012). REP-like sequences have been detected as participants in F′ plasmid formation, where the REPs form the junctions between the plasmid and chromosome sequences (Kofoid et al. 2003). Similar observations have been made for bacteriophage lambda derivatives that harbour chromosomal sequences (Kumagai and Ikeda 1991). BIME elements can induce pausing of RNA polymerase during the elongation phase of transcription, creating an opportunity for Rho-dependent termination to interrupt the expression of a polycistronic operon with an intergenic BIME (Espéli et al. 2001). In the absence of an IHF binding site, the REP cluster constitutes a BIME-2 element (Espéli and Boccard 1997). BIME-2 elements are targets for DNA gyrase binding and cleavage, allowing them to contribute to the management of local DNA topology (Espéli and Boccard 1997; Sutormin et al. 2019). A combination of transcription pausing, RNA polymerase backtracking, R-loop formation, and DNA gyrase binding (together with the impact of BIMEs on RNA turnover) may provide a mechanism for modulating gene expression at BIME-containing operons.

2.7 ERIC Sequences

ERIC sequences are 126-bp repeats that were found initially in the genomes of *E. coli*, *S*. Typhimurium, *Klebsiella pneumoniae*, *V. cholerae*, and *Yersinia pseudotuberculosis* (Hulton et al. 1991). They are also known as Intergenic Repeat Units (IRUs) (Sharples and Lloyd 1990). Despite their sequence conservation, ERICs are detected at different genomic locations in different species, although they are restricted to transcribed regions of the genome. ERICs have many features in common with REPs, despite not being related at the

level of nucleotide sequence. Like REP sequences, ERIC sequences have been exploited in PCR-based approaches to investigate genomic diversity.

2.8 Repeat-Mediated Rearrangements: Mechanisms and Frequency

The principal mechanisms that have been proposed for SSR-mediated genetic rearrangements are DNA-replication-associated strand slippage and recombination (Figure 2.1). The relative importance of the mechanisms reflects the sizes of the SSRs: recombination is more significant for rearrangements between larger repeats and strand slippage is more commonly used to rearrange small repeats. Support for the strand-slippage mispairing model comes from several studies showing a correlation between mutations in genes that encode components of the replisome and the rate of SSR-mediated rearrangement (Bichara et al. 2006; Gemayel et al. 2010; Pearson et al. 2005).

The rate of SSR-mediated rearrangements is influenced by factors internal and external to the cell. Internally, repeat copy-number and repeat size both correlate positively with rearrangement frequency (Bayliss et al. 2012; Lin and Kussell 2012). Repeat conservation has a positive impact on frequency of rearrangements and the GC content of the DNA is also influential due to the formation by repeats of non-B-DNA structures that encourage replication forks to collapse (Choudhary and Trivedi 2010; Wells et al. 2005). In agreement with this proposal, it has also been suggested that unusual DNA structures, such as triple-helical H-DNA, may be intermediates in some systems, such as the expansion and contraction of the numbers of 5′-CTCTT-3′ repeats in the *opa* gene of *Neisseria* spp. (Belland 1991). Repeat orientation with respect to replication fork movement is also a contributing factor to stability/instability (Hebert et al. 2004). Transcription across the repeats and the accompanying disturbance to local DNA structure may also exert an influence on rearrangement frequency. Environmental stresses originating outside the microbe may also exert an influence, perhaps by affecting DNA-based transactions such as DNA supercoiling levels, transcription, and replication.

2.9 Site-specific Recombination and Phenotypic Variety

Phenotypic variety in a population can arise from genetic mutation or through the regulation of gene expression in ways that do not produce uniform outputs across the population. Mutations can arise through a variety of mechanisms. For example, copying errors can introduce changes to the base sequence of the genome that are then inherited, with the new sequence passing vertically to later generations. These errors can be very modest, perhaps involving just a single base pair, or they can be more extensive, involving deletions or insertions of DNA of various lengths. Mutations can arise from illegitimate recombination due to errors in the operation of topoisomerases or the DNA repair machinery. Mobile genetic elements such as insertion sequences (IS) or transposons can produce heritable mutations and so can the arrival of genetic elements (linear DNA segments, plasmids, or phage) by HGT. Each of these genetic events is unpredictable, just as the environment is unpredictable, and

this adds a stochastic character to the appearance of the mutations and the associated phenotypic changes. Regardless of the source of genetic variety, the selective pressure inherent in the environment will then determine which new genetic arrangement is the most fit.

Regulatory events that lead to phenotypic change are environmentally responsive, distinguishing regulation from mutation, with its random character. In contrast to mutation, regulatory events are not only environmentally responsive, but also programmable, predictable (at least at the level of the population), and reversible. Genetic theories about the 'directability' of mutation, in the sense that regulation is subject to direction, are controversial (Brisson 2003; Cairns et al. 1988; Hall 1991; Lenski and Mittler 1993). One source of phenotypic variety that combines the predictability of regulation with the randomness of mutation is site-specific recombination. RecA-independent site-specific recombination plays an important role in the life cycles of bacteriophage and in the generation of diversity within bacterial populations. The process involves recombination between specific, short segments of DNA and is catalysed by a recombinase that specialises in recombining just those sites or other sites that closely resemble them. If the recombining sites are arranged as inverted repeats, the DNA segment between them is inverted; if they are arranged as direct repeats, the intervening DNA is deleted (reviewed in Dorman and Bogue 2016). The recombination pathways are molecularly precise and deterministic, but the timing of the reaction may be random. Environmental modulation of the recombination reaction can bias the outcome, favouring one recombination product over another. In this way, a process that seems at first to be stochastic can take on a stereotypic character. This ability to override stochasticity can aid the survival of the population by expanding the number of its members that contain the optimal outcome of the recombination event, optimal in terms of fitness within the prevailing environmental circumstances. Factors that impose directionality on the underlying randomness of the recombination reaction include architectural elements such as NAPs and variable DNA topology (Dorman and Bogue 2016).

2.10 Site-Specific Recombination: Bacteriophage Lambda

The site-specific integration and excision of the bacteriophage lambda genome into and from the chromosome of *E. coli* K-12 is one of the best-understood examples of the process in biology. The phage encodes the Int site-specific recombinase, together with a recombination directionality determinant called Xis (excisionase) that promotes excision and inhibits integration; it also harbours in its genome one of the two participating DNA sequences for integration: *attP* (Craig and Nash 1983; Han et al. 1994; Hoess et al. 1980; Kikuchi and Nash 1979; Tong et al. 2014). *E. coli* provides the other DNA sequence for the intermolecular recombination event, *attB*, as well as important co-factors, chief among which is IHF (the protein gets its name, Integration Host Factor, IHF, from the role it plays in the lambda lysogenic cycle) (Bushman et al. 1984; Seah et al. 2014) and the Factor for Inversion Stimulation (FIS) protein (Esposito and Gerard 2003; Papagiannis et al. 2007; Seah et al. 2014; Sun et al. 2006). The inserted phage genome, the prophage, is almost 50 kb in length and is flanked by directly repeated DNA sequences that are derived from the recombined *attB* and *attP* elements. Site-specific recombination between these direct repeats, *attL* and *attR*, excises the phage DNA from the bacterial chromosome and restores *attB* and *attP* (Figure 1.17).

Lambda integration and excision are parts of a story that has helped to deepen understanding of apparently random events in the creation of physiological variety. The bacteriophage infects *E. coli* cells that are expressing the maltose-inducible LamB lambda receptor protein in the outer membrane (Chatterjee and Rothenberg 2012; Schwartz 1975). This protein seems to be distributed across the cell surface roughly in a helical pattern and lambda uses this pattern to locate the cell pole, where DNA injection usually occurs (Edgar et al. 2008; Rothenberg et al. 2011).

The injected DNA consists of the entire viral genome and expression of its genes determines the fate of the phage and the host cell. Before gene expression can occur, the dsDNA phage genome becomes circularised through the base pairing of its G+C-rich cohesive (Cos) sticky ends and their ligation by *E. coli* DNA ligase (Friedman and Court 2001). The large circle of dsDNA is then negatively supercoiled by DNA gyrase. The circular DNA contains the *attP* site for integration into the chromosome and this will come into play if a decision is made to pursue the lysogenic pathway. Lambda does not embark on a search for the lambda attachment site and its *attB* core on the chromosome; instead, lambda waits at the cell pole for the *attB* site to come to it (Tal et al. 2014). The delivery of *attB* seems to happen as a result of the choreographed molecular ballet that the chromosome executes within the cell in the run-up to cell division (Espéli et al. 2008).

The options for lambda are to enter the bacterial chromosome using the site-specific recombination mechanism outlined here or to enter the lytic cycle in which the phage genome is replicated, packaged in viral heads that then mature and exit the host cell by lysing it (Friedman and Court 2001). In either event, the initial gene expression steps are the same in both pathways. This lysogeny/lysis decision has been examined in detail over several decades and is a multi-layered process (Casjens and Hendrix 2015; Hendrix 1983; Hershey 1971; Ptashne 2004). *E. coli* can be infected by more than one copy of lambda and the outcome of the decision is arrived at by consensus among the viruses. Just one vote for lysis is sufficient to bring about that outcome; lysogeny requires a unanimous vote (Zeng et al. 2010). The observation that the molecular events responsible for the decision are made locally and individually by each copy of lambda suggests that the molecules that each encodes do not mix and cross-control the process at other phage (Golding 2016).

2.11 The Lambda Lysis/Lysogeny Decision

The lysis/lysogeny decision is the outcome of the activities of the lambda-encoded DNA binding proteins CI and Cro. The CI protein acts as a transcriptional repressor of the genes in the lytic pathway and the Cro DNA binding protein opposes it in this endeavour by competing with CI to bind to operator site DNA that controls key promoters (Lee, S., et al. 2018). In its most simplified form, the genetic switch involves an attempt by Cro to repress the promoter for CI repressor maintenance, P_{RM}. The positively autoregulated CI protein, in turn, acts to stimulate the transcription of its own gene, *cI*, from the same P_{RM} promoter (Lee et al. 2018) (Figure 1.17). Stable expression of the CI protein from its autoregulated gene maintains the lysogenic state until something happens to remove CI, resulting in induction of the prophage and lysis. The establishment of lysogeny requires integration of the phage genome at *attB* through site-specific recombination with *attP* (Figure 1.17). Cell size and

cell physiology influence the lytic/lysogenic decision (St-Pierre and Endy 2008) and spatial constraints in the cytoplasm may influence the success of the search by circularised lambda for its integration site on the chromosome, especially as the onus seems to be on the bacterial chromosome to locate *attP* (Tal et al. 2014). This latter observation may indicate a link between lysogeny and the host cell cycle (Sergueev et al. 2002; Worsey and Wilkins 1975). When the bacterial host sustains DNA damage, the SOS response is induced and the RecA protein is activated (Little and Mount 1982). This triggers the degradation of the CI repressor, leading to a switch from lysogeny to lysis that can be rationalised as an attempt by the lysogenic virus to escape a host that may be doomed by fatal damage to its chromosome.

2.12 Tyrosine Integrases

The Int tyrosine integrase produced by bacteriophage lambda is the prototypic member of a large family of site-specific recombinases whose genes are found in temperate phage, prokaryotes, and in some archaea and yeast (Meinke et al. 2016). These proteins vary in size and amino acid sequence but have conserved catalytic sites and overall quaternary structures. Each binds to specific pairs of sites that have some DNA sequence identity. Two copies of the integrase bind to each site, with the site consisting of two sub-sites with one integrase protein binding at each. A short spacer region separates the protein-binding sub-sites and within this spacer, DNA cleavage, strand exchange, and ligation occur: the spacer regions in the two participating sites are identical in nucleotide sequence in classic examples of tyrosine integrase-mediated recombination. There are examples, such as the integration of the CTXϕ phage in *V. cholerae*, where strict homology is not involved (Rajeev et al. 2009). The reactions that are catalysed by integrases can be intermolecular (as in lambda integration) or intramolecular (as in lambda excision). The recombinase may work in association with directionality determinants (Xis in the case of lambda Int) and with architectural proteins (e.g. IHF) (Jayaram et al. 2015). In addition to phage integration and excision, integrases catalyse plasmid dimer and chromosome dimer resolution, decatenation reactions, and the reversible inversion of DNA segments that carry transcription start signals and transcription termination signals for the control of gene expression (Dorman and Bogue 2016; Grindley et al. 2006). Cross-regulation of distinct DNA inversion systems by different integrases in the same bacterium promotes a variety of gene expression outcomes, perhaps helping pathogenic bacteria to evade host defences and to maintain an advantage in terms of competitive fitness (Battaglioli et al. 2018). Tyrosine integrases may themselves directly interact with, and modulate, the host defences through molecular mimicry, as has been found for one recombinase in the human gut anaerobic bacterium *Bacteroides* spp. (Hebbandi Nanjundappa et al. 2017). *Bacteroides* spp. also encode IntDOT, a tyrosine integrase that is produced by the CTnDOT conjugative transposon, a carrier of antibiotic resistance genes (Section 2.20). IntDOT is an integrase that does not require perfect DNA sequence homology between the sites that it recombines, distinguishing it from prototypic integrases such as lambda Int (Ringwald et al. 2017). *Bacteroides* spp. rely on site-specific recombinases to generate physiological diversity both at the level of bacterial cell structure and in terms of secreted products. Tyrosine integrases are important

contributors to these processes, together with members of the serine invertase family of site-specific recombinases (Cerdeño-Tárraga et al. 2005).

Bacteriophage P1, whose prophage is a large single-copy plasmid, uses the Cre tyrosine integrase to resolve P1 plasmid dimers (Austin et al. 1981) and to circularise the newly injected, terminally redundant, P1 DNA (Sternberg et al. 1986). Like XerCD when acting at ColE1 *cer* plasmid site-specific recombination sites, Cre also requires the protein co-factors ArgR and PepA when acting at *loxP* in the P1 plasmid prophage (Paul and Summers 2004). Cre, and its specific target site *loxP*, have formed the basis of a system for the genetic modification of genomes that is used widely in biotechnology (Yarmolinsky and Hoess 2015).

2.13 Serine Invertases

Serine invertases use a distinct chemistry to catalyse site-specific recombination reactions that usually involve the inversion of DNA sequences in bacteria or phage (Rice 2015; Smith 2015). The prototypic example is Hin, the recombinase responsible for flagellar phase variation in *Salmonella* (van de Putte and Goosen 1992; Johnson 2015). This protein operates in a way that superficially resembles Int-mediated lambda excision, except that the recombining *hixL* and *hixR* sites are in inverted rather than direct orientation, as is the case with *attL* and *attR* in the lambda prophage (Figures 1.17 and 2.2). The *hin* gene, which expresses Hin, is bifunctional. In addition to encoding the serine recombinase, *hin* contains a *cis*-acting enhancer sequence that improves the frequency of recombination between *hixL* and *hixR* (Dhar et al. 2009; McLean et al. 2013; Merickel et al. 1998). The enhancer provides the surface on which the invertasome assembles and this complex includes the Factor for Inversion Stimulation, the FIS NAP (Bruist et al. 1987; Glasgow et al. 1989; Johnson et al. 1987). Assembly of the invertasome creates a large DNA loop whose formation is accommodated within the flexibility of the DNA and a smaller loop that requires the assistance of the HU protein to form (Figure 2.2) (Haykinson and Johnson 1993; Glasgow et al. 1989; Johnson et al. 1986). The genetic switch causes the connection/disconnection of a transcriptional promoter to/from the *fljBA* operon. The FljA protein is one of the two protein subunits used by *Salmonella* to synthesise its flagella; FljB is an inhibitor of the translation of the mRNA that specifies FliC, the alternative flagellar subunit protein in *Salmonella* (Scott and Simon 1982). FljB and FliC are antigenically distinct and flagellar phase variation may provide a means to evade the host immune system (Silverman and Simon 1980). Genetic switches related to the flagellar one are found widely in bacteria and phage. They differ from one another in the content of the inverted sequence: sometimes it is a transcription start signal, as in *fljBA*; in other cases it is a set of genes/truncated genes that are connected to, or disconnected from, a fixed promoter (Henderson et al. 1999; van der Woude 2011). Examples include some of the genes that determine the composition of the cell surface in *Bacteroides* spp. and genes that specify tail fibres in phages such as Mu and P1 (Cerdeño-Tárraga et al. 2005).

Serine invertases are interchangeable: for example, the Cin invertase that operates the tail fibre switch in phage P1 will also catalyse the inversion of the *hixL-hixR* system in the *Salmonella* chromosome (Smith 2015; Smith and Thorpe 2002). The members of the invertase family of site-specific recombinases are related by their amino acid sequences to the resolvases that are encoded by transposons of the Tn*3* family (Chang and Johnson 2015; Johnson 2015). Resolvases also use a serine-based chemistry to carry

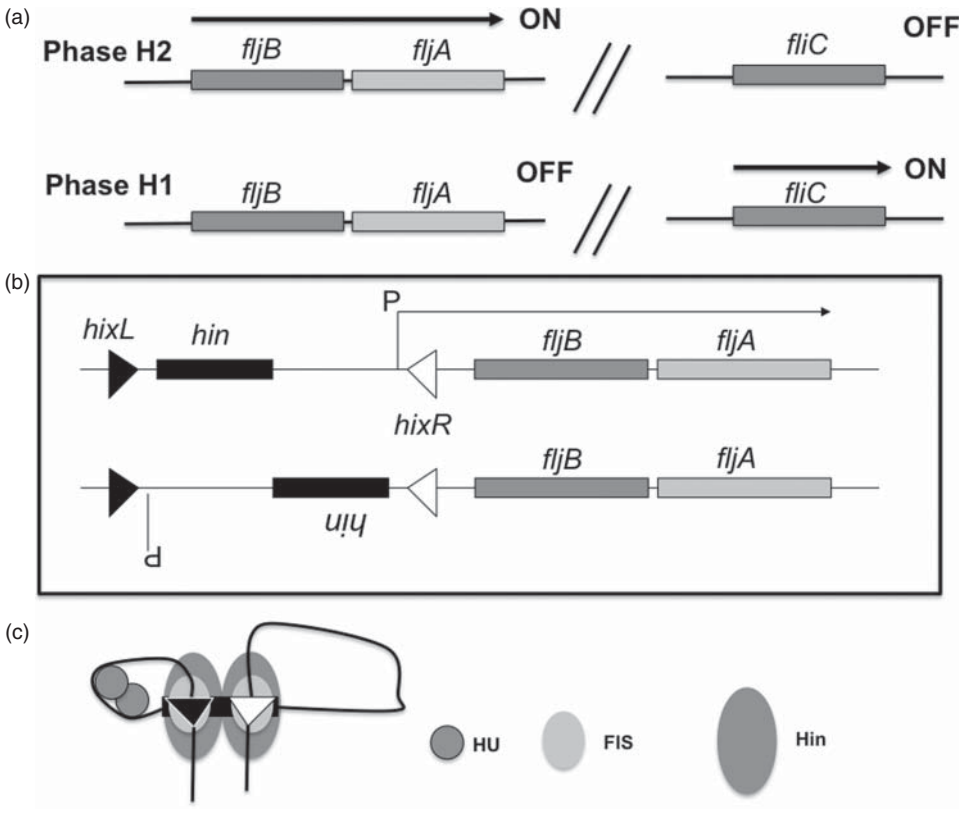

Figure 2.2 The Hin invertasome and flagella phase variation in *Salmonella*. (a) The H1 and H2 phases of flagellum expression are characterised by flagella composed of either the FliC or the FljB flagellar subunit, respectively. The two forms of flagellar subunit are mutually exclusive: FljA, produced from the *fljBA* operon, is an inhibitor of FliC production. (b) FljB and FljA are not produced when the promoter of the *fljBA* operon is disconnected from the genes by DNA inversion involving site-specific recombination of the *hixL* and *hixR* inverted repeats, catalysed by the Hin serine invertase. The *hin* gene is completely within the invertible region, as is the promoter for *fljBA* transcription, 'P'. (c) The Hin invertasome requires the DNA to form a small loop and a large loop and to align the inverted repeats at the *hin* gene, which serves as a recombination enhancer. The HU NAP assists the formation of the small DNA loop and FIS, binding to sites within the *hin* gene, and helps the four copies of the Hin recombinase to form a productive invertasome complex.

out their recombination reactions within the *res* sites of Tn*3* family members, but these proteins are not interchangeable functionally with the invertases: although resolvases from different transposons can substitute for one another (Rice 2015; Smith 2015). Collectively, resolvases and invertases are referred to as 'serine integrases', distinguishing them from the tyrosine integrases, of which bacteriophage lambda Int is the prototypic member.

2.14 Large Serine Recombinases

The large serine recombinases make up a family of site-specific recombinases that have been studied intensively in *Streptomyces* spp., *Mycobacterium* spp., and in nitrogen-fixing

cyanobacteria (Smith 2015). They are encoded by mobile genetic elements, including temperate bacteriophage, have no requirement for DNA negative supercoiling, and do not require host-encoded accessory factors such as NAPS. However, they do depend on directionality determinants that are encoded by the mobile genetic element and these influence the outcome of the recombination event (Singh, S. et al. 2014).

2.15 Transposition and Transposable Elements

Transposition is a form of illegitimate recombination in which the participating DNA sequences do not share homology and in which the reaction is catalysed by a transposase, usually encoded by the transposable element itself. Working within a complex known as the transpososome, two monomeric transposases each catalyse the nicking of one strand at either end of the transposon, the formation of a hairpin followed by its resolution and then the transfer of the nicked transposon strand to the cut target site (Bhasin et al. 1999; Kennedy et al. 1998, 2000).

Transposable elements vary greatly in complexity, ranging from IS that consist of the DNA needed to encode the transposase and the flanking sequences necessary for excision from the donor site and insertion into the target, to large elements that possess 'cargo' genes in addition to the basic transposition machinery (Siguier et al. 2014). IS can cooperate to create composite transposons in which two IS elements flank a segment of unique DNA and transpose as a unit. Over time, one of the IS may lose its independence due to mutation in the transposase gene, making its mobility contingent on the partner IS; Tn*5* (Figure 2.3) and Tn*10* (Figure 2.4), two of the most heavily-investigated transposons in molecular biology, are composite transposons of this type (Haniford and Ellis 2015).

Transposition can alter the genome and its functions in different ways. The transposon can interrupt a gene, depriving the cell of the gene product. Elements that have relaxed requirements for their sites of insertion are particularly prone to do this because they can insert almost anywhere. Bacteriophage Mu, which replicates via transposition, is known as the 'mutator' phage because it can target any part of the genome. Transposition into essential genes will kill the cell if the insertion causes a knockout mutation. It is also possible for an insertion to have indirect effects on the expression of several genes if it interrupts a regulatory gene. For example, transposition into the *hns* locus results in highly pleiotropic effects because the H-NS protein is a repressor of so many genes. Some transposable elements have promoters or partial promoters at their ends, and these can activate the transcription of genes located downstream from the site of insertion. For example, insertion sequence IS*10* has a strong, outward-reading promoter (P_{OUT}) that can transcribe an adjacent gene or operon (Figure 2.4). Transcription from IS*10* that resulted in expression of the *leuO* gene in *E. coli* revealed important information about the role of the LeuO protein, including its role in regulating the production of the DsrA *trans*-acting sRNA and hence the production of the RpoS sigma factor (Figures 1.20 and 1.21) (Klauck et al. 1997). Other elements have just a –35 box at their ends, but if this comes into register with a match to the consensus –10 box, a functional promoter may be created where none was present before. IS*911* is an example of a transposable element with such a –35 box; in the circularised IS*911*, it normally forms a strong promoter (P_{JUNC}) with a –10 that is

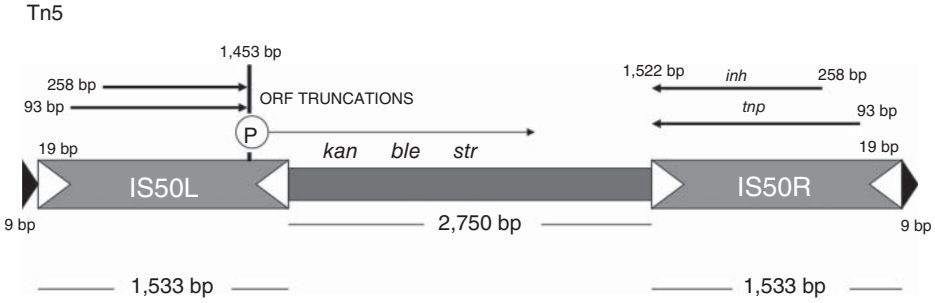

Figure 2.3 The structure of the composite transposon Tn*5*. This mobile element is composed of two almost-identical copies of IS*50*, arranged as inverted repeats: IS*50*L and IS*50*R. The cargo DNA between the IS50 copies contains an operon composed of genes expressing resistance to the antibiotics kanamycin/neomycin, bleomycin, and (in actinomycetes) streptomycin. The type II neomycin phosphotransferase encoded by the *kan* gene has been exploited as a selectable marker in many genetic tools used in recombinant DNA technology. It confers resistance to G418, the inhibitor of translation in eukaryotes. IS*50*R is an independent transposable element, expressing its own transposase from its *tnp* gene. IS*50*L is not capable of independent transposition due to a point mutation that has introduced a stop codon in its *tnp* gene. This mutation has also created the promoter for the transcription of the *kan-ble-str* operon. Transposition of Tn*5* depends on the transposase expressed by IS*50*R and the outer 19-bp inverted repeats. Tn*5* generates a 9-bp duplication of its target site upon insertion by the cut-and-paste transposition pathway. Expression of *tnp* is inhibited by methylation of a 5′-GATC-3′ at its promoter and by the activity of the nearby promoter of the *inh* gene. This gene is wholly contained within *tnp* and its product, the transposition inhibitor Inh, is a subset of the amino acid sequence of the transposase. Both proteins target the same inverted repeats but Inh is expressed to a higher level and usually manages to exclude Tnp.

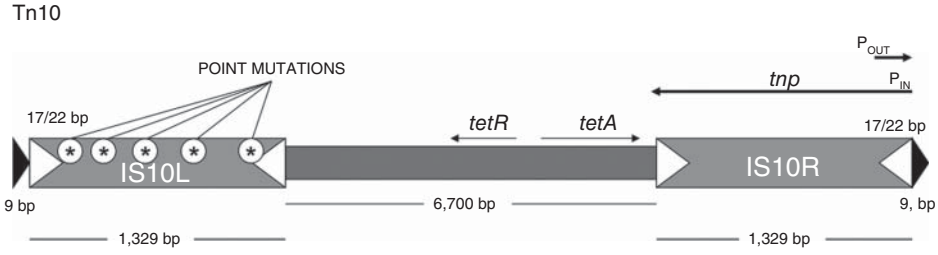

Figure 2.4 The structure of the composite transposon Tn*10*. Tn*10* is superficially similar to the composite transposon Tn*5* in being composed of two copies of an insertion sequence (IS*10*) arranged in inverted orientation, with one copy, IS*10*L, being fully functional and the other, IS*10*R, being non-functional as an independent transposable element due to several mutations (indicated by asterisks) that have rendered its transposase gene non-functional. The 6700-bp cargo DNA sequence between the IS elements encodes resistance to tetracycline: *tetR* expresses the TetR transcription repressor and *tetA* expresses the TetA cytoplasmic-membrane-located efflux pump that expels the antibiotic from the cell. TetR co-represses *tetR* and *tetA* by binding to a common operator sequence at both promoters: repression is relieved when tetracycline enters the cell and binds to TetR, altering its conformation and removing it from the operator. The transposase binds to the 17-to-22-bp inverted repeat sequences; repeat length varies from isolate to isolate. Insertion of IS*10*/Tn*10* generates a 9-bp direct repeat of the target site. The *tnp* gene is transcribed poorly from the weak P_{IN} promoter and the anti-sense transcript specified by the strong P_{OUT} promoter inhibits its translation.

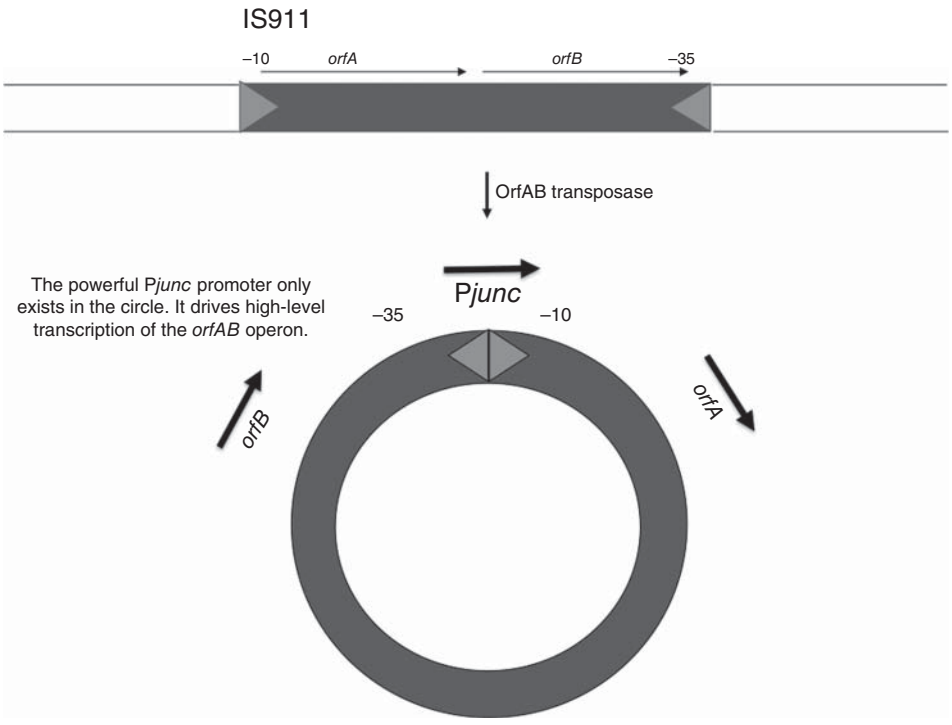

Figure 2.5 Formation of a hybrid promoter in the IS*911* circle transposition intermediate. When IS*911* is excised from the donor DNA molecule during transposition it forms a circular intermediate that includes a novel transcription promoter, P*junc*, whose −10 and −35 elements are brought into register at the junction of the transposon ends. This promoter can drive the transcription of the *orfAB* operon. In addition, the −13 box can become part of a new promoter at any insertion site that has an appropriately positioned −10 element next to the site. The creation of novel transcription units by incoming transposons is a useful way to alter bacterial gene expression patterns in a positive way (as opposed to altering them in a negative way by insertional inactivation of genes).

resident at the other side of the circle junction to transcribe IS*911*'s bipartite transposase gene (Figures 2.5 and 2.6) (Chandler et al. 2015). Identical copies of transposable elements are substrates for homologous recombination, and this can lead to novel arrangements in the genome, such as deletions, inversions, and amplifications, with associated changes to gene content, gene order, and gene copy number. These regions of sequence identity are also responsible for plasmid integration and excision, including the events that enable chromosome mobilisation by inserted self-transmissible plasmids in Hfr strains (Section 1.1).

2.16 Pathways of Transposition

Two transposition pathways have been described. In the replicative pathway, the transposon is copied during transfer to its target site, creating a copy at the target and another

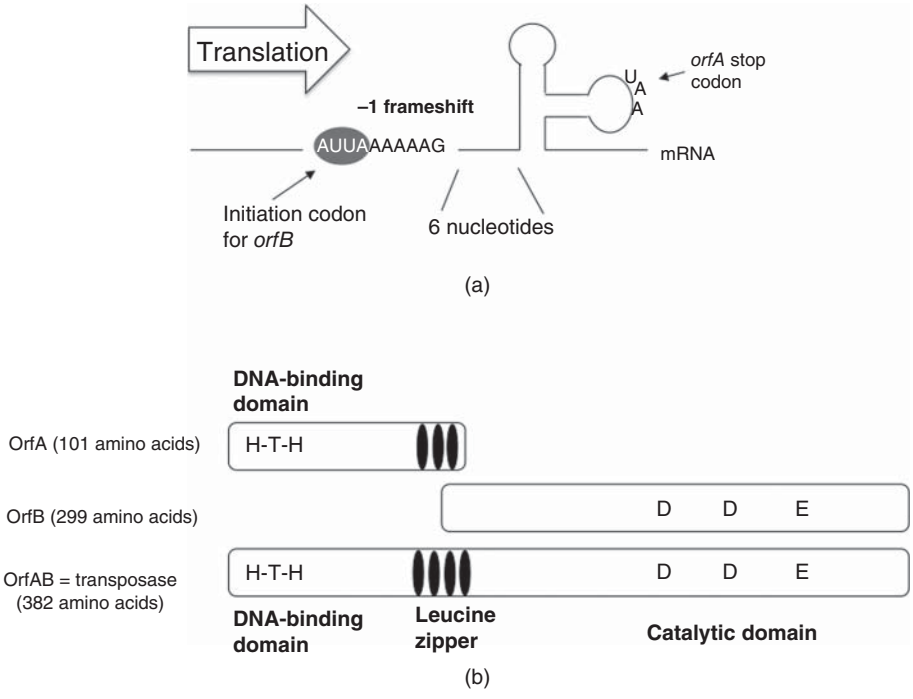

Figure 2.6 Control of transposase expression in insertion sequence IS*911*. The mobile element contains one transcription unit and two open reading frames, *orfA* and *orfB*. OrfA contains the helix-turn-helix DNA binding motif of the transposase and competes with the transposase for access to the ends of IS*911*. The 299-amino-acid OrfB is not produced as an independent protein. Instead, it is produced as part of the transposase when its open reading frame is brought into register with that of *orfA* by a rare frameshifting event that involves ribosome repositioning within an A-rich motif immediately before a region of secondary structure in the transposase gene's mRNA. If the −1 frameshift does not occur, translation of OrfA will terminate within the secondary structure. Translational slippage facilitates the expression of the full-length transposase, with the leucine zipper motif and the DDE catalytic domain being provided by the OrfB component. Possession of the same DNA-binding domain as OrfA and the low frequency of production of the transposase means that the number of successful transposition events of IS*911* is minimised.

copy at the donor site (Figure 2.7). This pathway doubles the copy number of the transposon. Targeting self-transmissible plasmids or bacteriophage provides the transposon with an opportunity to disseminate copies of itself laterally through the bacterial population, along with any cargo genes that it may possess. The Tn*3* family of transposons uses this pathway and Tn*3* itself carries as cargo a gene for ampicillin resistance (Figure 2.8). Its replicative transposition spreads resistance to this β-lactam antibiotic, making it a cause for concern in the context of antimicrobial resistance. The transposition pathway used by Tn*3*-like elements creates a physical connection between the donor and target molecules, linking them by two directly repeated DNA bridges that consist of transposon copies. This structure is known as a cointegrate and it can be resolved by RecA-dependent homologous recombination, using the direct repeats as the participating regions of homology. In practice, Tn*3*-like transposons typically use a transposon-encoded resolvase enzyme to resolve

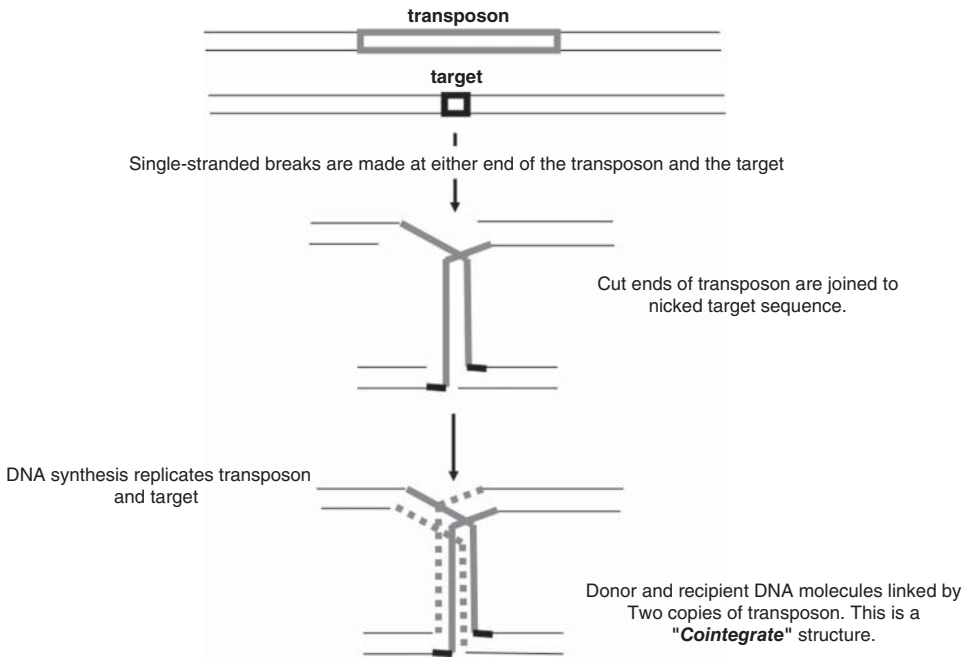

Figure 2.7 The replicative pathway of transposition. The transposon is represented by a grey rectangle in the donor dsDNA molecule. The target site is represented as a box in the recipient dsDNA molecule. The transposon-encoded transposase cuts the donor molecule at the junction between the transposon and the flanking DNA and makes a staggered cut at the target sequence. The top strand of the transposon is joined to the top strand of the recipient and the bottom strand of the transposon is joined to the bottom strand of the recipient, creating a bridge between the donor and recipient replicons. The bridging strands become templates for DNA synthesis, giving the pathway its name (the replicative pathway). The product of this transposition pathway is a cointegrate, composed of two previously independent replicons that are now components of a single molecule connected by two directly repeated copies of the transposon.

the cointegrate via a site-specific recombination mechanism at two directly repeated *res* sites located within the bodies of the transposons (Figure 2.9). This protein is a member of the serine invertase/integrase family discussed in Section 2.13.

The second transposition pathway involves a cut-and-paste mechanism with minimal DNA replication (the 'non-replicative' pathway of transposition) (Figure 2.10). Here, the transposon is excised from its donor site, leaving a double-stranded break in the DNA, and then inserted into its target site, with each step being catalysed by the transposon-encoded transposase. Creating double-stranded breaks is hazardous (especially in the single chromosome of a bacterium) and transposons using this pathway have evolved a number of strategies to restrict transposition to periods when it is relatively safe to do so. For example, the creation of hemimethylated 5′-GATC-3′ sites may be required in a transposase-binding site before the transposase can bind efficiently, or hemimethylated DNA may be needed before the promoter of the transposase gene can become active. These dependencies link transposition to the bacterial cell cycle and the generation of transiently hemimethylated chromosomal DNA, a period when a second chromosome copy is being

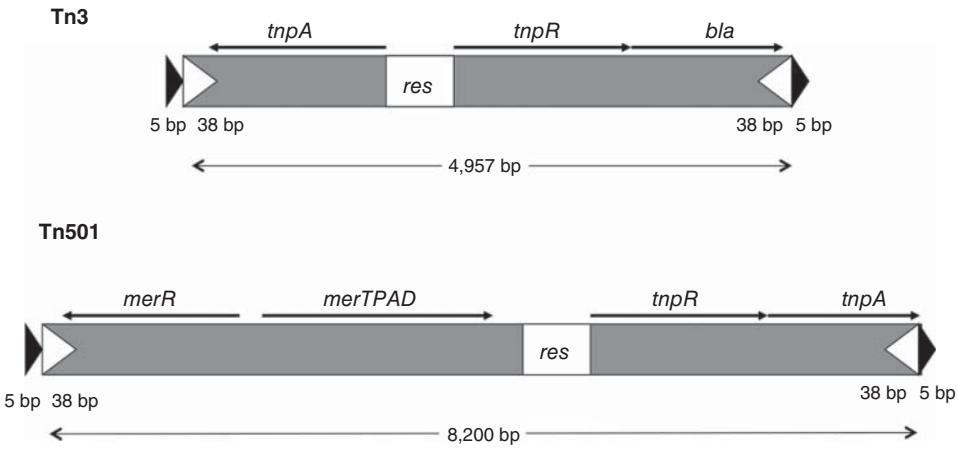

Figure 2.8 The structure of transposon Tn*3* and Tn*3*-family member Tn*501*. Tn*3* is almost 5 kb in length and is flanked by 38-bp terminal inverted repeats. It creates direct repeats of 5 bp upon insertion at its target site. Its two main transcription units are *tnpA,* encoding the transposase, and *tnpR bla,* a bicistronic operon encoding Tn*3* resolvase and β-lactamase, a periplasmic enzyme that confers resistance to β-lactam antibiotics such as penicillin. The divergently arranged promoters of the two transcription units share a binding site for the resolvase protein. This site, *res,* is bifunctional and acts as an operator for the resolvase when this protein is acting as a transcription repressor, and as a site for cointegrate resolution by resolvase-mediated site-specific recombination (Figure 2.9). Tn*501* is larger than Tn*3* and has three major transcription units: *merR, merTPAD*, and *tnpA tnpR*. The latter encodes the transposase (TnpA) and resolvase (TnpR) and its promoter is repressed when TnpR binds to the *res* site. As in Tn*3,* the *res* site in *Tn501* is used to resolve the cointegrated products of transposition by TnpR-mediated site-specific recombination. The terminal inverted repeats of Tn*501* and the extent of the target site duplication are the same as those of Tn*3*.

produced. The appearance of a double-stranded DNA break, one that may not be repaired quickly, may be tolerated in these circumstances, allowing the intact chromosome and its transposon insertion to have a future together.

2.17 Peel-and-paste Transposition

IS*200* was discovered in the histidine biosynthetic (*his*) operon of *Salmonella* and was thought initially to be a *Salmonella*-specific insertion sequence (Lam and Roth 1983). It has subsequently been detected in related Gram-negative bacteria and is unusual in that it transposes via an obligatory single-stranded DNA intermediate (He et al. 2014). IS*200* has one open reading frame and this encodes its transposase, TnpA, an enzyme with a structure that is distinct from that of the DDE transposases encoded by most insertion sequences. TnpA from IS*200* and related elements belongs to the HuH superfamily of enzymes that specialise in handling single-stranded substrates, a group that is distinct from the DDE transposase family (Chandler et al. 2013). IS*200* does not have inverted repeats at its ends and, because it uses a different transposition pathway to elements that rely on DDE transposases, it does not create a target site duplication following transposition. The *tnpA* gene is poorly transcribed, with about 80% of transcripts being terminated

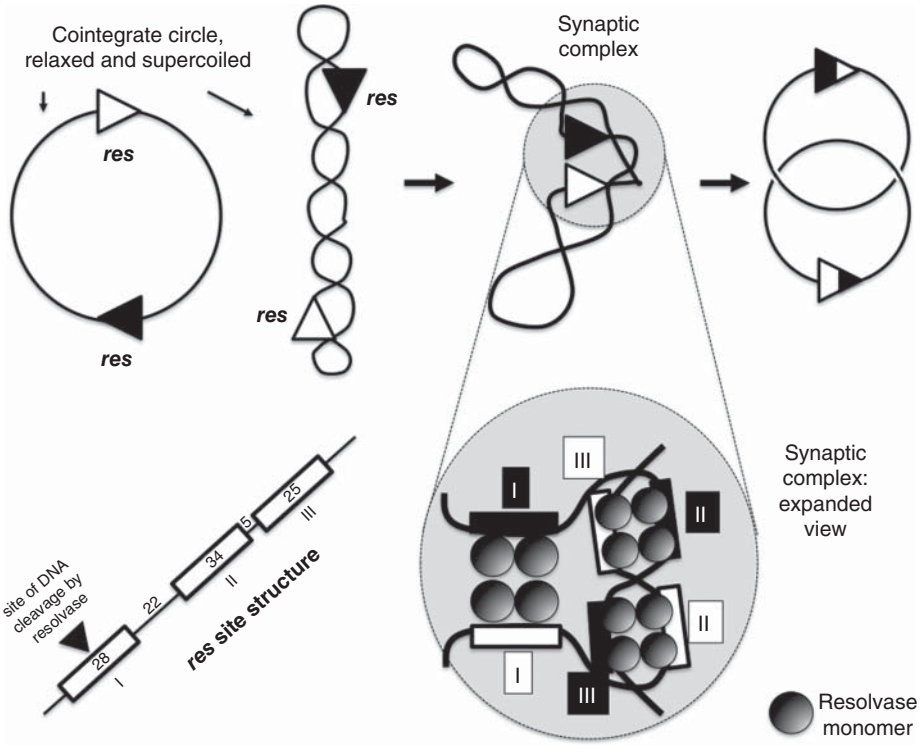

Figure 2.9 Cointegrate resolution in Tn*3* transposition. The cointegrate circle contains two directly repeated copies of the *res* site, each within one copy of Tn*3*. In the interwound form of the supercoiled circle, the *res* sites become aligned and form a synaptic complex when bound by the homotetrameric resolvase protein (encoded by the *tnpR* gene in Tn*3*). Each *res* site is composed of three subsites, I, II, and III. In the synaptic complex, sites I and I, II, and III and III and II align and are bridged by a resolvase tetramer. However, only the site-I-resolvase-site-I component undergoes DNA cleavage, strand transfer, and religation. The complexes involving *res* subsites II and III are simply architectural and add stability to the overall structure. The sub-structure of the *res* site is shown at lower left (the Arabic numbers refer to the length of specific features in base pairs) and the expanded view of the synaptic complex illustrates the alignment of the various subsites and their interactions with resolvase. The products of resolvase-mediated cointegrate resolution are catenated circles (top right), each with one copy of Tn*3* (and the *res* site). These are separated into unlinked circles by a topoisomerase.

prematurely by an intrinsic terminator near the 5′ end of the gene (Figure 2.11). The mRNA is poorly translated because of sequestration of the translation initiation signals in the folded transcript; there is also Hfq-dependent antisense RNA control that minimises TnpA production (He et al. 2014).

TnpA interacts with DnaN, the beta clamp (or processivity factor) of the replisome, targeting transposition to the replication fork (Lavatine et al. 2016). The TnsE protein encoded by Tn*7* performs a similar function (Parks et al. 2009) and many transposable elements may link their mobility to the moving replication fork (Chandler 2009). An advantage of this strategy is that excising/inserting the element behind the moving fork alters the genetic structure of only one of the two chromosome copies (Figure 2.12).

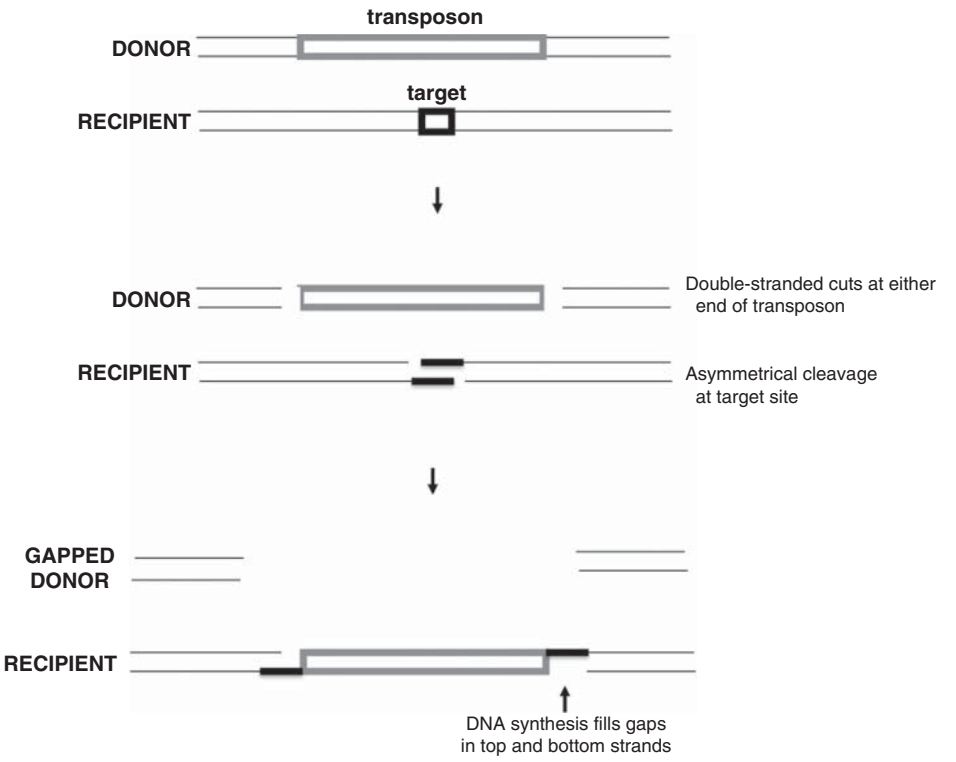

Figure 2.10 The cut-and-paste (or non-replicative) pathway of transposition. The transposon is represented by a grey rectangle in the donor dsDNA molecule. The target site is represented as a box in the recipient dsDNA molecule. The transposon-encoded transposase cuts the donor molecule at the junction between the transposon and the flanking DNA and makes a staggered cut at the target sequence. The transposon is pasted into the staggered gap in the recipient DNA molecule and the short gaps are repaired by DNA synthesis; this creates a direct repeat whose length is characteristic of that transposon. The donor molecule has a double-stranded break that must be repaired if this molecule is not to be lost.

The IS*200*-related element IS*608* has a preference for inserting at stalled replication forks. Its frequent appearance in the heavily transcribed *rrn* operons of *E. coli* has been interpreted as insertion sites being generated when heavy *rrn* transcription stalls replication forks as they transit the operons (Ton-Hoang et al. 2010).

In the peel-and-paste transposition mechanism, a copy of one strand of the mobile element in the lagging strand has its ends annealed prior to excision as a single-stranded circle (Figure 2.11). The excision event simultaneously repairs the gap in the donor strand. A target sequence is identified in the lagging strand of a replication fork and a synaptic event catalysed by TnpA inserts the mobile element. It is thought that no host-encoded cofactors are required to assist TnpA in its operations (He et al. 2014). Genes encoding HuH-type TnpA enzymes have been found associated with REP and BIME repeat elements in *E. coli* and other bacteria, leading to speculation that these elements may be (or have been) non-autonomous mobile elements (Ton-Hoang et al. 2012).

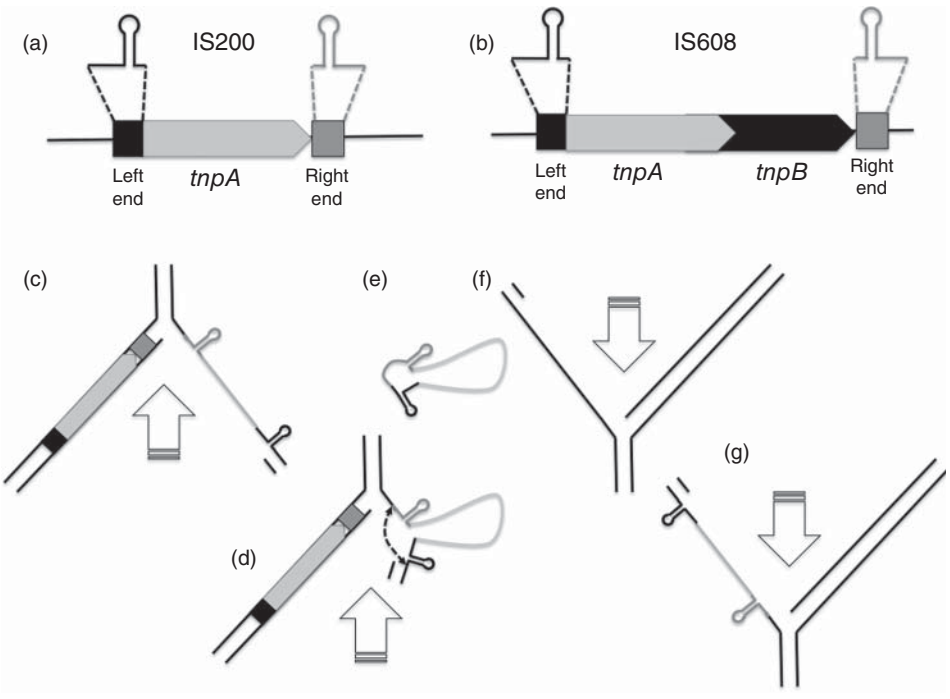

Figure 2.11 Peel and paste transposition. (a) Structure of IS*200* showing the position and orientation of the *tnpA* gene with respect to the Left and Right ends of the element. The extruded hairpins are shown about their respective ends. (b) Structure of IS*608* using similar symbols as for IS*200*. The *tnpB* gene is a second open reading frame that partially overlaps *tnsA* and whose function is unclear. (c) IS*200* at a moving replication fork (the direction of the moving replisome is indicated by the vertical arrow). The copy of IS200 on the lagging strand is mobilised for transposition by the TnpA enzyme and a synaptic complex is formed followed by excision of IS*200* (d) as a single-stranded DNA circle (e) that is then targeted to another replication fork (f) where it is inserted (g). Not to scale.

2.18 Control of Transposition

Transposable elements are generally characterised by very poor expression of their transposase enzymes. This arises from a variety of causes, such as weak transcription promoters, poor translation initiation signals, mRNA folding that interferes with translation initiation or elongation, interference by trans-acting RNA, or inhibitor proteins. Expressing an inhibitor protein efficiently from the same DNA template as the poorly expressed transposase sets up a competition between two DNA-binding proteins for access to the same sites that the inhibitor protein may be expected to win most of the time. Another strategy involves a need for infrequent translational slippage to bring into register two open reading frames to produce a mature transposase: most of the time the gene expresses only a truncated competitor protein, with DNA-binding activity but no transposase functions, that binds and blocks the site needed by the rarely expressed transposase if it is to catalyse transposition.

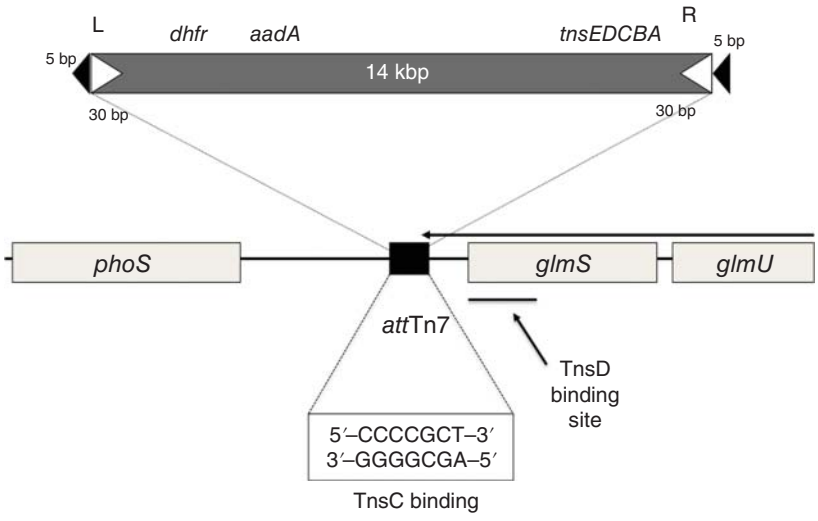

Figure 2.12 Transposon Tn*7* and *att*Tn*7*. The 14-kb transposon Tn*7* has a left (L) and a right (R) end and inserts into *att*Tn*7* in an orientation-specific manner as shown. Tn*7* encodes resistance to trimethoprim, an inhibitor of dihydrofolate reductase, by expressing a drug-resistant derivative of the enzyme from its *dhfR* gene. The *aadA* gene encodes an adenyltransferase that confers resistance to streptomycin and spectinomycin. The transposition proteins are encoded by the *tnsEDCBA* operon at the R end of Tn*7*. The TnsC protein binds to the target sequence within the 3′ end of the *glmUS* transcription unit. TnsC binds nearby within the *glmS* open reading frame. Insertion of Tn*7* at *att*Tn*7* is by cut-and-paste transposition and a 5-bp duplication of the target sequence is produced. During the transposition reaction, the TnsA and TnsB transposition proteins bind to the 30-bp inverted repeats at the termini of the transposon and to *att*Tn*7*.

Another impediment to frequent transposition is imposed by the poor *trans*-activity of transposases: these enzymes are 'sticky' and tend to act preferentially in *cis* at the element that encodes them, whereas their competitor inhibitor proteins or RNAs act efficiently in *trans*. *Cis*-preference may have a number of causes: in the case of the transposase encoded by IS*911* the explanation involves tethering of the nascent transposase to a ribosome that is translating the transposase mRNA in a process called cotranslational control (Duval-Valentin and Chandler 2011).

Many transposons rely on a single transposase protein to jump from site to site in the genome. Others have more sophisticated requirements and employ additional proteins as target detectors. Tn*7* has five transposition proteins and a special relationship with a unique site called *att*Tn*7* that is frequently located within the *glmS* transcription unit (between the transcription terminator and the end of the open reading frame) (Figure 2.12). Transcription of the *glmS* gene is inhibitory to the use of *att*Tn*7* by the transposon; this may represent a link between bacterial physiology and Tn*7* transposition (Deboy and Craig 2000). The name of this transposon insertion site is misleading, because it is not an 'attachment' site as used by temperate bacteriophage like lambda, where phage entry at *attλ* involves site-specific recombination. Tn*7* transposes into *att*Tn*7* and it does so in an oriented manner through a cut-and-paste mechanism (Choi et al. 2013). However, Tn*7*

can transpose to pseudo-*att*Tn*7* sites located elsewhere in the genome and, at a low frequency, to sites that are unrelated in sequence to *att*Tn*7*. These alternative target site choices require different Tns proteins. Tn*7* has five, of which TnsA and TnsB constitute the transposase. The adenosine triphosphate (ATP)-binding TnsC protein is a transposition regulator that interacts with TnsA, TnsB, and with TnsD at *att*Tn*7* (Choi et al. 2014; Ronning et al. 2004). TnsC is also a target immunity protein that interacts through space with TnsB and imposes target immunity, preventing colonisation of a region of DNA by Tn*7* if another Tn*7* is already installed there (Skelding et al. 2003; Stellwagen and Craig 1997). TnsD selects the *att*Tn*7* site, it binds within the open reading frame of the *glmS* gene; TnsE is involved preferentially in the selection of non-*att*Tn*7* on self-transmissible plasmids (Wolkow et al. 1996). This TnsE-mediated option allows Tn*7* to disseminate itself by lateral gene transfer; neither the TnsD- nor the TnsE-directed pathways is likely to interrupt chromosomal genes in the bacterial host, enhancing the survival of Tn*7* after transposition. The TnsE protein contains a motif for interaction with the processivity factor, or beta clamp (DnaN) of the replisome. This allows TnsE to direct Tn*7* to the moving replication fork and the beta clamp directs the orientation of the transposon ends (Parks et al. 2009).

The Tn*5090*/Tn*5053* elements resembles Tn*7* in using a transposase (TniA) and a target-selecting regulator protein (TniB) but they use the replicative pathway of transposition and encode a resolvase protein (TniR), and have a *res* site within the elements (Kholodii et al. 1993; Radstrom et al. 1994). Interestingly, Tn*7* can itself be made to use the replicative pathway of transposition if the active site of TnsA is mutated, relying on TnsB to catalyse the transposition reaction (May and Craig 1996). Bacteriophage Mu uses the cut-and-paste pathway of transposition to establish itself in the host chromosome and then switches to the replicative transposition pathway to multiply itself prior to cell lysis following induction of the prophage (Harshey 2014). Mu also resembles Tn*7* in having a transposase and a target-selecting regulator protein. However, Mu is also a bacteriophage so it can mediate its own horizontal transfer directly by forming infectious virus particles to package its genome.

Tn*7*-like transposons carry a variety of cargo genes, including genes for antibiotic resistance. These include *dfrA1*, coding for trimethoprim resistance; *sat2* coding for streptothricin resistance; and *aadA1*, encoding resistance to spectinomycin and streptomycin (Figure 2.12). These genes make up the cassettes of a type 2 integron, an integron whose tyrosine integrase gene, *intI2*, is interrupted by a stop codon. This genetic defect may explain why type 2 integrons seem to have a rather restricted distribution (Ramírez et al. 2010). The structure, function, and significance of integrons are summarised in Section 2.22.

One of the most interesting cargo gene sets carried by *Tn7*-like transposons consists of incomplete clustered regularly interspaced short palindromic repeat (CRISPR) elements (Peters et al. 2017). These lack the nucleases required to destroy target sequences but do contain arrays capable of specifying mature crRNAs that can interact with those targets, generating R-loops that may direct the transposon to insert there through RNA-guided transposition (Peters et al. 2017). This represents an interesting repurposing of a process that is used widely to eliminate invading phage and plasmid genomes to serve as an aid to transposition.

2.19 Host Factors and Transposition

Host factors play important parts in the transposition of numerous mobile genetic elements. One of these elements is temperate bacteriophage Mu, a virus that replicates via transposition (Section 2.18) (Harshey 2014) and uses the FIS-dependent Gin/*gix* serine-invertase-mediated site-specific recombination system to vary the composition of its tail fibres, extending its host range (Section 2.13) (Cerdeño-Tárraga et al. 2005). DNA gyrase is essential for the replicative transposition of bacteriophage Mu (Pato et al. 1990; Ross et al. 1986) but not the initial cut-and-paste transposition event following infection (Sokolsky and Baker 2003), and the binding site that is used by this type II topoisomerase at the mid-point of the Mu genome is the strongest known gyrase site (Pato 1994). Gyrase activity brings together the ends of the Mu prophage to form the transpososome (Pato and Banerjee 1996) and Mu becomes a stable microdomain within the bacterial chromosome (Saha et al. 2013). Mu also relies on the NAPs IHF and HU, and the ClpX protease, to complete its cut-and-paste transposition into its host's genome (Kobryn et al. 2002; Savilahti et al. 1995). Negative DNA supercoiling and the IHF NAP are important adjuncts to IS*10* (and Tn*10*) transposition (Chalmers et al. 1998; Crellin et al. 2004), with the transpososome being stabilised by the H-NS NAP and channelled towards intermolecular transposition and dissemination (Singh et al. 2008; Wardle et al. 2009). Downregulation of IS*10* transposase by the *trans*-acting RNA_{OUT} is enhanced by the Hfq RNA chaperone (Ross et al. 2010). Hfq has also been reported to upregulate the production of transposase in *Tn5* (IS*50*), but in this case the effect is exerted at the level of transcription (Haniford and Ellis 2015). Transposition of Tn*5* and (IS*50*) is enhanced by the FIS NAP, possibly indirectly, channelling increased transposition to the early exponential phase of the growth cycle, when FIS is abundant (Weinreich and Reznikoff 1992). However, FIS is inhibitory to IS*50* transposition through FIS binding to hemimethylated 5′-GATC-3′ sites at the inside end of the element within Tn*5*. The inhibitory role of FIS in IS*50*-only transposition may favour FIS-enhanced transposition of the complete Tn*5* (Weinreich and Reznikoff 1992). These observations suggest that FIS links Tn*5* transposition to the growth cycle and the cell cycle because FIS is usually abundant in early exponential phase and hemimethylated DNA is generated by DNA replication.

2.20 Integrative and Conjugative Elements (ICE)

Integrative and conjugative elements (ICE) were known formerly as conjugative transposons. However, they do not encode a transposase, using instead a form of tyrosine integrase that is tolerant of sequence divergence within its target sites (Cheng et al. 2000). Mobile elements that have the ability to transfer themselves from cell to cell by conjugation display a high degree of autonomy in their capacity for horizontal transfer through a bacterial population. CTnDOT of *Bacteroides* spp. is an example of a conjugative transposon that has been investigated in detail (Figure 2.13) (Wood and Gardner 2014). This large (65 kb) mobile genetic element can excise from the donor chromosome, circularise, and then promote its own transfer to another cell through a conjugation bridge encoded

by its *tra* genes. There are six target sites, or *attB* sites, for CTnDOT in the *Bacteroides* genome. Thus, this element combines the abilities of a temperate bacteriophage and a self-transmissible plasmid by being able to insert into, and excise from, selected target sites and to transmit itself horizontally from cell to cell. CtnDOT can mobilise conjugative plasmids and transposons (Li et al. 1995; Valentine et al. 1988). It carries genes for antibiotic (tetracycline and erythomycin) resistance, genes that are disseminated along with the CTnDOT element (Nikolich et al. 1992; Whittle et al. 2001). The tyrosine integrase encoded by CTnDOT is IntDOT, and this relies on CTnDOT-encoded directionality determinants when performing excisive recombination. These proteins, Xis2c, Xis2d, and Exc, are encoded by genes within CTnDOT; a host-encoded protein, BHFa, is also needed (Cheng et al. 2001). Their expression is upregulated following exposure of the bacterium to tetracycline through a regulatory cascade involving the *tetQ-rteA-rteB* operon and the *rteC* regulatory gene (Moon et al. 2005; Park and Salyers 2011). Tetracycline, a bacteriostatic antibiotic, will place the general population into stationary phase, enhancing its susceptibility to conjugative DNA transfer, while at the same time driving excision of the conjugative CTnDOT element from the donor chromosome.

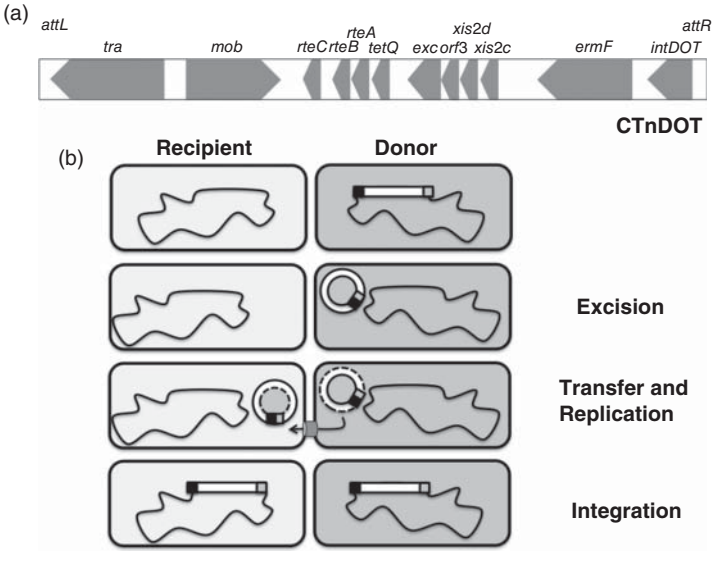

Figure 2.13 The CTnDOT Integrative Conjugative Element (ICE). (a) The rectangle represents the 65-kb mobile genetic element. The block arrows are the genes and contiguous arrows are members of operons: *tetQ-rteA-rteB, xis2c-xis2d-orf3-exc*. The *rteC* gene is an independent transcription unit and it encodes a positive regulator of the *xis* operon. The locations of the genes involved in CTnDOT conjugative transfer (*tra*) and mobilisation (*mob*) are indicated, as is the position of the erythromycin resistance gene (*ermF*) and the gene encoding the integrase (*intDOT*). The *ermF* gene is part of a genetically complex region that includes a mobilisable transposon and a composite transposon (not shown) (Whittle et al. 2001). The orientations of the block arrows show the direction of gene transcription. (b) A summary of the main steps in CTnDOT transfer. The element is excised from the donor chromosome and circularises. One DNA strand is then transferred by conjugation to the recipient cell where it integrates at an *attB* site. In the donor cell, the reintegration of the circularised element at *attB* restores the status of CTnDOT prior to excision. (The figure is not to scale.)

2.21 Integrons

Bacteria possess genetic elements called integrons that consist of a stable platform into which gene cassettes can be inserted to add new functions to the cell (Figure 2.14) (Stokes and Hall 1989). It is thought that integrons that were originally stably associated with bacterial chromosomes acquired mobility by associating with mobile genetic elements, leading to their widespread dissemination by HGT (Boucher et al. 2007; Cambray et al. 2010; Escudero et al. 2015; Mazel 2006). The typical integron platform has an *intI* gene, encoding a subtype of tyrosine integrases that is specialised for integrating gene cassettes into integrons by site-specific recombination. Integrons can grow to large sizes, as illustrated by the superintegron in *V. cholerae*, which, with 175 cassettes, represents 3% of the genome (Mazel et al. 1998). Cassettes can encode many functions, especially antibiotic resistance in the case of integrons on mobile genetic elements. The cassettes found in the *V. cholerae* superintegron

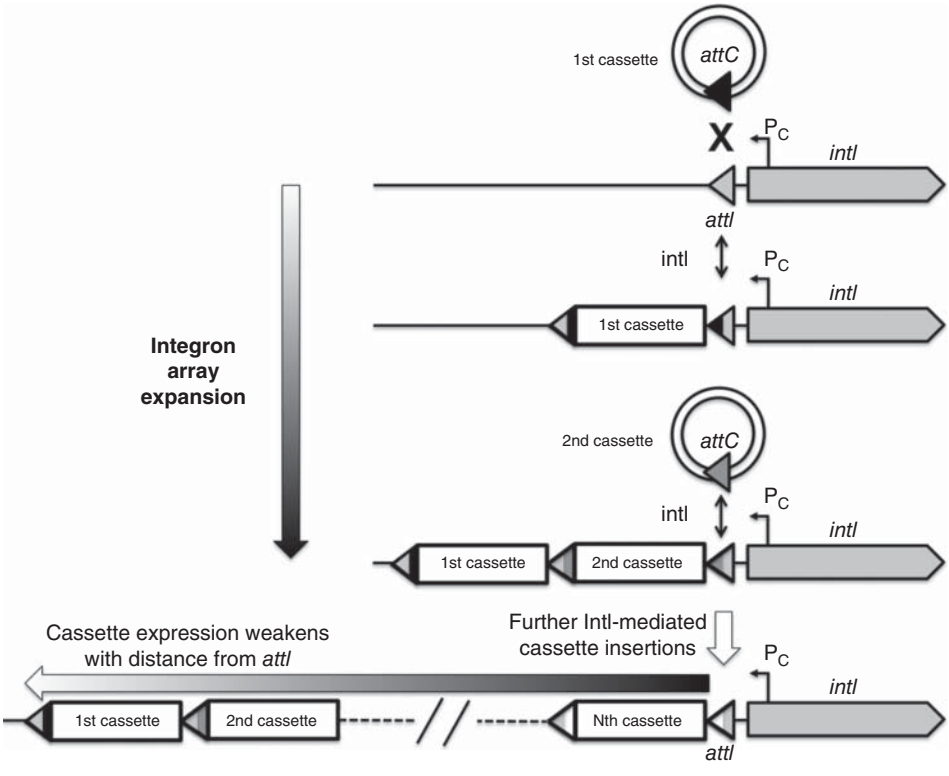

Figure 2.14 Integron structure. The basic platform of the integron consists of the *intI* gene encoding the integrase, the *attI* site at which cassettes are introduced by IntI-mediated site-specific recombination and the P_C promoter from which the cassette genes are transcribed. Recombination occurs between the *attI* site and the *attC* site on the circular cassette. This process is reversible. Additional cassettes are added to the integron at the *attI* site, moving the original cassettes further away from the P_C promoter. Those at the greatest distance are expressed weakly, or not at all, due to a defect in ribosome movement across *attC* structures along the integron transcript. IntI-mediated excision of cassettes and their reintroduction at *attI* brings silent cassettes back into use.

include some specifying toxin/antitoxin systems that promote the stability of the integron (Iqbal et al. 2015). Integrons are classified by their integrases and five classes have been identified. Classes 1 and 3 are associated with transposon Tn*402* (Escudero et al. 2015) while Class 2 is made up predominantly of Tn*7*-associated elements (Ramírez et al. 2010). Classes 4 and 5 are associated with *Vibrio* spp. (Hochhut et al. 2001; Sørum et al. 1992).

Cassette integration occurs at a specific site called *attI* that is usually located upstream of *intI*. A promoter, P_C, associated with *intI*, transcribes the inserted gene cassettes as an operon. As the array grows in length, gene cassettes that are at a distance from the P_C promoter are no longer expressed but continue to serve as a source of stored genetic memory; the order of the cassettes can be shuffled, bringing silent genes back into use (Collis and Hall 1995). Prior to integration, the cassettes exist as non-replicating DNA circles consisting of the open reading frame of the gene that is to be captured, and a site called *attC* that is the substrate for IntI-mediated integrative recombination at *attI* (Collis and Hall 1992). The presence of the *attC* elements between cassettes has been proposed as an attenuating influence on downstream cassette gene expression: the reduction in expression of cassettes downstream from P_C may reflect a negative impact of the *attC* elements interspersed between the cassettes on ribosome movement along the transcript (Jacquier et al. 2009). The special role of the *attC* elements in the production of the single-stranded DNA needed as a substrate for IntI-mediated recombination links the process to the forces involved in cruciform extrusion at *attC* (Lilley and Palecek 1984). In particular, DNA negative supercoiling is a key candidate for the driver of cruciform formation, and this links the recombination process to the physiology of the cell. The SSB protein suppresses cruciform extrusions that might interfere with DNA replication, conferring genetic stability on the integron (Escudero et al. 2015).

The IntI tyrosine recombinases expressed by integrons are unusual because they recombine one strand of the *attC* element with the double-stranded *attI* (Escudero et al. 2015). They differ from those tyrosine integrases that process double-stranded substrates in having an additional alpha helix inserted between conserved tyrosine integrase active site sequence patches, an addition that is essential for IntI activity in *attC* X *attI* recombination (Messier and Roy 2001). The reaction catalysed by IntI at cassette integration is reminiscent of that catalysed by the XerCD tyrosine integrases when the single-stranded DNA genome of filamentous bacteriophage CTXϕ integrates at the double-stranded *dif* site in the chromosomes of *V. cholerae* (Val et al. 2005) (Section 1.8). The *attC* X *attI* reaction is the most efficient of the various possible combinations, making integration at *attI* the most likely pathway for new cassette additions to the array, rather than at some random *attC* elsewhere in the integron (Collis et al. 1993, 2001). Excision of cassettes involves IntI-mediated *attC* X *attC* recombination, creating an excised, non-replicating circle with one *attC* site. If this is circle is not recaptured, it will be lost at cell division. Recapture by IntI-mediated insertion at *attI* brings the cassette back to the front of the array where its expression will be enhanced; insertion at a randomly chosen *attC* site reshuffles the order of cassettes in the array.

LexA, the master repressor of the SOS response (Section 2.32; Table 2.1), represses the *intI* promoter by binding to a LexA box that overlaps the –10 box of the promoter, linking *intI* transcription to DNA damage (Guerin et al. 2009). This damage increases the amount of single-stranded DNA in the cell and RecA detects this. Polymerisation of RecA causes autoproteolytic cleavage of LexA, leading to upregulation of members of the LexA regulon,

Table 2.1 The members of the LexA regulon in *E. coli*.

Gene	Comments
cadB	Cadaverine transport; identified by LexA ChIP analysis; Wade et al. (2005)
cho (*ydjQ*)	UvrC homologue; excision repair endonuclease; Moolenaar et al. (2002)
dinB	DNA polymerase IV
dinD (*pcsA*)	DNA-damage-inducible protein; Ohmori et al. (1995b)
dinF	Multidrug and toxic compound extrusion protein; Lackey et al. (1985); Rodríguez-Beltrán et al. (2012)
dinG	ATP-dependent helicase; Lewis and Mount (1992)
dinH	The LexA-repressed promoter of the *ftsK* gene; Dorazi and Dewar (2000b)
dinI	DNA-damage-inducible protein; interacts with the RecA filament and may regulate its activity; Galkin et al. (2011)
dinP (*dinB*)	Two names for the same gene (DNA polymerase IV); Kim et al. (1997); Ohmori et al. (1995a)
dinQ	A toxic inner membrane peptide; probably the toxin component of a TA system; expression controlled by sRNA; Weel-Sneve et al. (2013)
dinS	DNA-damage-inducible protein; putative transposase; Simmons et al. (2008)
ftsK	Molecular motor for directed movement of KOPS sequences during terminal stages of chromosome segregation; transcribed from *dinH*
hokE	Toxin component of a TA system; Pedersen and Gerdes (1999)
lexA	Master regulator of the LexA regulon; transcription repressor
molR (*dinO*)	Molybdenum metabolism regulator; Lee et al. (1990); identified by LexA ChIP analysis; Wade et al. (2005)
otsBA	Trehalose production; RpoS regulon; identified by LexA ChIP analysis; Wade et al. (2005)
polA	DNA polymerase I; identified by LexA ChIP analysis; Wade et al. (2005)
polB (*dinA*)	DNA polymerase II
recA	Homologous recombination; Co-protease for LexA
recN	DNA repair protein; SMC-like; substrate for ClpXP; Nagashima et al. (2006)
recX	Inhibitor of RecA activity
rmuC (*yigN*)	Recombination limiting protein
ruvAB	Helicase for Holliday junction branch migration
sbmC	Inhibitor of DNA gyrase activity
ssb	Single-stranded-DNA binding protein
sulA (*sfiA*)	Cell division inhibitor; binds to FtsZ and prevents septum formation until DNA damage is repaired; Hill et al. (1997)
symE (*yjiW*)	Toxin component of a TA system
tisAB (*ysdAB*)	Stress-inducible peptide toxins; Vogel et al. (2004)
umuDC	DNA polymerase V; error-prone DNA polymerase that is induced following DNA damage; trans-lesion DNA synthesis; SOS mutagenesis; Tang et al. (1999)
uvrA (*dinE*)	Nucleotide excision repair; excision nuclease, subunit A; Kenyon and Walker (1981)
uvrB	Excision nuclease, subunit B
uvrD	DNA dependent ATPase I and helicase II
ybfE	DNA-damage-inducible protein; Fernandez de Henestrosa et al. (2000)
ydjM	Cytoplasmic membrane protein; Daley et al. (2005)
yebG	DNA-damage-inducible protein; Lomba et al. (1997)

including the *intI* gene. This may help to couple recombination events at the integron to periods when the bacterium is experiencing events that are threatening to its survival, periods when testing new gene expression patterns may bring survival benefits (Cambray et al. 2011). In the *V. cholerae* integrons, the cAMP-CRP complex also plays a role in *intI* gene regulation, linking integron function to cAMP levels (Baharoglu et al. 2012). In this organism, cAMP-CRP works with TfoX and HapR to influence the import of single-stranded DNA into the cell via competence and transformation (Baharoglu et al. 2012) (Section 7.9).

2.22 Introns

The first prokaryotic introns to be detected were in bacteriophage T4. These self-splicing group I introns were in the *nrdB* (aerobic ribonucleotide reductase: small subunit), *nrdD/sunY* (anaerobic ribonucleotide reductase) and *td* (thymidylate synthase) genes (Chu et al. 1984; Gott et al. 1986). These are mobile genetic elements possessing a homecoming endonuclease gene (HEG) (Haugen et al. 2005). The endonuclease targets an intronless equivalent of its genetic home in another genome (e.g. an intronless version of its own bacteriophage). Insertion of the intron into the target interrupts the target sequence, making it immune to further intron insertion. The double-stranded break made in the insertion process is repaired through recombination-dependent replication repair in which the donor DNA molecule serves as the template (Mueller et al. 1996; Sandegren and Sjoberg 2004).

Group II introns are ribozymes that self-splice via a lariat RNA intermediate (Toro et al. 2007). They also transfer themselves to intronless copies of their home location (retrohoming) or to unrelated sites (retrotransposition) via a complex that consists of the RNA lariat and an intron-encoded protein. Group II introns are thought to have originated in bacteria before invading the nucleus of primitive eukaryotes (Lambowitz and Belfort 2015; Martin and Koonin 2006). Although bacterial group II introns can self-splice, the intron-encoded protein normally assists the process. This protein has a maturase function that assists the RNA splicing reaction and it has a reverse transcriptase activity to copy the intron into DNA during retrohoming or retrotransposition (Toro et al. 2007).

2.23 Horizontal Gene Transfer

Genetic information in bacteria is transmitted horizontally as well as vertically. HGT allows bacteria to acquire new traits through a single event, potentially accelerating the rate of genome evolution. For example, the arrival of a plasmid encoding antibiotic resistance in a bacterium makes a very real and material difference to the ability of the organism to survive if the relevant antibiotic appears in its environment.

HGT occurs through the processes of transduction, transformation, or conjugation. HGT was first described in *E. coli* in the 1940s (Tatum and Lederberg 1947) and has played a very important role historically in bacterial genetics research. In transduction, a bacteriophage mediates the transfer process following attachment to the bacterial surface. In transformation, naked DNA is taken up from the external environment by bacteria that are 'competent'

for transformation. In conjugation, a plasmid mediates its own transfer between bacterial cells, encoding the machinery for transfer in its own genome (Section 5.14).

Comparative studies of whole genomes have shown the extent to which HGT has influenced bacterial genome evolution: HGT is responsible for many apparent gene duplications (Dagan et al. 2008; Pál et al. 2005; Treangen and Rocha 2011), indicating that we are dealing with a *web,* or *net, of life* rather than a *tree of life* (Soucy et al. 2015; Williams et al. 2011). This complexity creates difficulties in applying the concept of 'species' to bacteria (Doolittle 2012) and has led to the use of the terms Pan Genome (the genome shared by a taxon), the Accessory Genome (genes found in just a few, or even one, member of the taxon), and the Core Genome (the set of genes found in all members of the taxon) (Collins and Higgs 2012; Gordienko et al. 2013; Kislyuk et al. 2011; Tettelin et al. 2005).

To be a successful incomer, the gene acquired by HGT should either exert a beneficial or a neutral effect on its new host. Neutral genes may evolve to confer an advantage over time; if they do not, they may be lost. Based on bioinformatic analyses of sequenced bacterial genomes, it has been proposed that the architecture of the chromosome imposes constraints on lateral gene transfer because it is important to preserve an advantageous distribution of 8-bp Architecture IMprinting Sequences, AIMS, on the leading strand, with an increasing frequency of occurrence towards the Ter region (Hendrickson et al. 2018). Incoming genes are favoured if they possess the correct AIMS sequence and take up a position in the appropriate orientation. It is thought that DNA inversions within replichores may not be well tolerated if they shift AIMS from the permissive to the non-permissive strand of the DNA. It is possible that possessing the right type of AIMS, and having it/them in the right orientation, could play a determining role in the successful outcome of an HGT event. Although the function of most AIMS is not known, the FtsK Orienting Polar Sequences (KOPS) located near the *dif* site in the Ter region of the chromosome have been proposed as examples of AIMS (Hendrickson et al. 2018). KOPS contribute to the directional loading of the FtsK translocase, a motor that drives chromosomes at the division septum into the correct daughter cell (Bigot et al. 2006, 2007; Sivanathan et al. 2009; Stouf et al. 2013).

The discovery that bacterial pathogens possess imported gene clusters that encode major virulence factors has revolutionised our understanding of pathogen evolution (Blum et al. 1994; Dobrindt et al. 2004; Groisman and Ochman 1996; Hacker et al. 1997; Strauss and Falkow 1997). In many (perhaps most) cases, the original source of the horizontally acquired genes remains obscure and the foreign nature of these genes is revealed by their unusual base composition when compared with the core genome.

Genomic islands, of which pathogenicity islands are one type, differ from the core genome in A+T/G+C content, GC skew and codon usage (Dutta and Paul 2012). They are inserted often at tRNA genes and are flanked by nearly perfect direct repeats of between 16 and 20 bp (Blum et al. 1994; Schmidt and Hensel 2004). Bacterial genomes exhibit skewed base composition in their chromosomal DNA strands, with the leading strand being richer in G and T bases and the lagging strand having a higher content of C and A (Francino and Ochman 2001; Lobry 1996; Lobry and Louarn 2003; Lobry and Sueoka 2002). In free-living bacteria, GC skew does not seem to have a biasing impact on codon usage by genes on the leading and lagging strands; obligate intracellular bacteria, with their reduced genomes, do show evidence of strand-linked codon bias (McInerney 1998; Romero et al. 2000). Perhaps in their predictable and supportive environments, with few competitors or opportunities

for HGT, obligate intracellular bacteria are exempt from many of the deleterious consequences of mutation that apply to free-living organisms. Intracellular bacteria that dwell in amoebae exhibit the opposite pattern. Examples include the human pathogens *Legionella*, *Listeria*, and *Pseudomonas*. Here, genome expansion rather than reduction is evident and this may reflect greater opportunities for HGT in an intracellular milieu that includes different bacterial species (Greub and Raoult 2004; Moliner et al. 2010).

Codon usage in the genes within islands and amino acid usage in the protein products may differ from those associated with genes in the core genome, perhaps indicating the application of different evolutionary pressures to the horizontally acquired genetic elements (Das et al. 2005). In addition to encoding functions related to pathogenicity, genomic islands can add metabolic functions such as the ability to utilise phenolic compounds (Ravatn et al. 1998), to enhance iron transport capabilities in pathogens (adaptive islands), to fix molecular nitrogen (symbiosis islands) (Sullivan and Ronson 1998), and to resist antibiotics such as methicillin, as in the case of *mecA* in *S. aureus* (resistance islands) (Ito et al. 1999), saprophytic islands that add colonisation functions (Hacker 2000). So-called 'fitness islands' add to the general fitness of the bacterium for its transmission from host to host (Preston et al. 1998) while 'defense islands' accumulate genetic elements that are dedicated to the detection and destruction of invading DNA (Makarova et al. 2011).

The high A+T content of the pathogenicity islands of *Salmonella*, the SPI elements, are described in Section 7.15, as is their relationship with the H-NS transcription-silencing NAP. It is attractive to consider H-NS as having played an important role in genome evolution by preventing the transcription of foreign genes through a mechanism that relies on the recognition of A+T-rich base composition. In this way, H-NS facilitates gene imports, but only of genes that fit the profile of those that can be silenced by this NAP. Once the genes are imported and established physically in the genome, the process of integrating them into the regulatory circuits of the cell can follow. In the case of well-studied pathogenicity islands, regulators that essentially are antagonists of H-NS and act as anti-silencers are encoded by genes that have themselves been imported by HGT. Consider, for example, the AraC-like proteins encoded by SPI1 in *Salmonella* and the ParB-like VirB protein that is encoded by the Entry Region pathogenicity island on the A+T-rich large virulence plasmid of *Shigella* (Dorman and Dorman 2017, 2018). By linking the expression of these H-NS antagonists to environmental signals that are encountered during the infection process, the transcription of the virulence genes is limited to those situations where expression can benefit the bacterium. Similar patterns can be observed among H-NS-silenced virulence genes in other pathogens such as *V. cholerae* where antagonists overcome transcriptional silencing in response to infection-relevant environmental cues (Dorman and Dorman 2018; Kazi et al. 2016).

To become firmly established in the genome, the horizontally acquired genes must possess a structural profile that makes them suitable by H-NS-mediated silencing. Presumably, their inappropriate expression would be deleterious to the cell and a novel gene-cell combination that was uncompetitive would be eliminated by natural selection. The new gene must also overcome other barriers to its establishment before becoming part of the genome that is transmitted vertically to future generations. These barriers involve the surveillance systems represented by CRISPR-*cas* and the restriction enzymes of the new host cell (Koonin and Makarova 2017; Pingoud et al. 2014). These represent formidable

obstacles to foreign gene entry because they can distinguish self from non-self at the level of DNA, coupled with their ability to destroy non-self sequences.

2.24 Distinguishing Self from Non-self

Bacteria have evolved (or have acquired and evolved) a plethora of defensive systems that protect against invaders, especially bacteriophage. A molecular arms race is underway between the bacteria and their far more numerous viral opponents and this results in the rapid evolution of defences in the bacteria and countermeasures in the invaders. Bacteria seem to mount a defense-in-depth, rarely relying on a single strategy to exclude or to destroy invaders. This is illustrated by the discovery of 'defense islands' in bacterial genomes (Makarova et al. 2011). Described by analogy with pathogenicity islands, these regions are rich in genes, mostly acquired by HGT, that protect the bacterium from DNA that is acquired by lateral transfer mechanisms. Restriction-modifications systems distinguish self and non-self based on chemical modification of the home DNA; invaders have their unmodified genomes cut into pieces by the site-specific action of endonucleases: an example of innate immunity. CRISPR-Cas systems also cleave foreign DNA, guided by RNA molecules that are a molecular memory of the system's previous encounter with that invader: an example of acquired immunity. Argonaute proteins cut foreign DNA into small pieces in both a guided and unguided fashion, but the nature of the self/non-self discrimination in this process is unclear. BREX systems allow phage to inject their genomes, but block their replication. Toxin–antitoxin (TA) systems are excellent exponents of the selfish genetic element strategy for self maintenance and propagation: they express an inhibitory or lethal agent that is chemically stable and a chemically unstable antidote; loss of the TA system soon leaves the cell without the protection of the antidote (Blower et al. 2011; Gerdes et al. 2005; Hayes 2003; Makarova et al. 2009; van Melderen and Saavedra De Bast 2009) (Section 1.5). Invasion of the cell by a phage can interrupt the supply of the antidote, eliminating that cell and its unwelcome passenger from the bacterial population. Toxins that puncture the bacterial membrane (holins) are typically lethal in their effect; those that cut mRNA that is associated with ribosomes may induce dormancy – a pathway to the emergence of persister cells in the population. CDI may also be considered as a mechanism for distinguishing self and non-self (for details, see Section 2.4).

2.25 Distinguishing Self and Non-self: CRISPR-Cas Systems

CRISPRs are clustered regularly interspaced short palindromic repeats (Jansen et al. 2002) (Figure 2.15). Early work showed that CRISPR-*cas* systems and H-NS are linked because H-NS silences transcription of the CRISPR-*cas* locus in *E. coli* (Mojica and Rodriguez-Valera 2016), and the LeuO wHTH LysR-like protein overcomes this silencing (Pul et al. 2010; Westra et al. 2010). The same is true in *Salmonella* (Dillon et al. 2010; Medina-Aparicio et al. 2011). It is interesting to note that the *leuO* gene is itself silenced by H-NS (Klauck et al. 1997), creating a scenario in which sporadic upregulation of *leuO* transcription can impose a stochastic, positive influence at the CRISPR locus. The carriage

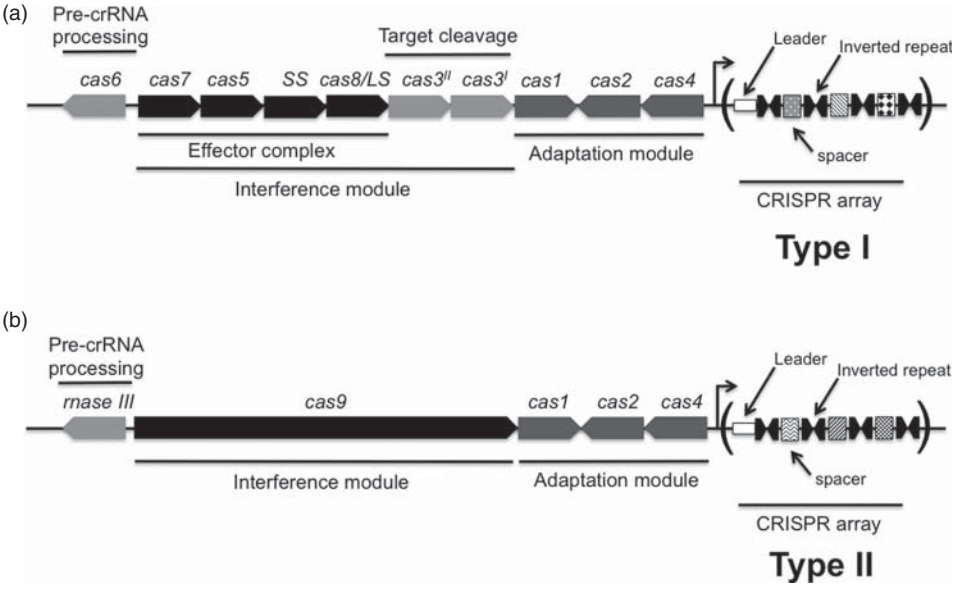

Figure 2.15 CRISPR-*cas* structure. Summary structures are provided for class I (a) and class II (b) CRISPR-*cas* loci. The principal difference between the classes is that several interference-related functions are distributed among a set of Cas proteins in class I systems and these are combined in a single protein (e.g. Cas9) in class II. The two classes are further subdivided into a series of types, making for quite a complex picture (see Koonin and Makarova 2017 and Mohanraju et al. 2016). The spacer sequences that constitute the memory of the CRISPR system are present in an array at the right end; with each spacer separated from its neighbours by copies of an inverted repeat. The spacers are inserted by the adaptation proteins and when the array is transcribed from its common promoter (angled arrow) the resulting pre-crRNA is processed to provide the crRNAs that will be used by the interference system to locate a complementary sequence in an invading mobile genetic element (typically a bacteriophage or a plasmid). *LS*: large subunit, *SS*: small subunit.

by self-transmissible plasmids and phage of genes encoding H-NS-like proteins (Dorman 2014a; Dorman and Ní Bhriain 2020; Doyle et al. 2007; Shintani et al. 2015; Skennerton et al. 2011) may enable these mobile genetic elements to downregulate CRISPR systems in bacteria that receive them via HGT. Some phages also produce anti-CRISPR (Acr) proteins that can neutralise the DNA-binding or nuclease activities of Cas proteins (Bondy-Denomy et al. 2015; Borges et al. 2018; Jiang et al. 2019; Rauch et al. 2017). Even more intriguing is the discovery that some phages produce a nucleus-like structure that contains the viral DNA together with proteins required for DNA replication and transcription (Chaikeeratisak et al. 2017a,b). Transcripts are exported to the cytoplasm of the infected bacterium for translation, with phage heads being assembled at the bacterial cytoplasmic membrane before migrating to the nucleus-like compartment for encapsulation of phage DNA prior to cell lysis (Chaikeeratisak et al. 2017b). These compartments protect the phage DNA (but not cytoplasmically located phage RNA) from attack by CRISPR-Cas systems and restriction enzymes (Mendoza et al. 2018).

The presence of a series of repeated sequences, usually with partial dyad symmetry and about 25–35 bp in length, separated by spacer sequences of about 30–40 bp, is a hallmark

of CRISPR elements. It was this structure that led to their original detection in *E. coli* at the *iap* gene, whose product is involved in the isozyme conversion of alkaline phosphatase (Ishino et al. 1987). The repeat sequences and their spacers form part of a transcribed region, with the spacers in the resulting RNA being used to target foreign DNA that contains complementary copies (Mojica et al. 1995, 2005, 2009). The spacers are derived from invading DNA sequences that have been captured, processed, and integrated into the CRISPR array and will be used to detect and eliminate DNA molecules that contain copies of those sequences, should these reappear in the cell in the future. Each spacer represents a memory, in molecular form, of a previous encounter with a specific phage or plasmid. If foreign DNA containing a sequence that is complementary to a spacer region should enter the cell, base pairing with a complementary RNA molecule (crRNA) expressed from the CRISPR locus leads to the eradication of the invader (Figure 2.15).

There are three stages to the CRISPR-Cas process of eliminating non-self DNA: adaptation, expression, and interference. The adaptation phase involves capturing the sequence that will become the next spacer to be inserted into the CRISPR-*cas* locus. A complex of Cas proteins, known as an adaptation module, binds and migrates along the target DNA molecule until it encounters a short motif known as a Protospacer-Adjacent Motif, or PAM. It then excises the protospacers and inserts them into the CRISPR spacer array between two repeats, creating a new spacer. Typically, this insertion is made at the front of the array (Amitai and Sorek 2016; Jackson et al. 2017). The rare integration events (Heler et al. 2017, 2019) require negative supercoiling of the CRISPR DNA and/or binding of the IHF DNA-bending protein in the leader region (Nuñez et al. 2016; Wright et al. 2017). In some systems, a reverse transcriptase activity encoded by the CRISPR-*cas* locus captures the spacer from RNA expressed by the invading genome (Silas et al. 2016). The expression phase involves transcribing the CRISPR array into a pre-crRNA that undergoes processing by a Cas protein complex or by a single, large Cas protein, to yield mature crRNAs. Each of these consists of a spacer with part of an adjacent repeat. The crRNA then guides the processing complex to its complement in an invading plasmid or phage genome, which is then cleaved by a Cas nuclease (Nishimasu and Nureki 2017; Plagens et al. 2015). Currently, two classes of CRISPR-Cas system are recognised: Class 1 is characterised by a complex of Cas effector proteins while Class 2 has a single, multifunctional Cas effector protein (Koonin et al. 2017). Cas9, which has been exploited widely in genome engineering, comes from Class 2.

During the interference process, the insertion of the spacer RNA into the double-stranded DNA target molecule creates an R-loop (Gong et al. 2018), a DNA:RNA hybrid structure whose formation is assisted by negative supercoiling of the DNA duplex (Drolet et al. 2003). It is for this reason that the Cas system requires a negatively supercoiled invader DNA template in addition to the presence of the PAM (Westra et al. 2012a,b). A dependency on negative supercoiling of the target DNA for the successful operation of the CRISPR-Cas defence links the process to the operations of topoisomerases and to processes such as transcription that generate supercoiling of the DNA at a local level (Section 1.32). It also makes successful destruction of the invader more likely to happen in a healthy, growing bacterium rather than one that is unable to maintain an optimal level of DNA supercoiling. Might this be a mechanism for preserving the fittest members of a bacterial population following infection by an invading mobile genetic element? Sensitivity to negative DNA supercoiling

is also a feature of the spacer recruitment step in CRISPR array development. Protospacers are inserted into the array at its leader end and the integration requires the DNA either to be negatively supercoiled or to be organised appropriately by the IHF DNA-bending protein (Nuñez et al. 2016; Wright et al. 2017). Spacer recruitment is a rare event, so only a small number of bacteria in the population will expand their CRISPR array following infection by an invading genome (Heler et al. 2017, 2019). The concentration of the IHF protein peaks at the transition between exponential growth and stationary phase (Bushman et al. 1984, 1985), just as DNA in the cell begins to relax. Perhaps IHF binding to the leader sequence of the array extends the period during which successful recruitment of protospacers can occur, linking the process to cell physiology (Dorman and Ni Bhriain, 2020).

CRISPR-Cas systems illustrate the difficulty of trying to organise genetic elements by placing them into neat categories. Koonin and Makarova (2017) have pointed out that the adaptive modules of CRISPR-Cas systems may have evolved from a 'Casposon', a transposable, self-synthesising mobile element that used a Cas1 protein as its integrase and collected additional *cas* genes, adding them to the evolving CRISPR-*cas* locus (Krupovic et al. 2017). The acquisition of spacer RNAs may have been facilitated by the recruitment of a reverse transcriptase (as found in modern type III CRISPR-Cas systems) (Silas et al. 2016, 2017). They have also described the similarities between the nucleases in Class II systems that recognise and cleave target DNA and the TnpB nucleases encoded by some transposons (Shmakov et al. 2017). Bacterial toxin/antitoxin systems have contributed RNases that assist the effector function of some CRISPR-Cas systems (Koonin and Zhang 2017). These examples show the modular nature of mobile genetic elements and genome surveillance systems. The appearance of CRISPR-*cas* components in modern Tn*7* family transposons is evidence that the exchange of modules happens in both directions (Section 2.18).

2.26 Distinguishing Self and Non-self: Argonaute Proteins

The arognaute family of proteins is found in archaea, bacteria, and eukaryotes. They are involved in RNA-guided or DNA-guided DNA silencing in which target DNA is cut up in a mechanism reminiscent of that used by Dicer in eukaryotes (Ipsaro and Joshua-Tor 2015; Koonin 2017). Typical targets are plasmids and phage genomes, making argonautes contributors to the defense against HGT (Olovnikov et al. 2013). How these proteins distinguish self from non-self in prokaryotes is still unclear but it has been suggested that association of self-DNA with other proteins such as NAPS may confer protection (Willkomm et al. 2018). Argonautes cleave double-stranded DNA into short segments that can be used as guides for further rounds of targeted attacks on DNA. Unlike CRISPR-Cas, argonautes do not create a memory bank of foreign DNA segments in the host genome, making them much less sophisticated in their mode of operation. In the argonaute literature, DNA silencing is often used interchangeably with DNA degradation; however, it is possible that argonautes and associated nucleases can interfere with transcription by binding in a guided way to DNA targets and blockading RNA polymerase (Willkomm et al. 2018). The discovery that argonautes associate with some CRISPR-Cas systems (Lapinaite et al. 2018) widens the possibilities for programming the targeting of nucleic acid of foreign origin by argonautes.

2.27 Distinguishing Self and Non-self: Restriction Enzymes/Methylases

Restriction endonucleases distinguish between self and non-self DNA using methylation patterns that are characteristic of self. These chemical modifications occur at sites in DNA that are recognised by the endonuclease and unmodified DNA is cut on both strands. The methylase and endonuclease operate as a partnership and these have been categorised as type I, type II, type III, and type IV restriction-modification systems (Loenen and Raleigh 2014; Loenen et al. 2014a; Pingoud et al. 2014; Rao et al. 2014). Of these, type II systems have received the most attention and have become a fundamental tool of modern molecular biology: EcoRI is a founding member of the type II group (Pingoud et al. 2014). The 'restriction' in the name refers to limits placed on the ability of invading phage to target certain bacterial hosts (Arber and Dussoix 1962; Luria 1953). An unmodified phage genome is cut up by the bacterial restriction enzymes; one that has become modified appropriately by the endonuclease-associated methylase will be treated as self by the endonuclease and will avoid cleavage (Loenen et al. 2014b).

Double-stranded cleavage of DNA is deleterious, so synchronisation of endonuclease and methylase production is required if the host is not to self-destruct. In the case of EcoRI, the genes encoding the endonuclease and the methylase are transcriptionally linked so that the transcription of the methylase gene produces an antisense RNA that silences the endonuclease gene. In contrast, the endonuclease gene cannot interfere with the transcription of the gene encoding the methylase (Mruk et al. 2011). Many restriction-modification genetic loci encode their own transcription regulator, called a C protein. This can act as both an activator and a repressor of transcription, depending on its intracellular concentration and the disposition of its binding sites at its target genes. Although the wiring of restriction-modification loci varies from one system to another, those that express C proteins use these regulators to ensure an optimal balance of endonuclease and methylase expression that is consistent with host cell survival and the destruction of invading genomes (Loenen et al. 2014b; Semenova et al. 2005).

2.28 Distinguishing Self and Non-self: BREX

The BREX system interferes with the replication of both temperate and virulent bacteriophage. Although BREX is characterised as a bacteriophage exclusion system, the viruses can adhere to the host cell and inject their genomes: it is later steps in the phage life cycles that are prevented. The nature of the interference is unclear, though it does not involve the physical destruction of the viral DNA. The BREX locus consists of a cluster of genes, with the system in *Bacillus cereus* being composed of two operons: *brxABCpglX* and *pglZbrxL* (Goldfarb et al. 2015). The *pglX* gene encodes a methyltransferase and this methylates the second A residue in the sequence motif 5′-TAGGAG-3′ in the host genome, distinguishing its DNA from that of invading phage: loss of the *pglX* gene correlates with a loss of phage resistance. BrxA is predicted to bind RNA, BrxB's function is unknown, BrxC contains an ATPase domain, PglZ is an alkaline phosphatase, and BrxL is a Lon-protease-like protein (Goldfarb et al. 2015). Systems of this type are widespread in prokaryotes, with

the *pglZ* gene being well represented in bacterial defense islands (Makarova et al. 2011). A BREX system from *E. coli* has a similar genetic composition to the *B. cereus* one and methylates the sequence motif 5′-GGTAAG-3′ on the second A residue. Bacteriophage lambda has 18 copies of this sequence hexamer, and lambda phage that become immune to BREX following infection of and induction in BREX$^+$ *E. coli* are methylated at all 18 sites. Thus, phage can develop immunity to BREX through an epigenetic mechanism involving BREX-dependent DNA adenine methylation (Gordeeva et al. 2019).

2.29 Self-sacrifice and Other Behaviours Involving Toxin—antitoxin Systems

TA systems consist of a toxin protein that inhibits bacterial cell growth and an antitoxin molecule that neutralises the toxin's activity (Harms et al. 2018). Toxins typically interfere with mRNA translation at ribosomes or with DNA replication, while antitoxins are RNA molecules or proteins that interact directly with the toxin or interfere with its expression at the transcriptional or post-transcriptional levels. TA systems are involved in (i) the process of 'plasmid addiction' where cells that fail to inherit a plasmid copy are subject to post-segregational killing; (ii) 'persister formation' in which individual cells in the population become dormant and thus insensitive to antibiotic action; and (iii) 'abortive infection' where individual cells infected by a phage die by suicide and do not pass on the infection. See Section 6.13 for TA systems and persister formation.

Bacterial cells can protect their populations from phage infection if individual infected cells commit altruistic suicide (Dy et al. 2014; Pecota and Wood 1996). Called abortive infection, this behaviour is the bacterial equivalent of throwing oneself on a grenade: it limits the damage to the infected cell by preventing the invader from using that cell as a vehicle in which to propagate. The strategy depends on the triggering by the infecting phage of the toxic function of a toxin/antitoxin system. Some phage can counter this strategy by expressing a general-purpose antitoxin of their own to suppress toxin activities in infected host cells (Alawneh et al. 2016). Others produce protease inhibitors that preserve antitoxins, preventing host cell suicide (Sberro et al. 2013).

2.30 Conservative Forces: DNA Repair and Homologous Recombination

Double-stranded chromosome breaks pose an existential threat to the bacterial cell and can arise, for example, due to the collapse or stalling of replication forks or errors made during the operation of type II topoisomerases. Such breaks must be detected quickly and repaired perfectly if the organism is to remain viable. These detection-and repair processes involve the activities of the RecBCD complex and the RecA protein. Single-strand gaps can arise in the daughter strand of replicating DNA due to UV irradiation (Wang and Chen 1992) or transcription-associated impediments due to collisions between DNA polymerase and RNA polymerase (Kogoma 1997). These are not processed by RecBCD (which needs a blunt-ended gap) but by RecQ (a helicase) acting in concert with RecJ (a nuclease).

These enzymes produce long ssDNA gaps or tails flanked respectively by two or one regions of dsDNA; the ssDNA is coated with the single-stranded DNA-binding protein, SSB (Morimatsu and Kowalczykowski 2014). RecBCD interacts directly with RecA but RecQ and RecJ do not. In the case of ssDNA that is processed by RecJ and RecQ, RecF, RecO and RecR load the RecA protein onto DNA (Morimatsu and Kowalczykowski 2003; Morimatsu et al. 2012; Umezu and Kolodner 1994). These proteins insinuate RecA into an SSB-ssDNA complex, a structure that otherwise leaves little room for newcomer proteins, and enhance the propagation of the RecA protein filament along the ssDNA. They do this by progressively remodelling the SSB-ssDNA complex as RecA replaces SSB (Bell et al. 2012, 2015). The RecA-ssDNA filament catalyses base-pairing and strand exchange with a homologous dsDNA molecule. The RecA-ssDNA pairs with its DNA complement, producing a 3-stranded DNA intermediate known as a displacement loop (D-loop). The strand that is the counterpart to the one within the RecA filament is displaced in this structure and this ssDNA strand is bound by SSB, stabilising the D-loop. RecA hydrolyses ATP while bound to the ssDNA and it stretches the DNA, exposing the unpaired bases in triplets that are available for pairing with their counterparts once the homologous sequence is detected at the start of D-loop formation (Chen, Z., et al. 2008). ATP hydrolysis is not required for the base-pairing interaction between RecA-ssDNA and the homologous sequence: this seems to involve a hunting process in which sequences are sampled until a good fit is obtained with a complementary DNA sequence (Menetski et al. 1990).

2.31 The RecA Protein

Early studies of F plasmid conjugation (Section 1.1) led to the discovery of the *recA* gene as important for recombination between an F- recipient and an Hfr donor strain (Clark and Margulies 1965). The *recB* and *recC* genes were identified as important factors in protecting bacteria from agents that induce double-stranded breaks in DNA (Barbour and Clark 1970; Willetts et al. 1969; Youngs and Bernstein 1973). It was discovered subsequently that RecB and RecC form an enzymatic complex with RecD that has nuclease and helicase activities (Braedt and Smith 1989; Rinken et al. 1992; Singleton et al. 2004) (Figure 2.16a). When RecBCD binds to double-stranded DNA it will unwind it and degrade it until it encounters a Chi site (nucleotide sequence: 5′-GCTGGTGG-3′; Chi = crossover hotspot instigator) at which point it recruits the RecA protein (Dixon and Kowalczykowski 1993; Ponticelli et al. 1985; Taylor et al. 1985). There is a Chi site approximately every 4–5 kb along the *E. coli* chromosome (Dillingham and Kowalczykowski 2008) with the sites being more densely distributed on the leading than on the lagging strand, consistent with a role for Chi and RecBCD in chromosome replication (Courcelle et al. 2015). If a linear dsDNA sequence lacks a Chi site, RecBCD can degrade up to 10 kb of it, contributing to the cell's defence against bacteriophage (Cockram et al. 2015). This RecBCD-mediated degradation process within dsDNA is called 'resecting'. The degraded products from RecBCD action can be fed as 'protospacers' to the CRISPR defence system, allowing it to identify foreign DNA during its adaptation phase (Levy et al. 2015) (Section 2.25). RecA loads at a free 3′ end that is created by the RecB nuclease within RecBCD, initiating the recombination/repair process (Arnold and Kowalczykowski 2000; Roman et al. 1991). The RecA protein is a central player

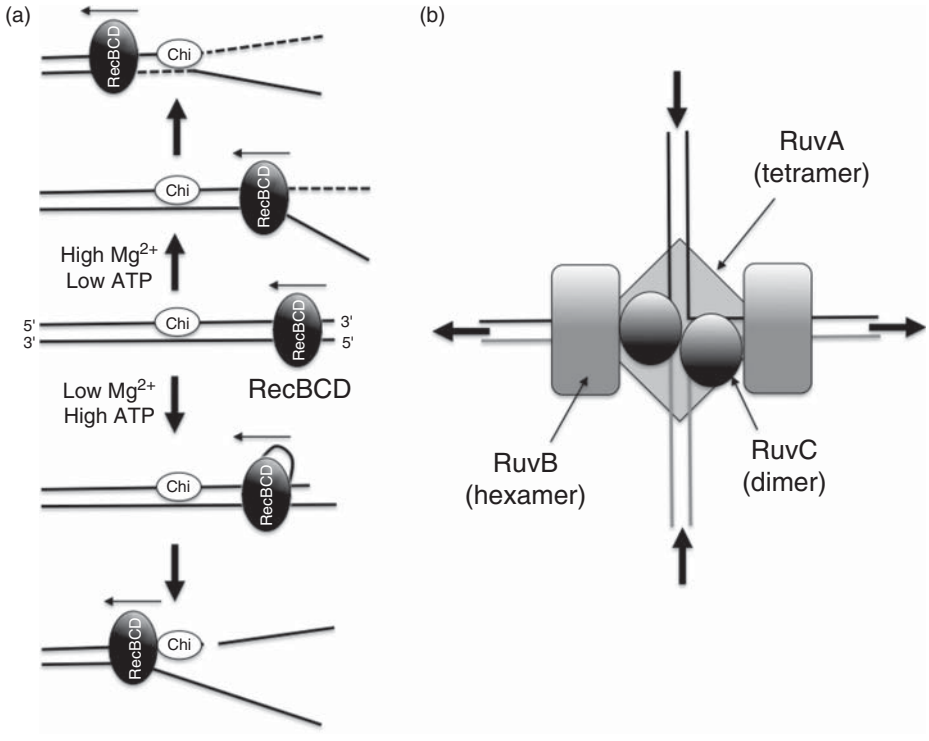

Figure 2.16 RecBCD activity and Holliday junction resolution. (a) The RecBCD complex is sensitive to magnesium and ATP concentrations. When magnesium is low and the ATP concentration is high, it unwinds DNA up to a Chi sequence and nicks the DNA strand there and then continues to unwind the duplex. If magnesium is high and ATP is low, RecBCD will unwind the DNA and degrade the strand containing the Chi site, up to the Chi site. Once RecBCD passes the Chi site it begins to degrade the other strand. (b) The RuvABC complex is shown at a four-way Holliday junction during strand exchange. The contributing DNA helices are being fed in from above and from below, as indicated by the upward- and downward-oriented arrows. The recombinant duplexes are being extruded to the left and right of the complex, as indicated by the arrows. The thin arrows show the positions of the members of the Ruv resolvasome complex.

in responding to the distress signal (single-stranded DNA) that initiates the SOS response and its associated DNA repair processes (Figure 2.17).

2.32 RecA, LexA, and the SOS Response

RecA uses the single-stranded DNA molecule that is generated by the RecB nuclease in RecBCD to search for a homologous sequence elsewhere in the genome. In haploid (or merodiploid) bacteria, the homologous sequence is typically located in the sister chromosome as this is being generated during chromosome replication. While cohesion may keep the sister chromosome nearby, RecA is capable of locating homologous sequences even when they are physically at a considerable distance from the double-stranded break (Lesterlin et al. 2014). The formation of the RecA-coated ssDNA filament (RecA*) triggers the

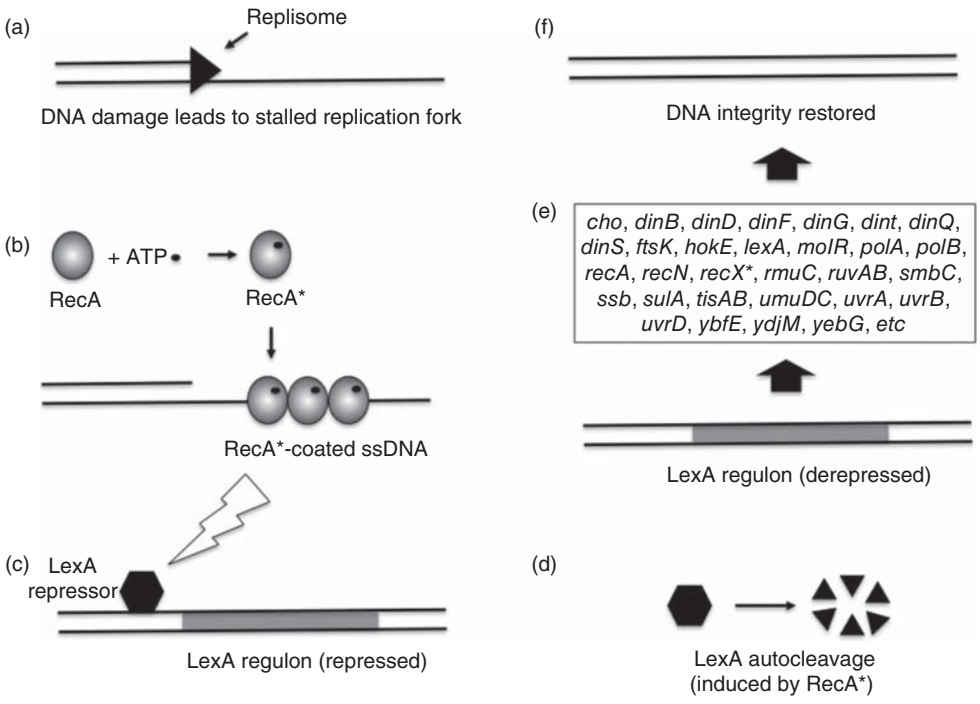

Figure 2.17 The SOS response. (a) DNA damage interrupts chromosome replication and leads to stalling of the replication fork. (b) The accumulation of single-stranded DNA induces the conversion of RecA to its active form. Here, RecA binds an NTP and polymerises along the ssDNA to become RecA*. (c) This nucleoprotein complex interacts with the LexA DNA-binding protein, acting as a co-protease to promote the auto-cleavage of LexA. (d) The elimination of the LexA transcription repressor results in expression of its large regulon. (e) The members of the regulon have been identified with varying degrees of certainty: although most have been confirmed experimentally, some have been assigned to the LexA-dependent group following bioinformatic detection of an SOS box (presumptive LexA binding site) in the promoter region. The functions of the gene products are summarised in Table 2.1. (f) The actions of the products of the LexA regulon restore the integrity of the damaged DNA, allowing chromosome replication to proceed.

SOS response via a complex that is composed of the LexA transcription repressor and the RecA-ssDNA complex (Figure 2.17). As the name suggests, the SOS response is an emergency response made by the bacterium following detection of DNA damage (Radman 1974). Activation of the SOS response involves the proteolytic autocleavage of LexA with upregulation of the LexA regulon consisting of genes that encode DNA repair proteins (d'Ari 1985; Courcelle et al. 2001; Fernandez de Henestrosa et al. 2000; Kreuzer 2013; Maslowska et al. 2018; Simmons et al. 2008). During the response, the chromosome continues to be replicated but cell division is blocked, leading to the characteristic filamentation that is seen in bacteria undergoing the SOS response.

SOS induction follows exposure to a wide range of physical and chemical stresses that damage DNA. The order and the level of production of the LexA regulon members (Table 2.1) reflects the architecture of the promoter regions of their genes, including the quality and positioning of the LexA operator sites (or SOS boxes). The first LexA-repressed

genes to be derepressed following SOS induction encode a high-fidelity DNA repair system. Then the SulA checkpoint protein interacts with the FtsZ protein to inhibit closure of the cell division septum, winning time for DNA repair to be completed (Bi and Lutkenhaus 1993). Bacteria can evade killing by antibiotics that target growing cells during the period of cessation of growth that accompanies SulA-FtsZ interaction (Blazquez et al. 2006; Cirz et al. 2006, 2007). This promotes the emergence of bacteria that are in a persister (i.e. dormant) state in the population. Induction of the SOS response also facilitates the mobilisation and transfer of pathogenicity islands, driving pathogen evolution (Maiques et al. 2006; Ubeda et al. 2005).

The later stages of the SOS response involve the expression and use of an error-prone DNA polymerase, UmuDC (DNA polymerase V) that drives mutation and evolution of the genome. This polymerase is produced from the LexA-repressed *umuDC* operon following SOS induction (Table 2.1). The functional form of UmuD is a dimer that has undergone proteolysis by RecA-mediated auto-cleavage to a truncated form, UmuD' (Shinagawa et al. 1988a). The resulting UmuD'$_2$ homodimer forms a complex with UmuC that is the active Pol V (Tang et al. 1999). If *E. coli* is deprived of either component of Pol V by mutation of either the *umuC* or the *umuD* gene, it no longer undergoes mutation when exposed to ultraviolet radiation, methyl methanesulfonate (MMS), or 4-nitroquinolone-1-oxide (Elledge and Walker 1983; Kato and Shinoura 1977; Shinagawa et al. 1983). This reveals the contribution of Pol V to the accumulation of mutations by the bacterium. Its ability to tolerate (i.e. bypass) lesions in single-stranded DNA requires the cooperation of RecA (Pham et al. 2002; Schlacher et al. 2005).

The SOS response plays an important role in processing DNA damage that arises due to transcription-replication conflicts where the replisome and RNA polymerase encounter one another in head-on collisions e.g. during expression of *rrn* operons (Boubakri et al. 2010). UvrD and the DinG ATP-dependent helicases are both SOS-induced and contribute to the resolution of the consequences of head-on conflicts (Table 2.1).

The LexA-dependent and error-prone DNA Polymerase IV (Table 2.1), encoded by the *dinB/dinP* gene, makes an important contribution to stress-induced mutagenesis. Pol IV is present in high concentrations following its induction and its error-prone mode of DNA synthesis represents a threat to the viability of the cell. This is managed through differential DNA synthesis rates that depend on the nature of the primer. Unlike high-fidelity DNA polymerases, Pol IV synthesises DNA poorly when using an RNA primer and its activity is further held in check through interactions with other proteins, including RecA (Tashjian et al. 2017).

2.33 Holliday Junction Resolution

RecA action creates Holliday junctions in which the participating DNA molecules are physically connected (Holliday 1964). The junction is processed by a 'resolvasome' complex composed of the RuvA, RuvB, and RuvC proteins (Kowalczykowski et al. 1994; West 1996; Wyatt and West 2014). The Ruv complex is responsible for branch migration and junction resolution to generate the separated, mature DNA duplex products from the RecA-generated junction (Kuzminov 1993; Rice et al. 1997) (Figure 2.16b). The genes encoding RuvA and

RuvB form an operon that is repressed by the LexA protein and forms a component of the SOS response (Benson et al. 1988; Shinagawa et al. 1988b). The RuvAB complex drives the process of branch migration while RuvC, encoded by a gene outside the SOS regulon, is the resolvase that cleaves the junction (Sharples et al. 1990; Takahagi et al. 1991). RuvA is a tetrameric DNA binding protein that binds Holliday junctions by recognising the structure of the four-way junction and not any specific DNA sequence (Wyatt and West 2014). RuvB is a hexameric ATPase with DNA binding activity. RuvA triggers ATP hydrolysis by RuvB and facilitates its DNA binding activity. Two oppositely oriented RuvB motors flank RuvA and rotate opposing arms of the Holliday junction so as to pump DNA through the RuvAB complex, converting homoduplex DNA into heteroduplex DNA. RuvA facilitates DNA unwinding and guides strand exchange. The dimeric RuvC protein scans the DNA for its preferred cleavage sequences (Bennett and West 1996; Fogg et al. 1999; Sha et al. 2000; Shida et al. 1996), cuts the DNA, and then the RuvABC complex dissociates from the DNA (Wyatt and West 2014). The products of homologous recombination are now physically separated from one another and can be segregated independently at cell division (Figure 2.16b).

The RecG helicase unwinds Holliday junctions and other forms of branched DNA structures, including D-loops, R-loops, and replication forks (Lloyd and Rudolph 2016). Loss of RecG reduces the rate of recovery of recombinants following Hfr mating by a degree that is similar to that seen in mutants deficient in the expression of the RuvABC complex (Lloyd et al. 1984; Storm et al. 1971). RecG has also been associated with chromosome replication initiation outside *oriC*, rescuing stalled or damaged replication forks (Gupta et al. 2014; Manosas et al. 2013), restarting replication (Lloyd and Rudolph 2016), the acquisition of mutations in cells undergoing stress (He et al. 2006), alleviation of CRISPR Cascade-mediated blocks to DNA replication (Killelea et al. 2019), and naïve adaptation in CRISPR-*cas* spacer arrays (Ivančić-Baće et al. 2015). It has been suggested that these different effects can be explained by invoking a role for RecG in preventing chromosome re-replication (Lloyd and Rudolph 2016).

2.34 Mismatch Repair

Mismatching of bases can occur during DNA replication and is repaired in a DNA-strand-specific manner where the new DNA strand is distinguished from the template strand by its methylation state: the duplex is hemimethylated behind the replication fork, with the newly synthesised strand being the unmethylated partner. Examples of mismatched base pairs are G-T and A-C; typically, these arise due to base tautomerization. The presence of mismatched base pairs in the duplex produces structural distortions that are detected by the mismatch repair machinery, beginning with the dimeric MutS protein, $MutS_2$ (Figure 2.18) (Iyer et al. 2006; Larrea et al. 2010; Marinus 2010). The MutS-mismatch nucleoprotein complex recruits the MutL dimer ($MutL_2$) and this in turn communicates with a MutH protein that is bound at a hemimethylated 5′-GATC-3′ site nearby. This contact activates MutH and it nicks the daughter DNA strand near the hemimethylated site. The UvrD helicase loads at this nick and unwinds the DNA, moving towards the mismatch and excising the mutated DNA strand for degradation by an exonuclease; RecJ or ExoIIV degrade single strands that excise on the 5′ side of the repair complex while ExoI or ExoX is used to degrade strands that excise on the 3′ side. Excision removes the mismatched base

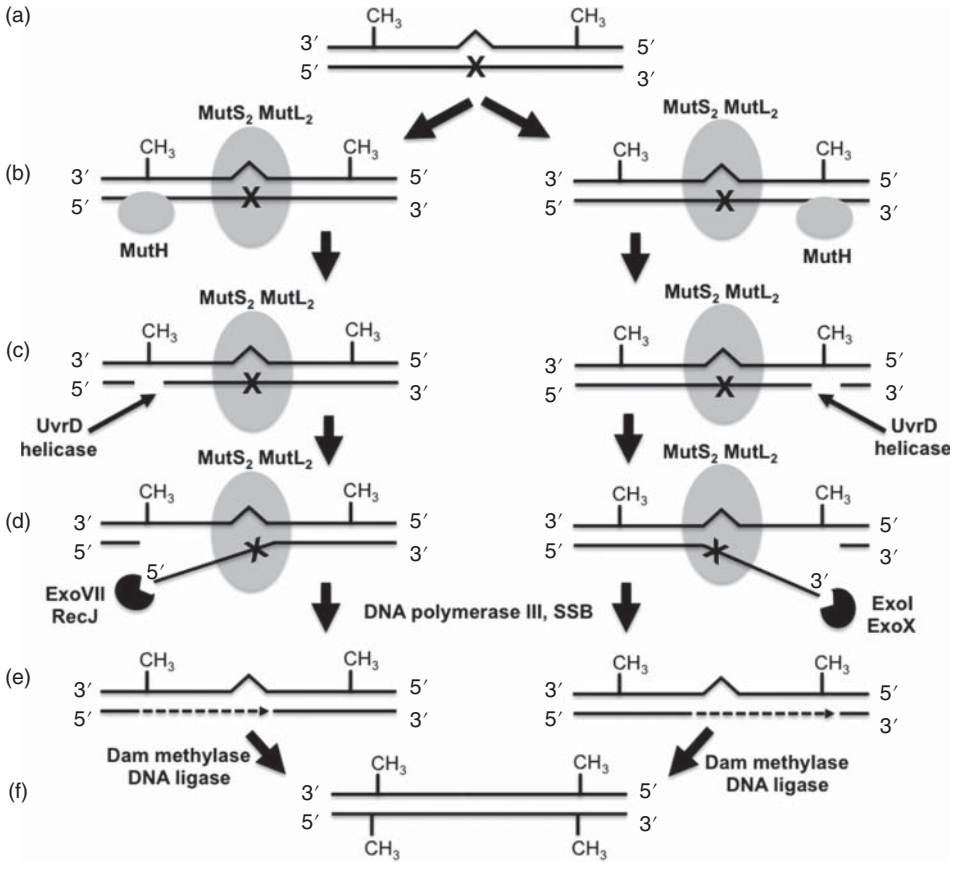

Figure 2.18 DNA mismatch repair. (a) A mismatched base pair at position 'X' causes a distortion in the DNA duplex (inverted "V") in freshly replicated DNA that is methylated (CH_3) only on the parent strand. (b). The dimeric MutS ($MutS_2$) and dimeric MutL ($MutL_2$) are recruited to the site of the distortion and interact with MutH which is bound to hemimethylated 5′-GATC-3′ sites nearby. (c) MutH cleaves the DNA on one strand and the UvrD helicase uses the free end to initiate unwinding of the duplex, moving towards the MutSL complex. (d) The free, single DNA strand is digested by exonucleases. RecJ or ExoVII digest the single strand if it has a free 5′ end (d, left); if it has a free 3′ end (d, right) it is digested by ExoI or ExoX. (e) DNA synthesis by DNA polymerase III, using the intact strand as a template, and assisted by SSB, replaces the digested strand and repairs the mismatched base. (f) DNA ligase seals the gap at the end of the newly synthesised strand and the hemimethylated DNA is then fully methylated by Dam methylase.

and part of the surrounding daughter strand; the resulting single-stranded gap is filled by DNA polymerase III working with SSB and using the opposite strand as a template; the nick is sealed by DNA ligase (Figure 2.18).

2.35 Non-homologous End Joining

Homologous recombination is the principal mechanism for repairing double-stranded DNA breaks in bacteria, but some bacteria can use non-homologous end joining (NHEJ),

a process normally associated with eukaryotes (Bertrand et al. 2019; Critchlow and Jackson 1998). NHEJ relies on an end-binding protein called Ku and an ATP-dependent DNA ligase, called LigD, that can catalyse double-strand break rejoining at blunt-ended breaks and at breaks with 5′ overhangs (Gong et al. 2005; Shuman and Glickman 2007). Homologues of Ku and LigD have been detected in *B. subtilis*, *B. pertussis*, *Mesorhizobium loti*, *Mycobacterium* spp., *Pseudomonas* spp., *Sinorhizobium loti*, and *Streptomyces coelicolor* (Aravind and Koonin 2001; Bertrand et al. 2019; Della et al. 2004; Weller et al. 2002). In *Mycobacterium* spp., a second ATP-dependent ligase, LigC, provides a backup for LigD (Gong et al. 2005) but this backup is not found universally (Shuman and Glickman 2007). The bacterial systems are associated with organisms that either sporulate or spend prolonged periods in stationary phase; these repair systems may help to protect the microbes from DNA-damaging host defences under conditions where just one copy of the genome is present (Shuman and Glickman 2007). During exponential growth, or in conditions of no growth were a second copy (or partial copy) of the genome is available, the homologous recombination system can use this copy as a template for DNA repair (Figure 2.19). In the absence of this resource, NHEJ may be the only option to repair a double-stranded break

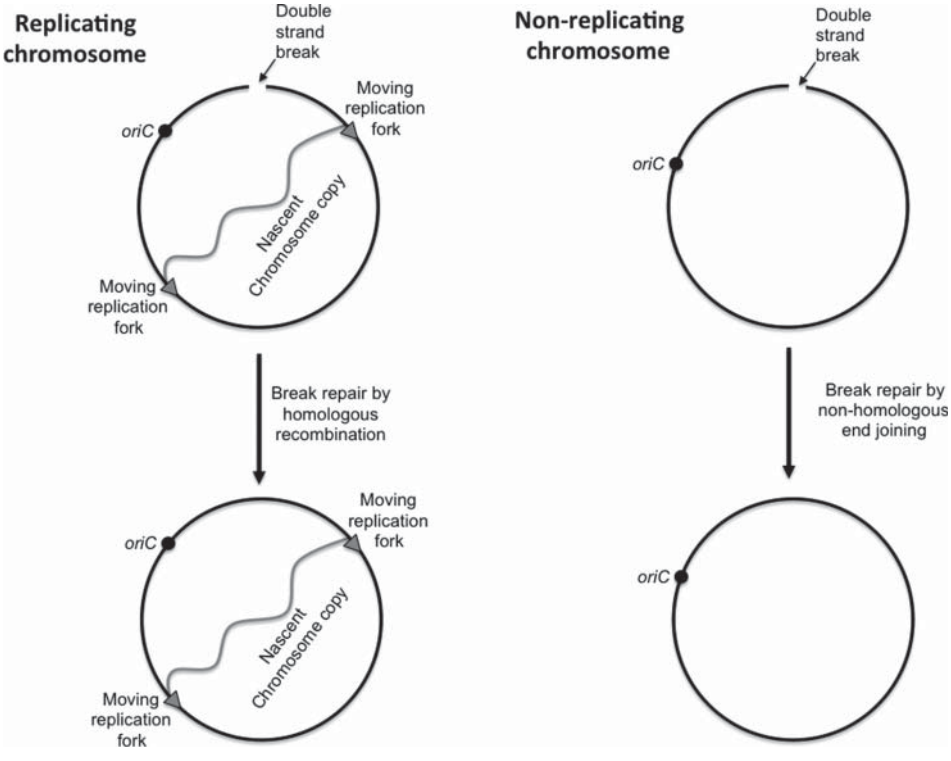

Figure 2.19 Double-stranded break repair. In a growing bacterium, when a double-stranded break occurs in a part of the chromosome that has already been copied, the nascent chromosome copy provides a DNA template with which RecA-dependent homologous recombination can effect a repair. In non-growing cells with just one copy of the chromosome, the only option is to use Ku- and LigD-dependent non-homologous end joining to repair the double-stranded break in the DNA.

in the chromosome (Figure 2.19). In support of this proposal, loss of Ku and LigD does not impede the growth of *B. subtilis,* but it is not tolerated by *B. subtilis* spores, which contain just one copy of the chromosome (Moeller et al. 2007; Wang, S.T., et al. 2006; Weller et al. 2002). This picture is made complicated by the finding that RecA is needed for spores to become resistant to double-strand breaks. Perhaps this reflects the role of RecA in the management of replication fork movement in the germinating spore so that the need for dangerous forms of DNA repair is minimised (Vlašić et al. 2014).

3

Gene Control: Transcription and Its Regulation

3.1 Transcription: More Than Just Transcribing Genetic Information

Transcription is not only a vital process in its own right but is also emerging as an important determinant of nucleoid structure in bacteria. Experiments using HiC methods have revealed that chromosome interaction domains are created and maintained by transcription, especially when long, heavily transcribed genes form the domain boundaries (Le and Laub 2016) (Section 1.33). For this reason, fluctuations in the level of transcriptional traffic may be expected to influence the organisation of the folded chromosome in living bacteria.

Transcription initiation would appear to be the most logical place in the process to make a decision on whether to proceed or not with gene expression, but it seems that all stages of transcription can be subject to regulation, with still further control being imposed at a post-transcriptional level. Thus, the mechanisms of gene regulation have the potential to influence genome structure, and vice versa. We will consider here bacterial RNA polymerase and the features in DNA with which it interacts before looking at the mechanisms by which the activities of RNA polymerase are regulated.

3.2 RNA Polymerase

Bacterial DNA-dependent RNA polymerase (RNAP) resembles its counterparts in eukaryotes and archaea at the levels of amino acid sequence, the structure and function of the subunits, and the mechanisms by which the polymerase carries out the different stages of transcription (Lee and Borukhov 2016; Werner and Grohmann 2011; Zenkin 2014). The striking similarities among the RNA polymerases from the different branches of life suggest that they have evolved from the polymerase of LUCA, the Last Universal Common Ancestor (Booth et al. 2016; Dorman et al. 2018; Koonin 2003). Its structure and function are outlined below.

Structure and Function of the Bacterial Genome, First Edition. Charles J. Dorman.

3.3 The Core Enzyme

The RNAP core enzyme of *Escherichia coli* consists of a 36.5 (kDa) α subunit (present in two copies), a β subunit (150.6 kDa), a β′ subunit (155.2 kDa), and an ω subunit (10.1 kDa) (Murakami 2015) (Table 3.1). The holoenzyme contains all of these proteins plus a sigma factor. There is a considerable division of labour among the subunits of RNAP. The sigma factor is responsible for recognising and binding to the promoter in DNA; the alpha subunits may also make contacts with DNA that are important for RNAP binding (Ross et al. 1993). In addition, the alpha subunits contact transcription factors (TF) that contribute to the recruitment of RNAP to the promoter and/or the initiation of transcription. The N-terminal domain of the alpha subunit is involved in core enzyme assembly; it has regions that interact with the beta subunit and it is connected to the C-terminal domain by a flexible linker (Figure 3.1). The beta subunit has an interaction domain in its C-terminus that contacts alpha and beta-prime. This part of the protein makes contact with the 5′end of the mRNA as transcription begins (Nudler 2009). A segment that is required for binding the alarmone (p)ppGpp, guanosine tetraphosphate/pentaphosphate, during the stringent response is located close to the midpoint of this protein while the C-terminal domain plays an important role in transcription termination (Landick et al. 1990). Amino acid substitution mutations giving resistance to the RNAP-inhibiting antibiotic rifampicin have been isolated along the N-terminal portion of the beta subunit (Jin and Gross 1988; Jin et al. 1988). The beta-prime subunit has segments that contribute to transcription termination along its entire length and there is a portion dedicated to interactions with the 3′ end of the mRNA located close to the C-terminus (Weilbaecher et al. 1994). Alpha subunit interactions occur at the N-terminal part of beta-prime and the same region includes a motif with strong similarity to DNA polymerase. Finally, a zinc-finger motif is located at the beta-prime N-terminus: this is a feature commonly found in DNA binding proteins in eukaryotes (Miller et al. 1985; Vilas et al. 2018). Together with the beta subunit, the beta-prime makes up the characteristic 'crab claw' structure of RNAP, connected at their bases to the N-terminal domains of the two alpha subunits. A cleft between the beta and beta-prime proteins is partitioned into a *primary channel* to accommodate the DNA ahead of RNAP, a *secondary channel* for nucleoside triphosphate (NTP) entry, and an *RNA exit channel* to handle RNA:DNA hybrid separation and RNA secondary structures encountered during transcription pausing or termination (Figure 3.1). DNA-template-directed RNA assembly takes place at an active centre in the primary channel at the centre of the crab claw. This zone contains a catalytic loop coordinating catalytic site magnesium, a trigger loop, and an F loop, together with an alpha helical bridge element that connects the beta and beta-prime subunits. The omega subunit is a beta-prime chaperone and is located at the lower surface of the beta-prime pincer (Nudler 2009). RNAP subunits have different designations that are derived from systems based on the names of the genes that encode them, on their Greek letter names, or on their molecular masses. These have been summarised in Table 3.1.

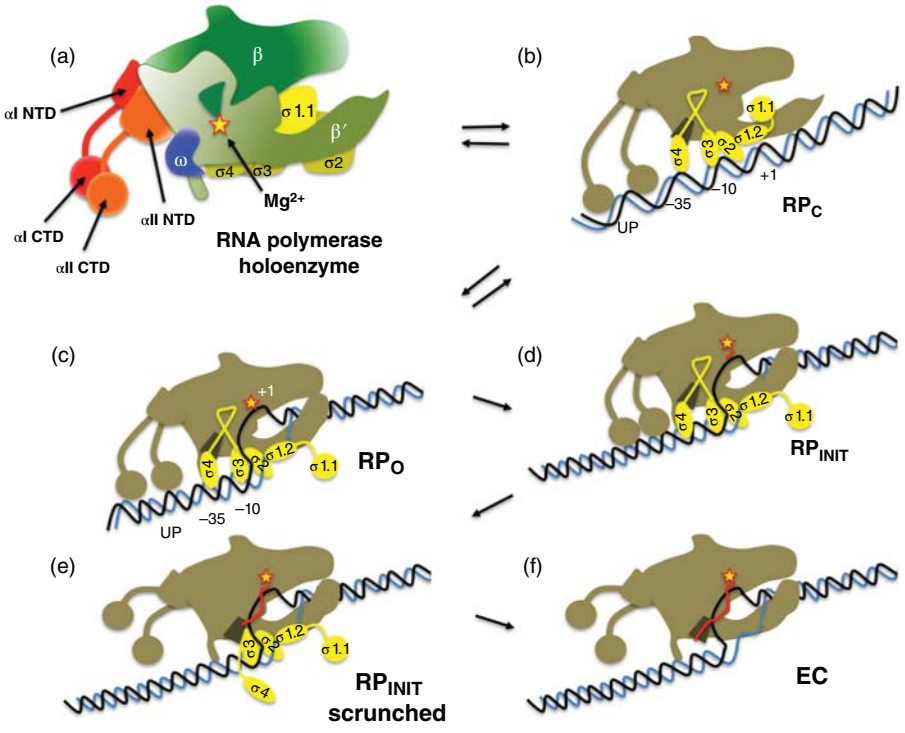

Figure 3.1 RNA polymerase and transcription initiation. (a) This gives a summary of the subunit structure of prokaryotic RNA polymerase, with each subunit shown in a different colour. The core enzyme consists of the α subunit, which is present in two copies (orange and red), the β subunit (green), the β′subunit (pale green), and the ω subunit (blue). The holoenzyme also has a σ subunit (RpoD, yellow) and its regions are labelled using the scheme from Figure 3.2. The σ subunit is partially obscured by the core enzyme. The active site magnesium ion is represented by a star and labelled Mg^{2+}. In (b–f), the core polymerase is shown as a simplified beige shape and the subdomain structure of the σ factor is shown in yellow. (b) The formation of a closed transcription complex, RP_C. The holoenzyme is engaging with a promoter (see Figure 3.2 for details) through its σ factor; contact is also being made at the UP element via the C-terminal domains of the two α subunits. The polymerase is not committed to initiating transcription yet and can disengage from the promoter, as indicated by the two-way arrows. (c) The closed transcription complex isomerizes to an open complex, RP_O, via an intermediate state RP_I (not shown). Here, conserved region 1.1 of σ^{70} has moved from the entrance to the major cleft and the DNA from approximately +2 to −12 has unwound, creating a transcription bubble. This step is part of a coordinated load-and-unwind mechanism in which cleft closure induces DNA melting (see Glyde et al. 2018). The presence of this bubble, detectable experimentally by potassium permanganate footprinting, is a signature of the open complex. The polymerase is still not committed irreversibly to transcription initiation, however. As shown by the two-way arrows, RP_O can revert to RP_C. (d) With the arrival of rNTPs, an initial transcribing complex (RP_{INIT}) forms. The linkage by phosphodiester bond formation of rNTPs positioned opposite the +1 and + 2 positions in the DNA template starts the process of RNA synthesis. (d–e) The next step is described as DNA scrunching. Here, the transcription bubble undergoes an expansion as the DNA is pulled into the cleft and the nascent RNA grows from a 2- to a 6-unit chain. The peptide linker that connects the σ3 and σ4 subdomains (d) of the σ factor prevents the escape of the nascent RNA chain from the complex by obstructing the exit channel (indicated in brown behind the yellow linker). This steric hindrance may result in several rounds of abortive transcription initiation until the roadblock is removed. (e–f) Finally, a transcript of between 11 and 15 nucleotides is synthesised, of which 9 remain in the RNA-DNA hybrid in the transcription bubble, the exit blockage is removed and the nascent transcript emerges. At this point, the polymerase escapes from the promoter and the associated conformational changes cause the sigma factor to be jettisoned and the core polymerase to enter the elongation phase of transcription. (*See colour plate section for colour representation of this figure*)

Table 3.1 Subunit composition of *Escherichia coli* RNA polymerase holoenzyme (with sigma factor RpoD).

Subunit name(s)	Number of amino acids	Molecular mass (kDa)	Number of subunits
Alpha (α, RpoA)	329	36.5	2
Beta (β, RpoB)	1342	150.6	1
Beta prime (β′, RpoC)	1407	155.2	1
Sigma (σ, RpoD)	613	70.2	1
Omega (ω, RpoZ)	91	10.1	1

3.4 The Sigma Factors (and Anti-Sigma Factors)

The sigma factor directs RNAP to the promoter, and sigma factors can come in a variety of forms, allowing RNAP to recognise a variety of promoter architectures (Table 3.2). *E. coli* and its close relatives have seven sigma factors, six of them belonging to one sigma factor family: that of sigma-70, RpoD (Alba and Gross 2004; Chan et al. 1996; Gruber and Gross 2003). Most of the RpoD sigma factor binds to the core RNAP at the entrance to the major cleft that is formed between the beta and beta-prime subunits, and intrudes into the primary channel as far as the catalytic centre before emerging at the exit channel.

Table 3.2 RNA polymerase sigma factors *(Escherichia coli*).

Sigma factor	Upstream recognition sequence (−35 equivalent, 5′-3′)	Spacer sequence length	Downstream recognition sequence (−10 equivalent, 5′-3′)	Function
Sigma-70 (σ^{70}, RpoD)	TTGACA	17	TATAAT	Housekeeping genes at exponential growth
Sigma-54 (σ^{54}, RpoN)	ttGGcaca	4	ttGCA	Nitrogen-regulated genes
Sigma-38 (σ^{38}, RpoS)	CCGGCG	17 + 1	CTATACT	Stress and stationary phase genes
Sigma-32 (σ^{32}, RpoH)	TNtNCCCTTGAA	13–17	CCCCATtTA	Heat shock genes
Sigma-28 (σ^{28}, RpoF/FliA)	TAAA	15	GCCGATAA	Flagella synthesis
Sigma-24 (σ^{24}, RpoE)	GAACCT	16	TCTGAT	Extreme heat shock; extracytoplasmic stress
Sigma-19 (σ^{19}, Rpo19/FecI)	AAGGAAAAT	17	TCCTTT	Ferric citrate transport

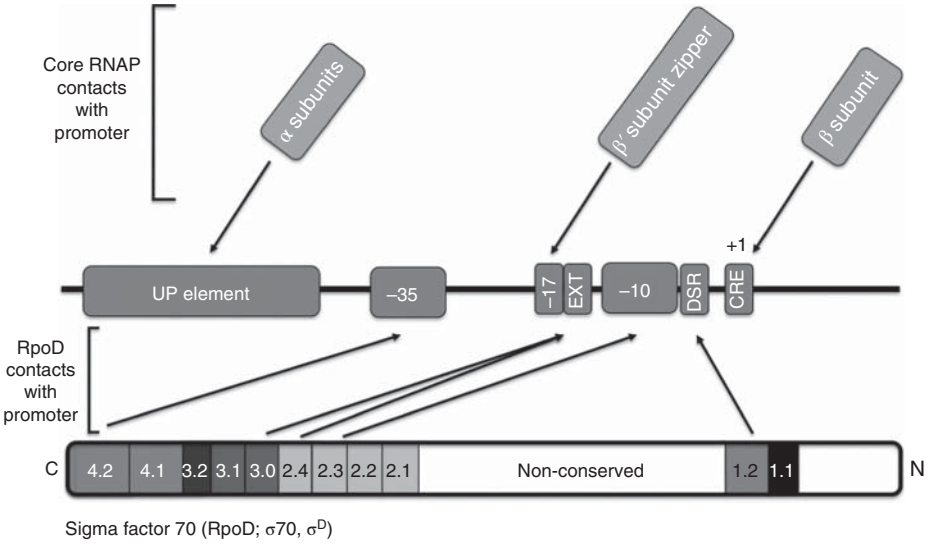

Figure 3.2 Structures of an RpoD-dependent transcription promoter and of the RpoD sigma factor (σ^D, σ70, sigma-70). The promoter is shown in the middle of the figure: the direction of transcription is from left to right, with the transcription start site designated as +1. Contacts with the core RNA polymerase subunits are summarised above the promoter and those with RpoD below. The CRE and DSR elements represent the core recognition element and the discriminator sequence, respectively. The former contacts the β subunit of the core polymerase and the latter is a feature of stringently regulated promoters that contacts region 1.2 of RpoD. The −10 and −35 hexamers are the central features of the promoter and contact regions 2.3 and 4.2, respectively. The extended −10 (EXT) is contacted by regions 2.4 and 3.0 of RpoD while the −17 position of the promoter comes into contact with the β′zipper. The carboxyl terminal domains of the α subunits of the core polymerase contact the UP element of the promoter, an A+T-rich region lying between −40 and −60. The non-conserved region of RpoD is absent from RpoS and it has been suggested that this might explain the lower affinity of the latter for core RNA polymerase, an important consideration when assessing competitions between RpoD and RpoS for access to the core. For further reading, see Maeda et al. (2000).

RpoD, the founding member of the family, is divided into four structural domains, with these domains being further functionally subdivided into conserved regions (Figure 3.2). Conserved region 1.1 prevents RpoD binding to DNA unless the sigma factor is part of the RNA polymerase holoenzyme (Camarero et al. 2002; Dombroski et al. 1992; Schwartz et al. 2008). It also plays a role in the formation of an open transcription complex (Vuthoori et al. 2001; Wilson and Dombroski 1997). In the holoenzyme, the conserved region 1.1 of RpoD is located in the active centre channel, but in the open complex the entering DNA relocates it to outside the channel (Mekler et al. 2002). Region 1.1 has to undergo significant repositioning during this process and a flexible linker of 37 amino acids that connects it to the rest of RpoD facilitates this (Bae et al. 2013). Region 1.2 contacts the discriminator sequence in stringently regulated promoters (Haugen et al. 2006). Domain 1 is separated from Domain 2 by a non-conserved region that is not found in RpoS and may result in RpoS having a lower affinity for core RNAP (Maeda et al. 2000). Domain 2 is subdivided into four conserved regions and these are required for binding the sigma factor to the core polymerase

(2.1), recognising the −10 (or Pribnow box) feature of the promoter and melting the DNA at the promoter to form an open transcription complex (2.3) (Figure 3.2) (Lee and Borukhov 2016). Conserved region 2.4 of Domain 2 and conserved region 3.0 of Domain 3 are responsible for recognising the TG motif of the extended −10 box. Conserved region 4.2 of Domain 4 contacts the −35 box of the promoter. Domain 3 is required for the crosslinking of RNAP to the initiating nucleotide triphosphate for the first step in mRNA/RNA synthesis. Region 3.2 is located in the inter-domain linker between domains 3 and 4. Like domain 2, domain 4 is subdivided into four conserved regions (Figure 3.2). Region 4.1 of RpoD binds to core polymerase, region 4.2 recognises the −35 motif of the promoter, while region 4.3 contains a helix-turn-helix (HTH) motif that places an alpha helix in the major groove of the DNA to read the base sequence (Sharp et al. 1999). Region 4.4 of RpoD is required to interact physically with transcription factors that bind close enough to RNAP to interact simultaneously with the alpha subunit and the sigma subunit (Figure 3.2).

Alternative sigma factors (Table 3.2) either resemble housekeeping sigma factors with 4 structural domains, or they contain domains 2, 3, and 4, or just domains 2 and 4 (Lin et al. 2019). The outlier among the *E. coli* sigma factors is RpoN (sigma-54) (Figure 3.3).

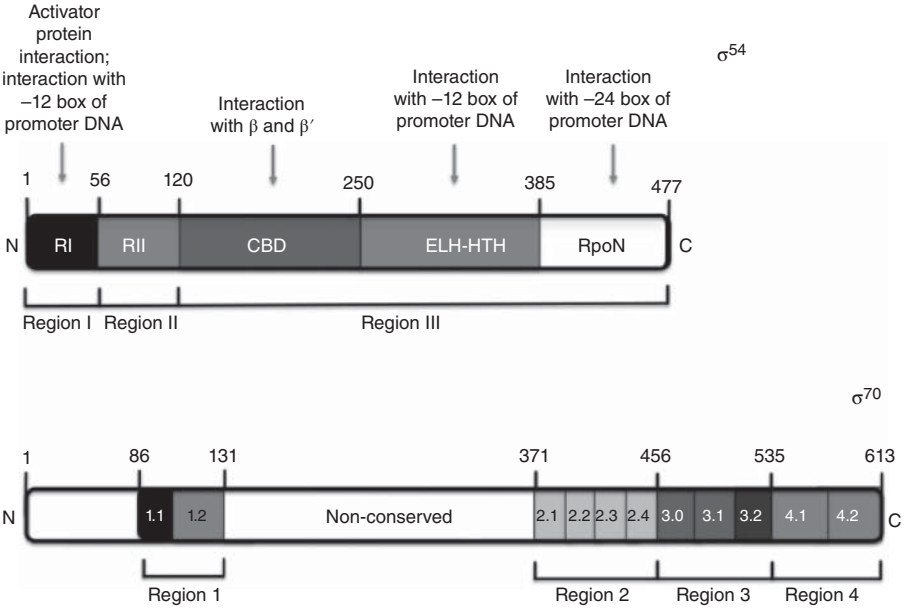

Figure 3.3 Domain structure of RpoN (σ^N, σ^{54}, sigma-54). The 477-amino-acid σ^{54} sigma factor is shown aligned with σ^{70} (613 amino acids) to allow the relative sizes and domain organisations to be compared. See Figure 3.2 for a summary of the functions of the domains and sub-domains in σ^{70}. The positions of regional boundaries are calibrated in terms of the amino acid sequences of the proteins. Region III of σ^{54} is composed of a core-binding domain (CBD), an extra-long helix (ELH), a helix-turn-helix (HTH) domain and the RpoN domain. Some of the functions of different segments of σ^{54} are given above the diagram. Promoters recognised by σ^{54} differ from those bound by σ^{70} in having −12 and −24 boxes, rather than −10 and −35 elements. Activator interaction with σ^{54} holoenzyme is essential for the isomerization of the closed transcription complex to an open complex.

This has a distinct structure and recognises promoters with a different architecture to those used by members of the RpoD family. These differences extend to the upstream regulatory regions in terms of scale (they are long) and structure (they may include enhancers) and include important differences in the mechanism by which transcription factors activate transcription (physical contact is essential if any activity is to occur: the promoters are otherwise silent) (Wedel and Kustu 1995; Zhang, N., et al. 2016). Sigma-54 has three domains: domain I is required for promoter melting and it is glutamine rich. This latter feature may be significant in that many sigma-54-dependent promoters are at genes involved in nitrogen metabolism (Glyde et al. 2017). Domain II is a region with many acidic amino acids, while domain III is responsible for binding sigma-54 to the core polymerase, for DNA-binding site recognition through a HTH, and for DNA crosslinking (Figure 3.3) (Yang et al. 2015).

A global analysis of sigma factor-binding patterns in *Salmonella* has revealed that when not in use, these proteins are found within transcriptionally silent segments of the chromosome (Cameron et al. 2017). Sigma factors are maintained in an inert mode through interactions with anti-sigma factors. The Rsd anti-sigma factor of *E. coli* binds the RpoD housekeeping sigma factor and prevents the formation of the RNA polymerase holoenzyme (Patikoglou et al. 2007; Sharma and Chatterji 2008; Yuan et al. 2008). This allows alternative sigma factors to bind to the core polymerase instead, reprogramming transcription. Rsd accumulates at the end of exponential growth and helps prepare the cell for the onset of stationary phase by switching RNA polymerase from an RpoD-dependent transcription initiation mode to one that relies on RpoS instead (Jishage and Ishihama 1998, 1999). The positive influence of Rsd on alternative sigma factor selection is not restricted to RpoS; the inhibition of RpoD by Rsd also favours RpoE, RpoH, and RpoN (Costanzo et al. 2008; Jishage et al. 2002; Laurie et al. 2003). Other examples of anti-sigma factors include FlgM, the inhibitor of the flagella regulon sigma factor RpoF (Section 6.21), RseA, which inhibits the envelope stress/extracytoplasmic function (ECF) sigma factor, and RpoE (Section 4.2), with ECFs making up the largest class of the alternative sigma factors in bacteria (Lonetto et al. 2019). Some anti-sigma factors contain a zinc ion that forms a redox-sensitive switch; on receipt of the appropriate redox signal, a conformational change causes the protein to release its target sigma factor from the inactive complex (Bae et al. 2004; Campbell et al. 2007; Kang, J.-G., et al. 1999). A variation on the anti-sigma factor theme is illustrated by the bacteriophage T4-encoded AsiA sigma factor 'appropriator'. This protein interferes with RpoD-dependent transcription initiation at host genome promoters and redirects a modified RNA polymerase holoenzyme containing an RpoD-AsiA complex to read viral T4 promoters instead (Colland et al. 1998; Nechaev and Severinov 2003).

Reprogramming of transcription in the later stages of stationary phase involves the *ssrS1*-encoded 6S RNA (Cavanagh and Wassarman 2014). The mature 6S non-coding RNA accumulates in stationary phase (Wassarman and Storz 2000) when it confers a growth advantage on the bacterium (Trotochaud and Wassarman 2004). 6S interacts with, and inhibits, RpoD-containing RNA polymerase holoenzyme, favouring a shift to the transcription of RpoS-dependent genes (Gildehaus et al. 2007; Trotochaud and Wassarman 2005; Wassarman and Storz 2000).

3.5 Promoter Architecture

The transcription promoters of bacterial genes have DNA sequences that attract those sigma factors of RNA polymerase that read them. RpoD (sigma-70) is the housekeeping sigma factor of *E. coli* and the promoters it recognises consist of two hexameric sequences separated by a spacer of approximately 17 bp (Browning and Busby 2016; Lee and Borukhov 2016). By convention, the base pairs in genes are assigned coordinates that give their position relative to the transcription start site. This site is always designated as +1, with downstream positions also having positive values and upstream ones being assigned negative numbers (there is no position 'zero'). The conserved hexamer closest to +1 is called the Pribnow box or –10 motif and is related to the consensus sequence 5′-TATAAT-3′ (Figure 3.2). The second hexamer is called the −35 motif and its consensus sequence is 5′-TTGACA-3′. The consensus sequences of the promoters that are read by the other six sigma factors in *E. coli* are presented in Table 3.2.

Some RpoD-dependent promoters have an A+T-rich DNA motif of about 20 bp called an UP element located immediately upstream of the −35 box (Figure 3.2) (Ross et al. 1993). The UP element is contacted in the minor groove of the DNA by the C-terminal domains of the alpha subunits of RNA polymerase (Ross et al. 1993, 2001). This is the same portion of the alpha subunit that contacts transcription factors (Dove et al. 1997; Ebright and Busby 1995; Ishihama 1992, 1993). These additional DNA contacts with RNA polymerase improve the efficiencies of promoters that have UP elements. However, because UP elements are a permanent feature of those promoters, they cannot affect RNA polymerase activity differentially. In contrast, transcription factors can do this because they are not permanently present at the promoters that they regulate.

Consideration of promoter architecture also takes into account the number, type, location, and orientation of binding sites for DNA-binding proteins that control the activity of RNA polymerase. The presence or absence of these sites links the activity of the promoter to the signals that control the activities of the DNA-binding proteins. Their disposition at and around the promoter determines, in part, the mechanism by which they influence the process of transcription initiation. They also dictate the regulatory 'clubs' to which the promoter belongs. Club membership can be gained or lost through the acquisition or loss of the binding site, allowing the regulatory network of the cell to evolve on a gene-by-gene basis without affecting the regulatory protein itself. Thus, regulatory regions at promoters play a central role in the evolution of gene control networks that is separate from events that affect the RNA polymerase binding sites or the open reading frame of a protein-encoding gene (Carroll 2005, 2008; Oren et al. 2014; Perez and Groisman 2009).

3.6 Stringently Regulated Promoters

Genes that are subject to control by the stringent response have a G -rich discriminator in the non-transcribed strand in the region between the −10 and + 1 (Figure 3.2). Stringent control occurs when the bacterium accumulates uncharged transfer RNA molecules, a sign that the organism should downregulate its translation apparatus. Genes and operons that encode parts of the translational apparatus are under stringent control and have promoters

with the G-rich discriminator (Figure 1.19) (Travers 1980). This sequence element interacts with the σ1.2 subdomain of RpoD, contributing to high stability in the open transcription complex, RP_O (Haugen et al. 2006). The discriminator-RpoD interaction is an important factor in rendering promoters sensitive to negative regulation by (p)ppGpp and DksA (Haugen et al. 2006). The G-rich sequence, with three hydrogen bonds in each base pair, may make the DNA more difficult to melt by the stringently modified RNA polymerase and so block the formation of open transcription complexes (Pemberton et al. 2000). In contrast, genes that are upregulated under stringent conditions by unmodified RNA polymerase have A+T-rich sequences in the corresponding segment of their promoters (Gummesson et al. 2013). The stringent response is not concerned exclusively with transcription: it also inhibits translation initiation (Milon et al. 2006; Mitkevich et al. 2010) and chromosome replication (Ferullo and Lovett 2008; Levine et al. 1991) (Figure 1.19).

3.7 Transcription Factors and RNA Polymerase

Regulating transcription initiation with DNA-binding proteins that respond to chemical or physical signals provides greater flexibility in the achievement of control than does reliance on fixed structures such as an UP element next to the promoter. A DNA-binding protein that recognises a specific nucleotide sequence can act positively or negatively on RNA polymerase, depending on the relative locations of the protein-binding site and the promoter. A binding site that overlaps the promoter may cause the bound protein to act negatively, repressing the initiation of transcription. In contrast a binding site in the immediate upstream region of the promoter allows the protein to act positively, recruiting RNA polymerase, assisting with the transition of a closed to an open transcription complex, or both (Browning and Busby 2016) (Figure 3.4).

Proteins that act in these ways are called transcription factors and they differ from nucleoid-associated proteins in being more restricted in the numbers of promoters that they control, in being responsive to signals, and in not usually being associated with the architecture of the nucleoid (Browning and Busby 2016, Dillon and Dorman 2010; Dorman 2013). This distinction does not hold up very well to close scrutiny. For example, the CRP transcription factor controls hundreds of promoters while being sequence-specific in its binding preferences and it responds to a signal: cyclic AMP (cAMP). The IHF NAP is sequence-specific, the Lrp NAP binds a signal (branched chain amino acids) and the FIS NAP is derived from the DNA-binding domain of the NtrC transcription factor family (Morett and Bork 1998). These examples show that the boundary between NAPs and transcription factors is both porous and blurred, possibly hinting at the evolutionary histories of both types of DNA-binding protein and their versatility.

The *lac* operon has guided our understanding of transcription factor action (Lewis 2013). There, the LacI repressor protein binds to its operator (binding site) upon recognising the base sequence in the major groove of the DNA via an HTH motif; there are additional contacts in the minor groove made by a hinge region that connects the HTH-containing module to the rest of the protein (Chuprina et al. 1993; Spronk et al. 1999). LacI is tetrameric (Gilbert and Müller-Hill 1966) and interacts simultaneously with two copies of the operator to prevent RNA polymerase from initiating the transcription of the *lacZYA* operon

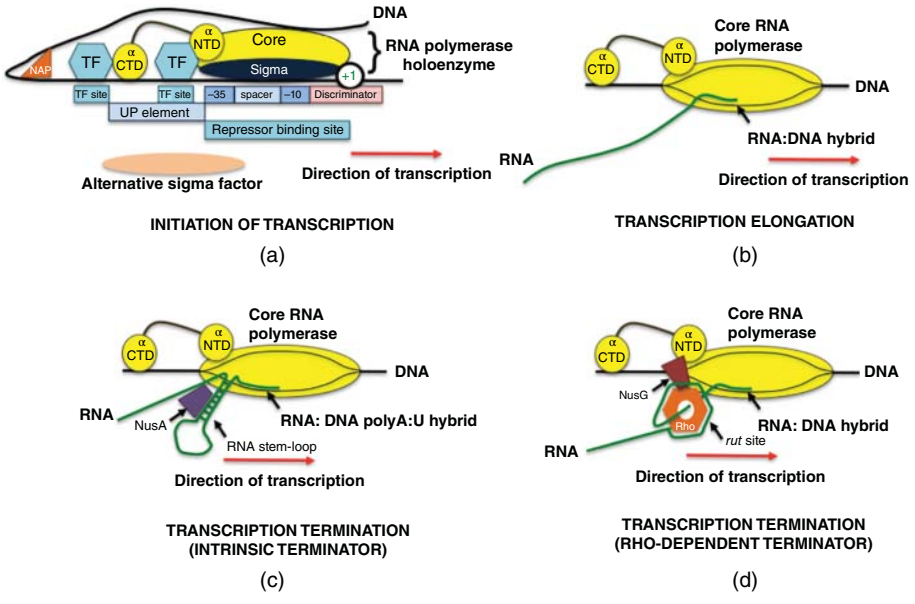

Figure 3.4 Transcription regulation at initiation, elongation, termination. (a) Opportunities to regulate transcription initiation. RNA polymerase holoenzyme is shown bound to an RpoD-dependent promoter. Changing the sigma factor will alter its promoter binding preference. Transcription factors (TFs) bound to specific sites (TF sites) help to recruit RNA polymerase. If ligand binding or other physiologically relevant signals control the activities of these TFs, then the activity of the promoter will be governed by those signals. TF binding upstream from the core promoter results in contact with the C-terminal domain of the α subunits of RNA polymerase, a hallmark of Class I activators. TF binding in a region corresponding to the boundary between the core promoter and the UP element (if present) involves contact with the N-terminal, the C-terminal domains of the α subunits, and contact with the σ factor, a characteristic of Class II activation. There may be additional contacts between the core polymerase and upstream DNA if the upstream DNA loops due to the action of a DNA architectural protein (e.g. the IHF NAP), DNA supercoiling, or DNA intrinsic curvature. Varying any of these parameters can add to the multi-layered control of the promoter. This is not achievable in the case of UP element contact, because this feature is present permanently. A binding site for a repressor that blocks access by RNA polymerase to the promoter prevents transcription initiation. The activity of the repressor, like that of an activator, is usually physiologically determined by ligand binding or some form of posttranslational modification. For stringently regulated promoters (Figure 3.2) the discriminator sequence links transcription initiation to the concentration of the (p)ppGpp alarmone and the DksA protein. (b) Transcription can be regulated during the elongation phase by stalling of the core polymerase or premature termination. Interactions between ribosome-free portions of the mRNA and the transcription bubble can result in R-loop formation. (c) An intrinsic terminator of transcription. This type of terminator consists of a stable RNA secondary structure followed immediately by a run of U residues. The NusA protein (purple) assists with the formation of the RNA hairpin, which cause RNA polymerase to stall and base pairing fails in the adjacent weak RNA:DNA U:A hybrid, separating the transcript from its DNA template strand. This separation results in transcription termination. (d) A Rho-dependent transcription terminator. The hexameric, ATP-dependent Rho helicase (orange) binds to the transcript and surrounds it, guided by a C-rich *rut* site. Rho does not require a stem-loop structure. The C-tract engages with a narrow groove on the surface of the Rho hexamer and, assisted by the NusG protein (brown), Rho detaches the RNA from the core polymerase, terminating transcription. (*See colour plate section for colour representation of this figure*)

(Oehler et al. 1990). Allolactose is the allosterically acting carbohydrate signal that binds to LacI, causing it to release the operator, relieving *lac* repression. Its presence shows that the substrate for the *lacZ*-encoded beta-galactosidase is available for uptake and metabolism. The cAMP-CRP complex binds as a dimer to a site upstream of the *lacZYA* promoter, recruiting RNA polymerase and enhancing transcription. It does this in the absence of glucose. If glucose is available, the *lac* operon will not be activated by cAMP-CRP because the cAMP second messenger will not be present (Section 6.17).

NAP biology was not considered during the pioneering investigations of *lac* operon regulation. However, it is now recognised that the ability of the HU NAP to encourage DNA curvature allows it to assist in the formation of the short DNA loops formed by the tetrameric LacI repressor protein (Becker and Maher 2015). Loop formation is also influenced by the topology of the participating DNA (Fulcrand et al. 2016; Normanno et al. 2008). The same is true in the cases of other classic examples of transcription control that involve DNA looping, such as GalR-mediated repression of the *gal* operon (Lewis et al. 1999) and the action of the lambda repressor protein (Ding et al. 2014).

Promoters that rely on the RpoN (or sigma-54) sigma factor have a distinct geographical layout. These promoters remain resolutely silent unless RNA polymerase holoenzyme containing RpoN is contacted physically by a transcription factor that is bound to an upstream enhancer sequence (Reitzer and Magasanik 1986; Studholme 2002). This gives RpoN-dependent promoters a eukaryotic character that distinguishes them from those read by members of the RpoD sigma factor class. The DNA between the promoter and the enhancer must bend or supercoil to promote the required protein–protein contact to initiate transcription. This change to the path of the DNA can be brought about by looping, by the introduction of a writhing turn or turns, or by protein-induced DNA bending (Brahms et al. 1995; Cheema et al. 1999; Lilja et al. 2004). In the latter case, the IHF NAP, with its characteristic DNA U-turning activity, may be employed (Santero et al. 1992; Wassem et al. 2000).

The NtrC protein is an example of an enhancer-binding transcription factor that governs the expression of RpoN-dependent genes (Stock et al. 2000). It belongs to the class of ATPase associated with diverse cellular activities (AAA) proteins (Rappas et al. 2007). NtrC is activated by phosphorylation in response to ammonia limitation, allowing it to play a central role in the management of nitrogen relations in the cell (Keener and Kustu 1988). It is a response regulator family member and its sensor kinase partner is NtrB. Like NtrC, NtrB is found in the cytosol, distinguishing it from the many other sensor kinases that are found in the cytoplasmic membrane (Stock et al. 2000). As was mentioned before, the DNA-binding domain of NtrC is related to the FIS NAP, indicating that the two have an evolutionary connection (Morett and Bork 1998). The receiver domain of NtrC is located in the amino terminal domain of the protein and this segment is connected to the C-terminal DNA-binding domain by a glycine-rich region that has ATPase activity (Weiss et al. 1991). Activation of NtrC requires phosphorylation at a conserved aspartic acid in the receiver domain. This enables the formation of NtrC hexamers from the unphosphorylated dimers. Hexamers are the active form and their ATPase activity is essential for the conversion of the closed transcription initiation complex to an open complex (De Carlo et al. 2006; Vidangos et al. 2013).

3.8 Transcription Initiation

The initial stage of transcription is an important target for regulation because it represents a commitment to the expression of the gene. The go/no-go decision at this checkpoint can be governed by negative control acting through transcription repression, or it can be controlled by positively acting regulators such as the transcription factors described in the preceding section. Promoter recognition is thought to involve a combination of indirect readout by RNA polymerase as it surveys the shape of the DNA and direct readout that involves base-specific recognition at the −10 element of the promoter by the σ2 subdomain of the RpoD σ factor (Figures 3.1 and 3.4) (Feklistov and Darst 2011; Feng et al. 2016; Zhang et al. 2012). Once RNA polymerase is recruited to the promoter, a closed transcription complex (RP_C) is formed. This undergoes a reversible isomerization to an open complex (RP_O) in which a transcription bubble opens in the double-stranded DNA molecule (Figure 3.1). An initiation complex forms next as NTPs are polymerised to form an RNA molecule guided by the digital information in the DNA template strand. The template strand is drawn into the complex in a process known as scrunching, in which the transcription bubble expands in size (Kapandis et al. 2006). This initiation step can be interrupted and the nascent RNA molecule ejected as an abortive transcript, showing that the process is not yet fully committed to the production of the full message; holding the polymerase at the promoter in the scrunched complex results in rounds of abortive transcript production (Revyakin et al. 2006). The RNA exit channel must be cleared by a rearrangement of the σ factor so that the nascent transcript can grow in length and escape from the polymerase (Basu et al. 2014; Bae et al. 2015b). The transition from the initiation to the elongation phase of transcription involves a conformational change in RNA polymerase that is accompanied by a loss of contact with promoter DNA and the dissociation of the σ factor from the core enzyme (Murakami and Darst 2003). The departure of the sigma factor from the polymerase moves the process to its elongation phase, characterised by the processive action of the polymerase-DNA-RNA complex (Figure 3.1) (Belogurov and Artsimovitch 2015). Once the elongation stage of transcription is underway the process cannot be reversed, although it can still be interrupted. Elongation will proceed until the termination phase is reached. Here, an intrinsic terminator or a factor-dependent terminator will bring the process of transcription to an end (Figure 3.4) (Browning and Busby 2016; Lee, D.J., et al. 2012; Zhang et al. 2012).

RNAP in *E. coli* and its gamma-Proteobacteria relatives can form a stable open transcription complex without the assistance of additional stabilising proteins. This is not the case with RNAP in many other organisms, where the open complex is intrinsically unstable, even at promoters with a strong similarity to the consensus, unless an additional, stabilising factor is provided. The CarD protein of *Mycobacterium tuberculosis* is an example of a stabilising factor and it is widely distributed among bacteria outside the gamma-Proteobacteria group (Davis et al. 2015). CarD operates by wedging the open complex in its open configuration, preventing collapse of the transcription bubble (Bae et al. 2015a).

3.9 Transcription Elongation

The elongation phase of transcription involves the ternary elongation complex made up of RNA polymerase, the DNA template, and the nascent transcript (Figure 3.4) (Korzheva et al. 1998, 2000; Nudler et al. 1997). The newly transcribed RNA and its DNA template strand remain base-paired over approximately 10 nucleotides within the complex. To facilitate this, the DNA becomes unpaired and the non-template DNA strand is moved aside. This short region of melted DNA (10–12 nucleotides in extent) constitutes the transcription bubble. Downstream of the transcription bubble, a sliding clamp encloses the still double-stranded DNA. Two features, described as zip locks, move ahead of and behind the RNA:DNA hybrid. As the elongation complex moves along, these zip locks guide and maintain the conformation of the RNA:DNA hybrid. Translocation of the elongation complex relies on the energetics of base pairing, with the 3′ end of the RNA moving rearwards from the active centre i site so that the next NTP can be engaged in the i + 1 site of the active centre (Mustaev et al. 2017). The pre-translocated complex has a DNA:RNA hybrid of 10 nucleotides and the translocated complex has a 9-nucleotide DNA:RNA hybrid (Washburn and Gottesman 2015). Translocation is not exclusively a steady and unidirectional process: pausing, and even backtracking, of the complex can result following encounters with certain features in the DNA template (Figure 3.5) (Larson et al. 2014; Vvedenskaya et al. 2014).

Transacting factors also influence pausing and backtracking, while yet other factors can suppress them. For example, the NusA protein enhances transcription pausing and this effect is counteracted by NusG (Strauß et al. 2016). The UvrD protein is associated with DNA repair, especially following UV damage, and it has a role in backtracking of the transcription complex to reveal patches of damaged DNA (Epshtein et al. 2014). During the process of transcription-coupled DNA repair, the Mfd protein (or transcription repair coupling factor, TRCF) works to move the transcription complex forwards (Chambers et al. 2003; Deaconescu et al. 2006). The GreA and GreB proteins work on backtracked transcription complexes to restore the 3′ end of the nascent RNA to the complex's active centre. This involves cleavage of the extruded RNA to create a new 3′ end (Figure 3.5) (Borukhov et al. 1993, 2005).

Another factor that reduces the probability of backtracking is the coupling between transcription and translation in bacteria (Figure 3.5). The ribosomes queuing along the nascent transcript exert force on core RNA polymerase in the elongation phase of transcription to prevent it from stalling and backtracking (Demo et al. 2017; Fan et al. 2017; Kohler et al. 2017; Proshkin et al. 2010). An extreme case involves segments of mRNA with consecutive codons for the amino acid proline. This amino acid uses C-rich codons and C-rich mRNA is the preferred substrate for interaction with the narrow groove on the surface of the hexameric Rho transcription terminator, leading to transcription pausing/termination and possible backtracking (Section 3.10). The translation elongation factor EF-P intervenes to ensure efficient translation through poly-proline codons, preventing translation interruptions that lead to a decoupling of translation and transcription and a removal of translating ribosome pressure on RNA polymerase to keep transcribing the DNA template (Elgamal et al. 2016).

Misincorporation at the 3′ end of the nascent transcript is an important source of pausing and backtracking of the transcription elongation complex (Gamba et al. 2017).

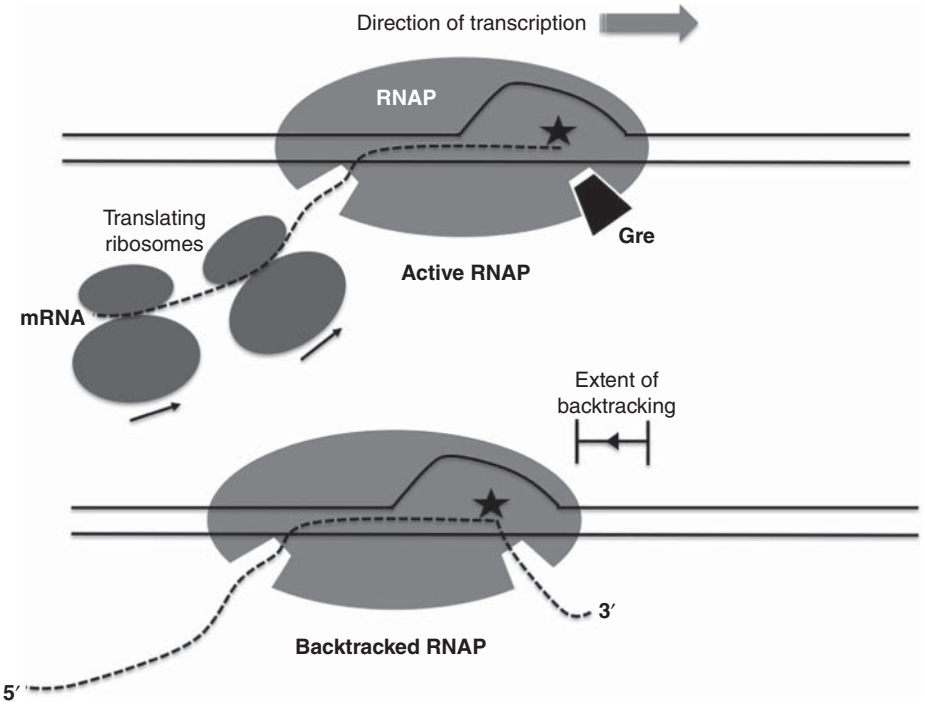

Figure 3.5 Backtracking of RNA polymerase during transcript elongation. The RNA polymerase core enzyme is represented by the grey oval labelled 'RNAP'. The black star represents the catalytic centre. During active transcript elongation, the Gre transcript cleavage factor in the secondary channel and the translating ribosomes on the nascent mRNA prevent backtracking. Following pausing and backtracking of RNA polymerase, the 3′ end of the transcript is extruded via the secondary channel. Transcript cleavage by Gre creates a new 3′ end at the catalytic centre, reactivating the elongation process. The translating ribosomes push RNA polymerase along, reducing the potential for pausing and backtracking.

Once pausing/backtracking has occurred, there is potential for conflict with the DNA replication machinery. Conflicts are inevitable anyway because the transcription elongation complex moves much more slowly than the replisome (Helmrich et al. 2013). Co-directional conflicts seem to be resolved readily with the replisome displacing the transcription elongation complex and even using the transcript to prime DNA synthesis (Pomerantz and O'Donnell 2008). However, if co-directional collisions occur in the presence of a backtracked elongation complex, the result can be a double-stranded break in the DNA with severe consequences for the cell (Dutta et al. 2011). Conceivably, a stalled elongation complex in a heavily transcribed gene could produce a 'traffic jam' of following complexes that exacerbate the negative impact on conflicts with the replisome (Yuzenkova et al. 2014). In the case of head-on collisions, the consequences are much more severe and can lead to replication fork arrest (Pomerantz and O'Donnell 2010). Collisions of this type are associated with mutation (Belogurov and Artsimovitch 2015) and this is one of the reasons that long, heavily transcribed, and essential genes are thought to be located on the leading strand of the chromosome (Vilette et al. 1995).

If ribosomes stall during translation due to failure to charge tRNAs (an event that triggers the stringent response), the result can be pausing of the transcription elongation complex. The DksA protein intervenes to prevent pausing through a mechanism in which it binds in the secondary channel of RNA polymerase (Zhang et al. 2014). DksA is structurally similar to GreA and GreB but differs from them in not being able to induce RNA cleavage by RNA polymerase in backtracked elongation complexes (Lee, J.H. et al. 2012).

Research with bacteriophage has provided important insights into the major steps in transcription. For example, the process of transcription elongation is a target for the Nun protein encoded by the HK022 coliphage. This protein specifically stops RNA polymerase translocation within genes of phage lambda, allowing HK022 to exert super-infection immunity against this competitor coliphage (Vitiello et al. 2014). Nun achieves its effect by inserting into spaces in the transcription elongation complex between the nucleic acids and RNA polymerase to jam the machinery, blocking further forward movement by the complex (Kang et al. 2017).

3.10 Transcription Termination: Intrinsic and Rho-Dependent Terminators

Transcription termination that releases a full-length, mature transcript occurs after the transcription elongation complex has read the complete open reading frame of a protein-encoding gene (Figure 3.4). Termination occurs at a specific site at the end of the gene, or at the end of the last gene in a polycistronic operon. Terminators are classified either as 'intrinsic' or Rho-dependent. The intrinsic type appears in DNA as a G+C-rich inverted repeat followed by a T-tract. When transcribed, these form a stem-loop structure in RNA followed immediately by a run of Us (Figure 3.4). The high G+C content lends stability to the stem while the run of U bases assists with the separation of the U-rich RNA from the A-rich DNA template that encoded it. We have seen above that the NusA protein can prolong the period for which a transcription elongation complex stalls: in intrinsic termination the same protein assists in the formation of the stem-loop structure (Nudler and Gottesman 2002) (Figure 3.4).

Rho-dependent terminators do not have specific secondary structures. The hexameric Rho interacts with a Rho utilisation site (or *rut* site) in RNA, engaging it with an RNA binding cleft on the surface of each of its monomer subunits (Grylak-Mielnicka et al. 2016). Steric limitations in this surface cleft mean that Rho engages preferentially with C-rich tracts in RNA (Burgess and Richardson 2001; Skordalakes and Berger 2003). Rho threads the RNA through a hole in the centre of the hexamer and then translocates along the RNA using an ATP-dependent mechanism until it encounters a stalled transcription elongation complex (Richardson 1982), catalysing the release of the transcript (Epshtein et al. 2010) (Figure 3.4). In the case of genes that encode proteins, Rho must compete with ribosomes for access to the *rut* site in the transcript, introducing an opportunity for regulation. While transcription and translation remain tightly coupled, the ribosomes will exclude Rho. However, if translation stalls, Rho can intervene to terminate transcription (Cardinale et al. 2008).

3.11 Rho and Imported Genes

Rho, together with the NusA and NusG proteins, ceases to be essential for the survival of *E. coli* if the genome is modified to remove its horizontally acquired genes (Cardinale et al. 2008). These findings implicate these transcription co-factors as playing a special role in the control of transcription of foreign genes. This observation is made even more interesting in the context of evidence that the H-NS protein both silences horizontally acquired genes and contributes to Rho-dependent transcription termination efficiency (Boudreau et al. 2018; Dorman 2004; Kotlajich et al. 2015; Saxena and Gowrishankar 2011).

3.12 Rho, R-Loops, and DNA Supercoiling

R-loops arise when the transcription elongation complex backtracks in G+C-rich DNA that has elevated levels of negative supercoiling (Figure 1.16) (Harinarayanan and Gowrishankar 2003; Leela et al. 2013). Under natural growth conditions, the negative supercoiling can be generated by the tracking activities of the transcription elongation complex (or DNA polymerase) with the topoisomerases of the cell being enlisted to resolve the topological bottlenecks. In keeping with this model, R-loop formation can be induced experimentally in mutants deficient in the DNA-relaxing activity of topoisomerase I (Drolet et al. 2003). In addition, mutants of *E. coli* carrying the *rho-15* allele display reduced levels of negative DNA supercoiling (Fassler et al. 1986). Presumably, this loss of supercoiling helps to suppress R-loop formation and its associated deleterious effects (Dorman et al. 2018). These effects can be quite serious, leading to genome instability as a result of hyper-recombination (Nudler 2012; Wimberly et al. 2013). Potentially lethal double-stranded DNA breaks can be encouraged by the presence of R-loops when collisions occur between moving replication forks and transcription elongation complexes (Dutta et al. 2011; Gan et al. 2011; Lang et al. 2017). Here, the protective effect of Rho lies principally in its ability to suppress backtracking by RNA polymerase (Nudler 2012). RNase H eliminates R-loops by degrading the RNA component of the RNA:DNA hybrid (Zhao et al. 2018).

3.13 Rho and Antisense Transcripts

Antisense transcripts are usually not translated because they lack appropriately positioned translational signals that are in register with any open reading frames that they may contain. For this reason, antisense transcripts are available for binding by Rho, thus preventing RNA polymerase backtracking with associated R-loop formation in G+C-rich DNA templates that are appropriately supercoiled. The action of Rho also prevents extension of these transcripts: under conditions where Rho is incapacitated and a suitable helicase suppresses R-loop formation by keeping the RNA and DNA separate, transcripts of many thousands of nucleotides in length can be detected at many genomic sites in *E. coli* (Raghunathan et al. 2018).

Pervasive transcription involves transcription start sites buried within genes and it can be directed in the sense or the antisense orientations, although most attention has been

paid to transcription running in the antisense direction (Lybecker et al. 2014; Wade and Grainger 2014). Spurious, intragenic antisense promoters that arise due to point mutations are thought to drive much of pervasive transcription (Hahn et al. 2003; Lybecker et al. 2014; Stone and Wray 2001). Whole genome analyses suggest that most spurious promoters are Sigma-70-dependent and subject to silencing by the H-NS nucleoid-associated protein (Singh, S.S., et al. 2014). Conservation of antisense transcripts is poor even between closely related model organisms *E. coli* and *Salmonella,* leading to speculation that perhaps most antisense transcripts are non-functional (Lybecker et al. 2014).

Pervasive, antisense transcript formation is suppressed by the Rho factor, binding C-rich untranslated nascent RNA as it emerges from the elongation complex. In a minority of cases where the C-content of the RNA is lower, NusG enhances the action of Rho (Botella et al. 2017; Peters et al. 2012).

3.14 Anti-Termination: Insights from Phage Studies

The N protein of bacteriophage lambda acts as an anti-terminator at the transition between the early and later stages of phage infection (Echols 1971). The N protein binds to nascent transcripts to override Rho-dependent termination, allowing RNA polymerase to transcribe the later-stage-specific genes. N binds to specific sites in the transcript and carries out a nucleation function for a complex of host-encoded proteins consisting of NusA, NusB, NusG, and the S10 ribosomal protein. This complex modifies RNA polymerase, converting it to a state that is resistant to termination (Friedman and Court 2001). In this modified state, RNA polymerase can overcome both Rho-dependent and intrinsic transcription terminators (DeVito and Das 1994).

The Q anti-termination protein of phage lambda is required for the expression of genes late in the infection (Roberts et al. 1998). These genes are transcribed from the $P_{R'}$ promoter and the target of the Q protein is RNA polymerase within a stalled transcription elongation complex at the $P_{R'}$ promoter. The NusA transcription elongation factor protein assists Q in this function (Wells et al. 2016). Modification by Q allows RNA polymerase to read through subsequent transcription terminators, with Q remaining stably associated with the transcription elongation complex as it reads over 22 kilobases of the lambda genome (Deighan and Hochschild 2007). Lambda Q also renders RNA polymerase resistant to transcription pausing during elongation (Deighan et al. 2008). Q-dependent escaping from pauses involves a scrunching process similar to the one observed at transcription initiation where stationary RNA polymerase draws downstream DNA into itself, using the energy to break the bonds that hold the complex in its paused state (Strobel and Roberts 2014).

3.15 Transcription Occurs in Bursts

Historically, studies of gene expression in bacteria (and other types of cell) have relied on bulk experiments, with gene expression outputs being averaged across populations. Single-cell-level investigations have allowed higher-resolution pictures of gene expression

patterns to be assembled, and work at the single-cell and single-molecule levels has revealed the extent of cell-to-cell variation in the expression of a given gene (Ancona et al. 2019; Raj and van Oudenaarden 2008; Thattai and van Oudenaarden 2001). Initially, this approach was informed by probabilistic models of gene expression where randomness in gene expression outputs was thought to be due to stochastic events affecting mRNA translation (Berg 1978; McAdams and Arkin 1997; Rigney 1979a,b; Swain et al. 2002) or transcription (Blake et al. 2003; Kepler and Elston 2001; Peccoud and Ycart 1995; Raser and O'Shea 2004; Sasai and Wolynes 2003; Talaswi et al., 1987). The experiments that followed tended to confirm the expectations that had arisen from the modelling: variations in *Bacillus subtilis* or *E. coli* gene expression arose from random translation of a small number of mRNA molecules (Elowitz et al. 2002; Ozbudak et al. 2002; Swain et al. 2002). Chromatin remodelling was offered as an explanation for randomness in gene outputs in the yeast *Saccharomyces cerevisiae* (Blake et al. 2003). Transcriptional bursting was additionally proposed as a possible explanation in further yeast work (Raser and O'Shea 2004). Transcriptional bursting was identified as a contributory factor in *E. coli* when single-cell transcription was analysed by measuring mRNA levels in individual living bacterial cells (Golding et al. 2005; So et al. 2011; Taniguchi et al. 2010; Zong et al. 2010). Not only is gene output randomised from cell to cell, cell division randomises the relationship between transcripts and protein products, with the effect being most pronounced in recently divided cells (Golding et al. 2005).

Factors that might contribute to transcription bursting are those that bias the probability that a given gene is transcribed in one cell in the population rather than another, isogenic, cell in the same population. Examples might include: stochastic events affecting the supply of a sigma factor needed for the transcription of a specific gene; stochasticity in the relationship between RNA polymerase holoenzyme and the target promoter, or between a regulatory protein and the promoter, or both; variation in the availability of NTPs to initiate transcription in one cell compared with an isogenic neighbour; near-neighbour effects where transcription of an upstream, or of a downstream, gene affects the transcription of the target gene; the fact that the cell cycles of the bacteria in the population are not in synchrony, so replisome passage will happen at each target gene at a different time, leading to variation in the timing of the removal of DNA-binding proteins and their rebinding; stochastic differences in chromosome folding between isogenic cells such that interference with gene activation is exacerbated or alleviated in unpredictable ways; the formation of DNA–protein–DNA bridges in the vicinity of the target gene promoter such that different bridgeheads, and hence bridges of different robustness, are erected; global and local differences in DNA topology may result in one gene copy in one cell being active while other copies are silent; barriers to supercoil diffusion between transcription units may be imposed and removed at random; stochastic events at the level of translation may feedback onto transcription initiation; etc. This non-exhaustive list indicates the wide variety of sources of stochasticity that can have an impact on transcription initiation and produce population-wide differences that are difficult to predict.

One model for a mechanism that might underlie transcription bursting in bacteria takes into account the creation of positive supercoils by the tracking activity of RNA polymerase core enzyme during elongation in a topologically closed system such as a chromosome loop (Ancona et al. 2019; Chong et al. 2014). The same phenomenon would apply in a plasmid

(Chen et al. 1992; Dorman and Dorman 2016). The variable here is the availability of DNA gyrase, the type II topoisomerase that eliminates positive supercoils using the same mechanism by which it introduces negative ones. As predicted by the twin-domain model of Liu and Wang (1987), a build-up of positive supercoils in the DNA template retards transcript elongation in the single-molecule experimental system and severely impedes transcription initiation. The blockage is relieved when gyrase is added to the *in vitro* assay (Chong et al. 2014). In the complex milieu of the living cell, the list of factors contributing to transcription stochasticity given above will come into play, making DNA topology just one influence among many.

4

Gene Control: Regulation at the RNA Level

RNA biology is central to the expression of genetic information and to the regulation of that process. The central dogma of molecular biology tells us that *DNA makes RNA makes protein* and the RNA in question here is messenger RNA, mRNA. This molecule is produced by transcription (Chapter 3) and it transmits the information coded in DNA to the protein-making machinery via a sequence of triplet codons. Transmission of the information requires an adaptor molecule called transfer RNA, or tRNA, to direct the polymerisation of a nascent polypeptide, usually within a molecular machine called a ribosome, via the process of translation. The tRNA molecules deliver the amino acids to the ribosome. By matching each tRNA to its cognate codon in the mRNA, the correct amino acid is added to the growing peptide chain in the correct sequence.

Transfer RNAs fold into a characteristic structure that is common to their class and they are chemically stable (unlike bacterial mRNAs which have a short half-life). They belong to the wider class of stable RNAs that includes ribosomal RNA, or rRNA. In association with the ribosomal proteins, rRNAs form the ribosomes. The genes that encode the stable RNAs are under complex control to ensure that the supply of translational machinery components matches demand. RNA turnover represents an important regulatory process in the governance of cellular physiology and homeostasis.

Messenger RNA will be unable to participate in the process of translation if the requisite signal elements needed for translation initiation are sequestered. This can arise from intramolecular base pairing when the mRNA folds back on itself or it can be due to intermolecular base pairing with another RNA. Either type of base-pairing reaction can be assisted or impeded by chaperones and these are usually RNA-binding proteins.

The binding of small signal molecules can also influence RNA folding and this can provide the basis of a regulatory switch. Typically, the RNA can fold into one of two alternative secondary structures and one of them results in formation of a transcription terminator. The terminator interferes with transcription via a process known as transcription attenuation and blocks the expression of the genetic information that is encoded by the DNA. Many signals can operate RNA-based switches (or 'riboswitches') and include complex molecules (e.g. ATP, sugars, amino acids) and metal ions.

Non-coding RNA (ncRNA) molecules with regulatory roles can operate by binding and sequestering translation signals in target mRNAs and/or by making the target RNA adopt an alternative secondary structure that changes its stability or its proficiency for translation. These ncRNAs are often relatively small (hence 'sRNA' [small RNA]) and chemically stable

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and work well *in trans*. Their production is subject to complex regulation and we are still at an early stage in appreciating the pervasive nature of their influence on the expression of genetic information in bacteria. This chapter will describe the principal types of regulation at the RNA level that have been described in bacteria.

4.1 Antisense Transcripts and Gene Regulation *in cis*

The control of gene expression by RNA in modern bacteria is not simply a vestige of an earlier, hypothetical, 'RNA World': it plays a central role in governing the flow of genetic information in the cell (Figure 4.1) (Wagner and Romby 2015). Some of the earliest examples of gene regulation by RNA involved antisense transcripts that base paired with their sense counterparts to affect the expression of the genetic information carried by the latter (Storz et al. 2011; Wagner and Simons 1994). For example, the 69-nucleotide RNA-OUT encoded by the insertion sequence IS*10,* a component of transposon Tn*10,* is expressed antisense to the transposase mRNA, also known as RNA-IN (Figure 2.4) (Simons and Kleckner 1983). RNA-OUT is highly stable and acts efficiently when expressed *in trans*. Its stability depends on adoption of a simple stem-loop structure that is resistant to exoribonuclease attack (Pepe et al. 1994). RNA-OUT inhibits the translation of RNA-IN by base pairing with it to sequester the translation initiation signals of the transposase gene (Kittle et al. 1989). The RNA chaperone protein Hfq assists this interaction (Ross et al. 2013). Furthermore, RNA-OUT base-pairing with RNA-IN results in destabilisation of the latter, further downregulating expression of transposase (Case et al. 1990). Although RNA-OUT works well *in trans*, exerting multicopy inhibition of IS*10*/Tn*10* transposition, it is classed as a *cis*-encoded sRNA because it is expressed from the DNA strand that is complementary to the strand encoding its RNA target (Dutta and Srivastava 2018; Storz et al. 2011).

4.2 RNA that Regulates *in trans*

Trans-encoded sRNA is expressed at a distance from the gene that encodes its RNA target and usually the two will have a lower level of base pair complementarity than typical *cis*-encoded sRNAs and their targets (Figure 4.1) (Gottesman 2005). The *trans*-encoded type of sRNA may interact with multiple targets, giving it control of a regulon. An early example was the 174-nucleotide *micF* sRNA that inhibits the translation of the mRNA specifying the outer membrane porin protein OmpF in *Escherichia coli* (Mizuno et al. 1983, 1984). The OmpF porin is downregulated in bacteria experiencing osmotic up-shock and is replaced by the OmpC porin, a protein whose expression is stimulated by osmotic stress in *E. coli* (Pratt et al. 1996). The rationale that has been proposed for this is that the OmpC porin excludes a wider range of solutes based on charge and size from the periplasm and that this may have a survival advantage in the mammalian gut, where toxic molecules such as bile salts are found. In contrast, the lower level of discrimination exerted by OmpF may facilitate scavenging in an external, low-osmolarity environment (Nikaido et al. 1983). Expression of *micF* is enhanced by osmotic up-shock and the *micF* gene is transcribed divergently from

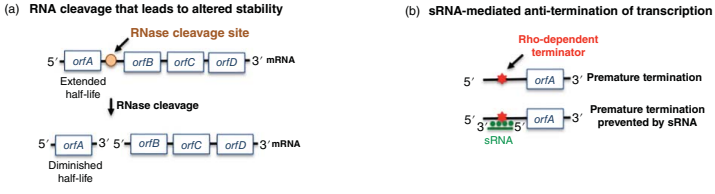

Figure 4.1 Gene control at the RNA level. (a) Cleavage of a polycistronic mRNA by an RNase endonuclease (e.g. RNase E) exposes the part of the transcript that was originally furthest from the 3′ end to early degradation by 3′-to-5′ exonuclease activity. This causes a part of the transcript (*orfA*) that was initially its most stable to become one of its least stable components. The differential degradation of the *orf*-specific transcripts will influence the relative numbers of the gene products that can be expressed from the mRNA. This is a useful way to adjust the stoichiometry of subunits in, for example, a multi-component complex. (b) The *orfA* gene has a Rho-dependent terminator (red star) in the 5′ end of the untranslated leader of its transcript, allowing premature termination of transcription in advance of the protein-encoding part of the gene. Termination is blocked when a small regulatory RNA (sRNA, green) binds and sequesters the terminator sequence. Thus, the sRNA is acting in the role of an anti-terminator. Its mechanism of action may involve interference with the movement of Rho along the transcript. (c) An intrinsic (i.e. Rho-independent) terminator (red) and an anti-terminator (green) overlap in the transcript, where they can form mutually exclusive hairpin structures. The binding of a protein (such as a ribosomal protein, purple) or an appropriate, uncharged, tRNA can shift the RNA folding equilibrium in favour of the anti-terminator. (d) The outcome of the competition to form the mutually exclusive terminator (red) and the anti-terminator (green) secondary structures is affected by the translation of a small leader peptide. If the leader peptide is expressed, the terminator (red) forms and this blocks the expression of the *orfA* gene. This is because ribosomes translating the leader prevent the formation of the alternative anti-terminator (green) hairpin in the RNA. (e) In the riboswitch, a signal molecule (yellow star) that is linked to the biological function of the *orfA* gene is bound by a receptor in the folded RNA. Binding of the signal molecule stabilises the riboswitch in its terminator conformation, preventing the expression of *orfA*. (f) The translation initiation signals of *orfA*, consisting of the ribosome-binding site (RBS), the translation initiation codon (usually AUG) are sequestered *in trans* by the binding of an sRNA (green), silencing the *orfA* gene at the level of translation. In an alternative version of the strategy, the translation initiation signals are sequestered *in cis* by the folding of the transcript. Here, an sRNA binds to the transcript in a region that overlaps with part of the secondary structure, preventing its formation. This liberates the translation initiation signals and *orfA* can be translated. (*See colour plate section for colour representation of this figure*)

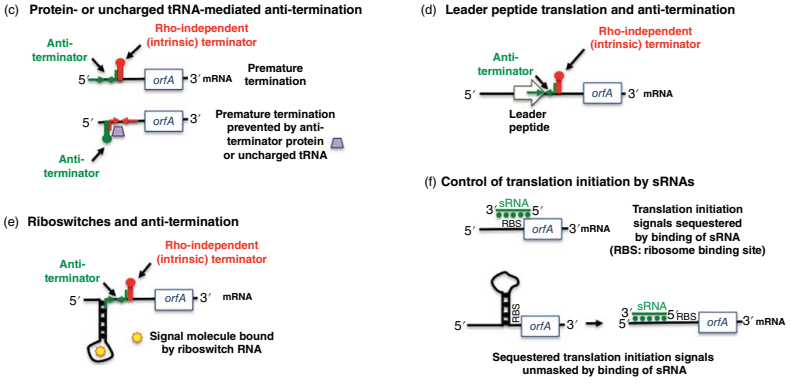

Figure 4.1 (*Continued*)

its genomic neighbour, *ompC*, with both genes being regulated by the OmpR DNA-binding protein (Pratt et al. 1996). The H-NS NAP (Nucleoid-associated protein) also controls the expression of *micF* (Suzuki et al. 1996b). The H-NS paralogue, StpA, which is an RNA chaperone, plays an important part in promoting the interaction between the *micF* sRNA and the *ompF* mRNA and in stabilising the sRNA (Deighan et al. 2000). A *trans*-acting sRNA that inhibits OmpC expression, *micC*, has also been described (Chen et al. 2004).

These early observations of *cis*- and *trans*-encoded sRNAs in the 1980s prompted experiments in genetic engineering that aimed to create artificial 'mic' sRNA molecules (where micRNA = 'mRNA-interfering complementary RNA'). These experiments proved to be successful and illustrated the enormous versatility and potential of gene control through sRNA that have some complementarity to their mRNA targets: they also helped to identify some of the key features of a successful sRNA–mRNA partnership, such as the importance of targeting the translation initiation signals in order to achieve an inhibitory effect (Coleman et al. 1984). In recent years the central role played by sRNA in governing gene expression throughout the genome has become apparent as a result of a combination of whole-genome and single-gene investigations. These studies have revealed the extent to which sRNA influences bacterial physiology (Melamed et al. 2016; Water et al. 2017) and details of many of the regulatory mechanisms (Dutta and Srivastava 2018).

Small regulatory RNAs can target proteins as well as RNA molecules. For example, the CsrB and CsrC sRNAs of *E. coli* sequester the CsrA (RsmA) RNA-binding protein, making it unavailable for binding to low-affinity mRNA targets (Pannuri et al. 2016). CsrA (Csr: carbon storage regulator, also referred to in *Erwinia* as Rsm, repressor of stationary-phase metabolites) plays a central role in governing the switch between a motile and a sessile lifestyle, with the onset of biofilm production in the latter case (Chatterjee et al. 1995; Mukherjee et al. 1996; Jackson et al. 2002; Romeo et al. 2013). It is also involved in switching metabolism from glycolysis to gluconeogenesis and glycogen biosynthesis (Romeo et al. 1993; Sabnis et al. 1995). These influences run counter to those of cAMP-CRP in metabolism, with the exception of motility where both exert a positive influence, and reveal a critical regulatory node in the physiology of the bacterium (Romeo et al. 1990; Wei et al. 2001). Their effects are modulated not only by carbon source but also by growth cycle stage and stress (Pannuri et al. 2016) (Figure 4.2). The CsrB and CsrC sRNAs are transcribed by the envelope-stress-response sigma factor, RpoE; they are also part of the stringent response and respond positively to ppGpp at the level of transcription (Figure 4.2). In a feedback loop, translation of the *rpoE* mRNA is blocked by the CsrA RNA-binding protein, and in yet a further layer of complexity, CsrA autoregulates *csrA* transcription positively but controls *csrA* mRNA translation negatively (Yakhnin et al. 2017) (Figure 4.2). Transcription of the *csrA* gene from its P_3 promoter is RpoS-dependent, linking its expression to stress and stationary phase. There is also evidence that CsrA can enhance RpoS expression during exponential phase when bacteria grow at low temperature (Romeo et al. 2013). Unlike RpoS, which controls stationary-phase-specific genes positively, CsrA downregulates these genes and upregulates genes involved in growth (Romeo et al. 2013).

RpoE is expressed in bacteria undergoing heat stress or other environmental insults that result in improperly folded cell envelope proteins. It is used to transcribe genes encoding envelope components and RseA, an anti-sigma factor that is located in the cytoplasmic membrane, keeps the activity of RpoE in check (Figure 4.3). The RseB protein binds to

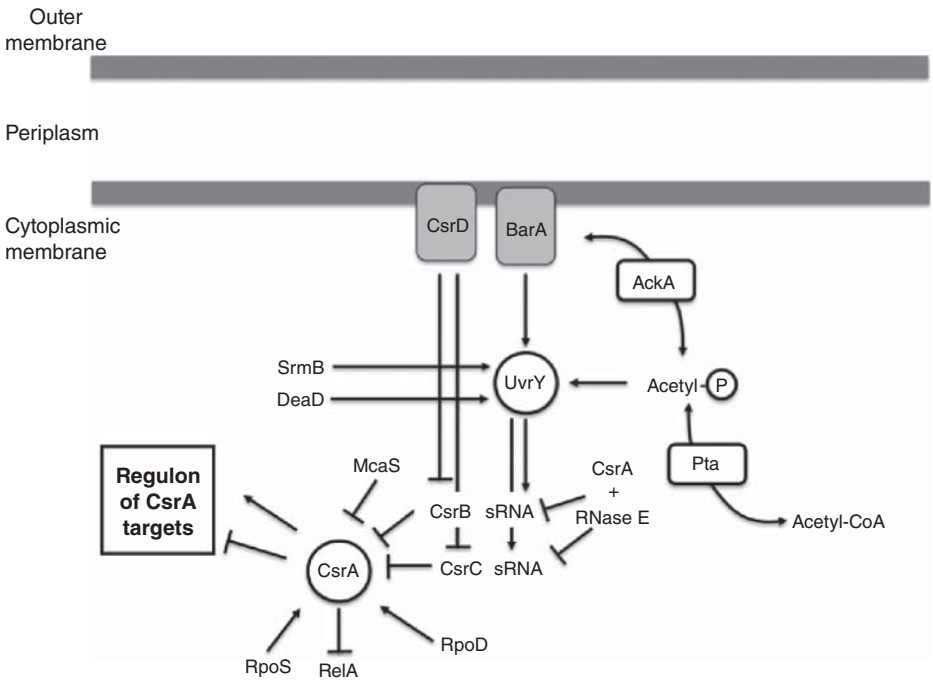

Figure 4.2 CsrA regulon. The CsrA RNA binding protein co-purifies with hundreds of RNA molecules, making it very influential, both directly and indirectly, in controlling regulatory circuits and protein production. CsrA is inhibited by the CsrB and CsrC sRNAs, and CsrA inhibits these in turn when acting in concert with RNase E. The CarD protein inhibits CsrB and CsrC via RNase E. The biofilm-associated sRNA McaS is also an inhibitor of CsrA. The BarA sensor kinase responds to short-chain fatty acids by phosphorylating the UvrY response regulator. UvrY then stimulates the production of CsrB and CsrC. In addition to BarA, UvrY can be phosphorylated by acetyl-phosphate, linking the CsrA system to the acetyl kinase (AckA) phosphate acetyl transferase (Pta) pathway.

RseA on its periplasmic surface and enhances its performance. The anti-sigma factor is turned over by proteolysis in a cascade that is initiated by the DegS periplasmic protease. In this way, envelope stress eliminates the anti-sigma factor and RpoE is made available for interaction with core RNA polymerase. The accumulation of outer membrane proteins and LPS in the periplasm during envelope stress is a signal for RseA-RseB separation, leading to degradation of the RseA protein and initiation of the RpoE-dependent damage response pathway (Lima et al. 2013). The sRNAs transcribed by RpoE-programmed RNA polymerase act in part to shut off the oversupply of unneeded envelope components, including outer membrane proteins (Chaba et al. 2011).

4.3 DsrA and the RpoS/H-NS Link

DsrA is one of the most intensively studied of the sRNAs in *E. coli* (Gottesman 2004). Despite this, DsrA has a relatively restricted number of mRNA targets. Chief among them are the transcripts for the RpoS sigma factor and the H-NS NAP (Figures 1.20 and 1.21); DsrA also

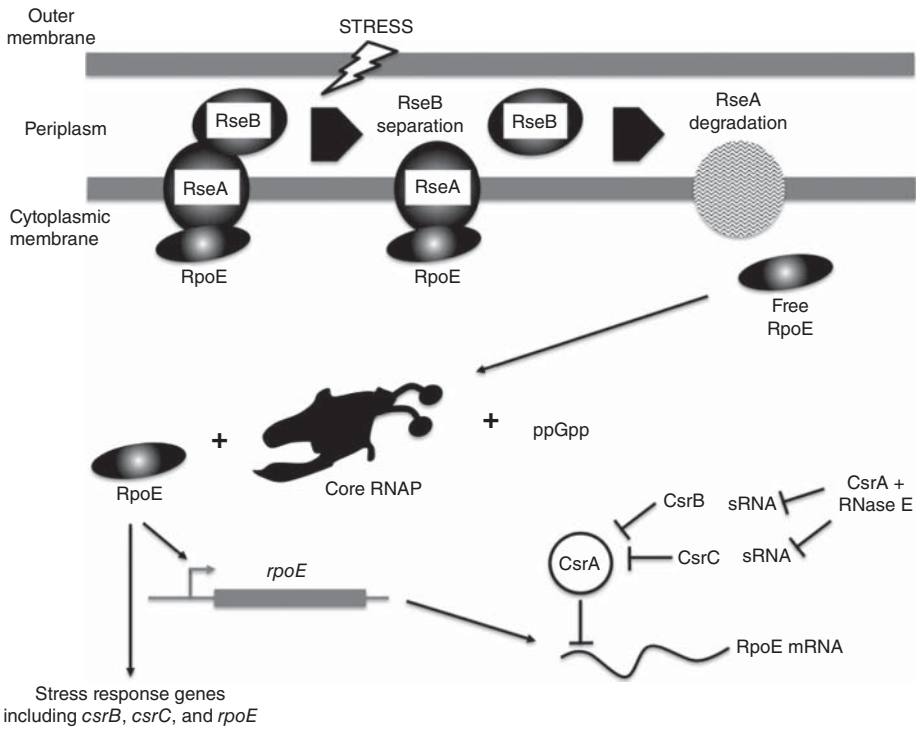

Figure 4.3 Envelope stress, the RpoE sigma factor and sRNAs. The RpoE sigma factor is found in a complex with the RseA anti-sigma factor in unstressed bacteria. RseA, in turn, is bound on its periplasmic face by the RseB protein, protecting RseA from proteolysis by periplasmic proteases such as DegS (not shown). Envelope stress leads to an accumulation in the periplasm of outer membrane proteins and LPS components, and these in turn trigger the separation of RseB and RseA. The unprotected RseA protein is degraded and RpoE is liberated, making it available for binding to core RNA polymerase. Together with the alarmone ppGpp, RpoE-programmed RNA polymerase holoenzyme transcribes the stress response regulon that includes the *rpoE* and the genes encoding the sRNAs CsrB and CsrC. Translation of the *rpoE* mRNA is blocked by the CsrA regulatory protein while CsrA is inhibited by CsrB and CsrC. The CsrB and CsrC sRNAs are targeted in turn by CsrA working in combination with RNase E. Turnover of the sRNAs helps to reset the system to its pre-activation state once the envelope stress response is complete, or the stress is removed.

targets the *mreB* (cell wall biosynthesis) and *rbsD* (ribose metabolism) mRNAs (Lalaouna and Massé 2016). The name DsrA is derived from its genetic location in *E. coli*: downstream of *rcsA* (Sledjeski and Gottesman 1995). Expression of the 85-nucleotide sRNA is induced in *E. coli* growing at low pH (Bak et al. 2014) or low temperature (Repoila and Gottesman 2001). The LysR-like regulatory protein LeuO represses transcription of *dsrA*; the H-NS NAP, in turn, silences transcription of the *leuO* gene (Klauck et al. 1997) (Figure 1.21).

DsrA has a positive influence on the expression of RpoS and acts negatively on the expression of H-NS, MreB and RbsD. The impact on RpoS and H-NS levels is of particular physiological significance because each controls the expression of hundreds of other genes, giving DsrA, indirectly, enormous influence over global gene expression patterns (Figures 1.20 and 1.21).

The RpoS mRNA has the potential to form a hairpin secondary structure at its 5′ end that will sequester the translation initiation signals (Brown and Elliott 1997). Base pairing between the DsrA sRNA and one segment of the inhibitory hairpin leaves the ribosome-binding site (RBS) and translation initiation codon of RpoS mRNA available, allowing high-level expression of the sigma factor (Majdalani et al. 1998). Following the identification of DsrA as a regulator of RpoS translation, three more sRNAs have been discovered that also control the expression of this important sigma factor. These are ArcZ (Mandin and Gottesman 2010), OxyS (Zhang et al. 1998) and RprA (Majdalani et al. 2001) and their contributions have been assessed as fine-tuning RpoS expression (Soper et al. 2010).

DsrA regulates the translation of the mRNA that specifies MreB, a protein that determines the rod shape of the *E. coli* cell. The mechanism involves a translational block, leading to degradation of the *mreB* message and is thus quite similar to that by which DsrA affects H-NS expression (Cayrol et al. 2015). The *rbsD* gene encodes ribose pyranase and is important for ribose metabolism. DsrA makes contact with the downstream portion of the *rbsD* message, setting up the mRNA for degradation (Lalaouna et al. 2015).

The *hns* gene is under multifactorial control at the level of transcription; it is also controlled negatively by the DsrA sRNA at the level of translation (Lease et al. 1998; Sledjeski and Gottesman 1995) (Figure 1.21). The sRNA–mRNA interaction occurs within the five codons immediately following the *hns* translation initiation codon and results in a translation block due to ribosome exclusion (Lalaouna et al. 2015).

4.4 sRNA Turnover

Small regulatory RNAs typically have secondary structures that should protect them from exonucleases. When they bind to their target mRNAs they become targets for attack by the endonucleases RNase E and RNase III, with the Hfq RNA chaperone exacerbating the effect (Afonyushkin et al. 2005; Aiba 2007; Morita et al. 2005). RNase E activity increases the instability of the RyhB, SgrS, and CyaS sRNAs in the absence of PNPase (De Lay and Gottesman 2012; Sinha et al. 2018). In contrast, other sRNAs do not seem to be targets for RNase III or RNase E, but instead are degraded by PNPase, an enzyme that tolerates folded RNAs (like most sRNAs) poorly (Andrade et al. 2012). It has been pointed out that PNPase can have both a protective and a destructive relationship with sRNAs (Bandyra et al. 2016; Cameron et al. 2018) and suggested that the action of DEAD-box RNA helicases may prepare the sRNA for degradation by PNPase (Mohanty and Kushner 2018; Viegas et al. 2007).

4.5 DEAD-box Proteins

DEAD-box proteins are named after a conserved amino acid sequence motif that they share. These proteins are ATP-dependent RNA helicases and *E. coli* has five: DbpA, DeaD, RhlB, RhlE, and SrmB, none of which is essential for life. However, loss of DEAD-box proteins DeaD and SrmB makes *E. coli* cold-sensitive for growth (Iost et al. 2013). The principal

roles of DEAD-box helicases are in ribosome biogenesis (see Section 5.3) and in mRNA degradation (Section 4.8). The RhlB helicase is a component of the degradosome and has a well-established role in mRNA degradation (Section 4.8). Three other DEAD-box proteins, DeaD, SrmB, and RhlE may also be involved in RNA breakdown (Iost et al. 2013). DeaD is also known as CsdA and has been described as being a member of the Cold Shock Degradosome in which CsdA (DeaD) replaces RhlB in the degradosome during the early stages of adaptation to low temperatures (Prud'Homme-Généreux et al. 2004). DeaD is reported to assist the translation of RpoS mRNA at low temperature in its folded state (Resch et al. 2010), a state that sequesters the translation initiation signals (Gottesman 2004). In general, RNA molecules fold slowly and poorly at low temperatures and DEAD-box proteins play a valuable role in folding them correctly and promptly (Cartier et al. 2010).

4.6 RNA Chaperone Proteins

The case of sRNA and the RNA-binding protein CsrA is one that involves molecular mimicry: the CsrB and CsrC sRNAs titrate the CsrA protein, preventing interaction with CsrA's mRNA targets (Romeo et al. 2013). Other proteins play wide-ranging roles in the activities of sRNA by acting as chaperones, and four prominent examples are Hfq, FinO, ProQ, and StpA (Olejniczak and Storz 2017).

Hfq was discovered originally as an essential host factor for bacteriophage Q-beta in *E. coli* (Franze de Fernandez et al. 1968). It is a homohexameric protein that binds to sRNAs, stabilising them and promoting interaction with their mRNA targets, interactions that involve limited intermolecular base pairing (Updegrove et al. 2016; Vogel and Luisi 2011).

The RNA chaperone ProQ emerged from studies of the osmotic stress response transporter gene *proP* (Chaulk et al. 2011). Among the other sRNA targets of ProQ is RaiZ, an inhibitor of translation of *hupA* mRNA, encoding the alpha subunit of the HU NAP (Smirnov et al. 2017). Given the wide influence of HU on gene expression, recombination, and DNA replication and repair, ProQ, and RaiZ have the potential to exert considerable influence on bacterial physiology through HU alone. The influence of this RNA chaperone is even wider because it has over 50 sRNAs among its clients and these affect the translation of hundreds of mRNA targets (Smirnov et al. 2016). ProQ shows structural similarity to FinO and to Hfq: it has a FinO-like domain with high-affinity RNA-binding activity while its Hfq-like domain performs RNA strand exchange and duplexing (Chaulk et al. 2011). The FinO RNA chaperone controls expression of the *traJ* gene on the F plasmid, and hence F transfer by conjugation. 'Fin' is derived from Fertility Inhibition and the *finO* and *finP* genes were originally designated as *traO* and *traP* (Finnegan and Willetts 1971; Gasson and Willetts 1975). FinO functions by facilitating interaction of the FinP sRNA with the *traJ* transcript, blocking TraJ translation (Mark Glover et al. 2015; van Biesen and Frost 1994). Like RNA-OUT from IS*10,* FinP is encoded by a mobile genetic element (IncF plasmids) and was among the first regulatory RNAs to be characterised (van Biesen et al. 1993). Continuing the theme of mobile genetic element associations, it has been observed that genes involved in high frequency lysogenization by bacteriophage are often located adjacent to the *hfq* genes in different bacterial species (Olejniczak and Storz 2017). Bacteriophage also played a role in the discovery of the StpA RNA chaperone, as described in Section 4.7.

4.7 StpA, H-NS, and RNA Binding

The StpA protein (Section 1.43) is a paralogue of the H-NS NAP and it was discovered during an investigation of mRNA splicing in bacteriophage T4. The *stpA* gene suppressed the Td^- phenotype when the bacteriophage td^- gene was expressed from a plasmid in *E. coli* (Zhang and Belfort 1992; Zhang et al. 1995). This phenotype concerned a failure in a self-splicing reaction in the mRNA encoded by the thymidylate synthase (*td*) gene of T4. Purified StpA was found to promote intron splicing *in vitro* by binding to RNA non-specifically. It could also resolve misfolded mRNA precursor molecules and promote base pairing. The StpA protein did not contribute catalytically to the splicing reaction; instead its role was as an RNA chaperone, assembling the mRNA precursor to the spliced products into an active conformation (Zhang et al. 1995). The unrelated *E. coli* ribosomal protein S12 can also act as an RNA chaperone in this T4 splicing reaction (Coetzee et al. 1994).

StpA shares 58% amino acid sequence identity with H-NS (Zhang and Belfort 1992) and shares a common functional domain structure (Cusick and Belfort 1998; Dorman et al. 1999). Although H-NS is usually considered from the point of view of its DNA-binding activity and transcription silencing, it is also an effective RNA-binding protein that can influence RNA-based processes such as translation. For example, H-NS, acting within an A + U-rich region of the transcript, can reposition a ribosome on the *malT* mRNA to stimulate translation. This seems to be a general property of H-NS and is not confined to the *malT* example (Park et al. 2010). Interestingly, the StpA protein can also stimulate *malT* translation (Johansson et al. 1998).

The mechanism by which StpA operates as an RNA chaperone has been examined by means of fluorescence resonance energy transfer, FRET (Rajkowitsch and Schroeder 2007). StpA has both RNA annealing and strand-separation activities *in vitro*. Unlike Hfq, which can perform only annealing, StpA is able to promote both RNA annealing and strand displacement. The annealing activity of StpA is not dependent on RNA sequence complementarity between the participating molecules. Instead, it relies on the polynucleotide bridging activity of the protein, a property that StpA shares with H-NS (Rajkowitsch and Schroeder 2007).

H-NS has RNA-binding activity (albeit with weaker RNA chaperone activity than StpA) and StpA can bind to DNA, indicating that there is a substantial overlap in function between the two proteins (Zhang et al. 1996). Unlike H-NS, the StpA protein is a substrate for Lon-protease-mediated turnover, but it can be protected from proteolytic cleavage by forming a heterodimer with H-NS. The two proteins differ in primary structure at amino acid 21, with StpA having a phenylalanine and H-NS a cysteine at that position. Substituting a cysteine for the phenylalanine at position 21 removes the sensitivity of StpA to proteolysis (Johansson and Uhlin 1999).

StpA governs the timing of the appearance of the stress and stationary phase sigma factor RpoS by acting on the expression of the anti-adaptor protein RssC in *Salmonella* (Lucchini et al. 2009). RssB is an adaptor protein that binds to RpoS and thus promotes its proteolysis by the ClpXP protease (Hengge-Aronis 2002). RssC (IraM in *E. coli*) antagonises this process by binding RssB, making it unavailable for interaction with RpoS (Bougdour et al. 2008). The StpA protein represses the expression of the *rssC* gene and, at least in *Salmonella,* this restricts RpoS expression to periods when StpA is absent. An extreme situation arises if StpA

protein expression is blocked: here RpoS appears prematurely in rapidly growing bacteria (Fitzgerald et al. 2015; Lucchini et al. 2009).

Expression of the H-NS protein increases following a cold shock and this seems to arise from a stimulation of *hns* gene transcription by the CspA cold shock protein (Brandi et al. 1994; La Teana et al. 1991) (Figure 1.21). CspA is an important RNA chaperone and it has RNA-binding activity as well as an ability to bind to DNA (Goldstein et al. 1990; Jiang et al. 1997; Rennella et al. 2017). These observations indirectly place the H-NS-repressed *stpA* gene within the wider cold-shock-responsive stimulon, reinforcing the importance of thermal control to RNA metabolism. Transcription of the *stpA* gene responds positively to increases in temperature (Free and Dorman 1997), which is consistent with increasing and decreasing levels of StpA with rising and falling temperatures, respectively.

E. coli mutants that lack both H-NS and StpA are defective in growth but faster-growing derivatives that harbour suppressor mutations in the *spoT* gene indicate that these NAPs are linked to the stringent response (Johansson et al. 2000). The *hns stpA* double mutant expresses a reduced level of CRP and over-expression of CRP offsets the mutant's growth defect (Johansson et al. 1998, 2000; Sonden and Uhlin 1996). The *spoT* suppressor mutant fails to produce (p)ppGpp and among the consequences of this deficiency is a restoration of the expression of the CRP protein due to relief of negative stringent control at the P2 promoter at the *crp* gene. The *hns stpA* double mutant also fails to express the FIS (Factor for Inversion Stimulation) protein: the single *hns* or *stpA* mutants have reduced levels of FIS compared with the wild type, but the double mutant does not express detectable levels of FIS (Johansson et al. 2000). This illustrates the widely pleiotropic effects of loss of both H-NS and StpA among fundamental regulatory circuits in the bacterium. It also emphasises the highly networked mutual control among NAPs, other global regulators, and their genes: FIS is a positive regulator of *hns* transcription (Falconi et al. 1993) and FIS is a negative regulator of *crp* transcription (Gonzalez-Gil et al. 1998). The impact on FIS levels is also interesting given the reciprocal expression patterns of FIS and RpoS throughout the growth cycle: when FIS is abundant, RpoS is undetectable and vice versa (Ó Cróinín and Dorman 2007).

4.8 Degradation of mRNA

Endonucleases play a key role in mRNA turnover and RNase E is among the most important of these and is essential in *E. coli*. RNase E is associated with the cytoplasmic membrane (Khemici et al. 2008) where it forms a complex with PNPase (polynucleotide phosphorylase, an exonuclease with 3′-to-5′ activity), the RhlB RNA helicase, enolase (in the *E. coli* complex, an enzyme from the glycolytic pathway), and aconitase (in the *Caulobacter crescentus* complex, a TCA cycle enzyme) (Bandyra and Luisi 2018; Bandyra et al. 2013). This complex is referred to as the degradosome (Carpousis et al. 1994; Py et al. 1996). RNase plays essential roles in RNA quality control and remodelling, in addition to mRNA degradation. It seems that neither association with the membrane nor complex formation with the other enzymes is crucial for mRNA turnover by RNase E (Ow et al. 2000). However, the spatial organisation afforded by membership of the complex and membrane attachment is likely to enhance the choreography of the reactions performed in the cell by the intact degradosome.

Many mRNA molecules were thought to have a triphosphate at the 5′ end with conversion to a monophosphate enhancing the activity of RNase E. This conversion may happen in two steps, with an unidentified enzyme removing the γ-phosphate and the subsequent β-phosphate being removed by RppH (Deana et al. 2008; Luciano et al. 2017). RNase E can, however, process RNA substrates via a mechanism that does not depend on their 5′ chemical structure (Bandyra et al. 2018). RNase G (or CafA), a paralogue of RNase E, works with RNase E and has similar substrate specificity and some functional overlap (Li et al. 1999; Ow et al. 2003; Tock et al. 2000). RNase G differs from RNase E in not being either membrane- or degradosome-associated (Lee et al. 2002). Both enzymes cleave RNA regions that are A/U-rich and adjacent to secondary structures. RNase LS makes a contribution to mRNA decay and is part of a toxin/antitoxin system (Koga et al. 2011; Otsuka and Yonesaki 2005). RNase Z (or RNase BN) also contributes to mRNA decay as an endonuclease, but it has 3′-to-5′ exonuclease activity too and is involved in processing tRNA precursors (Perwez and Kushner 2006).

RNase II is the principal 3′-to-5′ exonuclease in *E. coli* and other Gram-negative bacteria, accounting for the bulk of the mRNA hydrolytic activity (Kelly and Deutscher 1992). Secondary structures impede the progress of RNase II (Spickler and Mackie 2000) so possessing them extends the half-lives of mRNAs, especially when the stem-loop structures occur at the 3′ end of the message. RNase R (encoded by *vacB*) also has 3′-to-5′ exonuclease activity but is not impeded by secondary structures in mRNA (Cheng et al. 1998; Hossain et al. 2016). This enzyme seems to be especially important for RNA metabolism during stationary phase (Andrade et al. 2006) and, in the case of pathogens, infection (Cheng et al. 1998).

PNPase has 3′-to-5′ exonuclease activity, but uses a phosphorolytic mechanism that consumes inorganic phosphate and generates nucleoside diphosphates, a reaction that is reversible. This allows PNPase both to synthesise and degrade RNA (Mohanty and Kushner 2000). In addition to being a component of the degradosome, PNPase belongs to the polyadenylation complex that also consists of PAP I (a poly-A polymerase) and Hfq (the RNA chaperone) (Mohanty et al. 2004). Exonuclease activity does not go to completion and the leftover oligonucleotides are hydrolysed by oligoribonuclease (Datta and Niyogi 1975, Ghosh and Deutscher 1999; Niyogi and Datta 1975).

4.9 RNA Folding and Gene Regulation

The folding of the 5′ end of an mRNA molecule can influence the expression of the genetic information that it contains. Returning to the example of the IS*10*/Tn*10* transposase gene, its transcript, RNA-IN, undergoes so-called fold-back inhibition (FBI), creating a secondary structure that sequesters the translation initiation signals from ribosomes (Kleckner 1990) (Figure 2.4). A related RNA-IN folding process prevents translation initiation in cases where an external promoter reads into the IS*10*R, the rightward insertion sequence of the composite Tn*10* element (Davis et al. 1985) (Figure 2.4).

Intramolecular folding, with associated base pairing, affects the stability of RNA molecules (Naville and Gautheret 2009). The nucleotide sequence of the RNA can promote folding as the molecule is synthesised, dictating which segments will pair with which

depending on the order in which the segments emerge from the transcription elongation complex and the intrinsic stabilities of the different base paired options (Ma et al. 1994). It is also possible to guide the RNA folding process using a protein or another RNA, or by using a protein acting in combination with an sRNA molecule (Gottesman 2004).

Intramolecular and intermolecular RNA-RNA strand annealing and displacement are important factors in RNA-mediated mechanisms of gene regulation. Depending on the nature of the interactions between them, the RNA-RNA contacts can make the molecule(s) more stable or more labile (Barquist and Vogel 2015; Wagner and Simons 1994). The stability of the RNA can also be altered by RNase-mediated endonucleolytic cleavage. Such cleavage of a polycistronic message, creating products that differ in stability, can allow different levels of protein to be expressed from a common precursor transcript (Nilsson and Uhlin 1991).

4.10 Transcription Attenuation

Alternative folding of RNA that reveals or sequesters transcription termination signals provides a basis for transcription attenuation (Lee and Yanofsky 1977; Henkin and Yanofsky 2002). The RNA can fold to create two mutually exclusive secondary structures: an intrinsic terminator and an anti-terminator. The anti-terminator is so-called because it shares a segment of RNA with the terminator. Since both structures cannot form in the same molecule at the same time, the appearance of the anti-terminator precludes the formation of the transcription terminator (Figure 4.1). The likelihood that one or the other will form is biased by additional processes, such as ribosomes binding to the RNA, or sRNA interventions, or yet further folding activity within the same RNA molecule.

RNA folding can be guided by ribosome binding and translation within a short open reading frame, with the ribosomes making one or more partners for base pairing in the anti-terminators unavailable (Figure 4.1). The regulatory switch is dependent on the ability of the ribosomes to translate the short open reading frame, something that may be governed by the availability of a specific tRNA charged with a critical amino acid. Ribosome stalling creates an opportunity for the anti-terminator structure to form, allowing transcription read-through into the downstream operon. For example, in the histidine biosynthetic operon, the important amino acid required for translation of the leader peptide is histidine itself: when this is in short supply the resulting stalling of ribosomes during leader sequence translation causes the downstream *his* operon to be expressed and histidine biosynthesis to proceed (Figure 4.1) (Artz and Broach 1975; Kasai 1974).

The process of RNA folding can be modulated by physical signals such as temperature (Krajewski and Narberhaus 2014) and pH (Nechoostan et al. 2009) and these can influence the operation of RNA switches based on transcription read-through/termination control (Figure 4.1).

4.11 Riboswitches

In attenuator systems called riboswitches, a physiologically relevant signal molecule is bound directly within the untranslated *cis*-acting RNA sequence, directing the folding of

the RNA as it emerges from the transcription elongation complex (Figure 4.1) (Coppins et al. 2007; Fürtig et al. 2015). This guides the terminator/anti-terminator decision. Riboswitches usually contain intrinsic terminators, although Rho-dependent ones have also been reported (Hollands et al. 2012, 2014). A multitude of signalling molecules have been identified that operate on riboswitches, often to switch off expression of an uptake system for that molecule or of enzymes that synthesise it (Sherwood and Henkin 2016). Examples of these signals include amino acids, ions (including magnesium), nucleotides (including ATP), coenzymes, and sugars (Cromie et al. 2006; Dann et al. 2007; Sherwood and Henkin 2016; Watson and Fedor 2012). The efficiency of the switch will be influenced by the rate of production of the *cis*-acting RNA sequence by the transcription elongation complex and by the folding dynamics of the RNA, as well as by the binding kinetics of the signal molecule. Folding may occur in more than one step, introducing the possibility of intermediate RNA species with their own thermodynamic properties. Each of these thermodynamic and kinetic factors represents yet another opportunity for tuning or modulating the performance of the riboswitch and its relationship with its ligand. It is also possible for riboswitches to respond to multiple ligands and to be sensitive to both thermodynamic and kinetic influences, producing a highly versatile means of controlling gene expression at the level of RNA (Coppins et al. 2007; Sherwood and Henkin 2016).

Riboswitches have been described that bind both the charged and uncharged forms of tRNA, with each binding mode producing a distinct output: typically, binding by an uncharged tRNA results in upregulation of the production of the cognate amino acid for that tRNA (Figure 4.1). These are the T-box riboswitches and typically they control the expression of genes involved in amino acid metabolism (Grundy and Henkin 1993; Henkin 2014). The switches exploit the anti-codon of the charged and uncharged forms of the tRNA by offering them an opportunity to base pair with a codon-like triplet in the riboswitch at a structure known as the Specifier Sequence (Figure 4.1). These switches monitor the ratio of charged to uncharged tRNA and when the uncharged population begins to expand, this triggers the expression of the relevant amino acid biosynthetic pathway. The system works well because the two forms of the tRNA compete for access to the riboswitch (Henkin 2014; Yousef et al. 2005).

4.12 RNA as a Structural Component in the Nucleoid

Early observations indicated that RNA, in some way, contributed to the organisation of the bacterial nucleoid (Pettijohn and Hecht 1974; Pettijohn et al. 1970). Other early work showed that nucleoid DNA is attached to the cell envelope at multiple points, with many connections being eliminated following treatment with rifampicin, an inhibitor of DNA-dependent RNA synthesis by RNA polymerase (Dworsky and Schaechter 1973; Guillen and Bohin 1986). These latter observations link the *process* of transcription, rather than RNA per se, to nucleoid architecture, and are consistent with many more recent results (see Sections 1.33 and 3.1), including studies of the effects of transertion (Murphy and Zimmerman 2002). Treatment with RNase was found to decompact the nucleoid, consistent with RNA playing a structural role (Worcel and Burgi 1972). More recently, Foley and colleagues studied the effect of RNA removal from nucleoids that were released

gently from *E. coli*: they concluded that the removal of RNA changed the DNA branching density, and hence the compaction, of the nucleoid (Foley et al. 2010). This study also found that the detergent Brij 58, commonly used in nucleoid preparations, had a strong molecular crowding effect and could induce compaction of the nucleoid following RNA removal. These two observations seem to implicate bulk RNA as playing a molecular crowding role in nucleoid compaction.

In *E. coli,* ncRNAs and the HU NAP have been found to interact, with two of the ncRNAs having sequence similarity with bacterial interspersed mosaic elements (BIMEs) or *boxC* repeat elements in DNA (Section 2.6) (Macavin et al. 2012). This has led to suggestion that the DNA repeats, HU, and the ncRNAs could form a structural scaffold within the nucleoid. The role of HU is thought to be transient, acting to facilitate the productive interaction of the DNA and RNA partners (Qian et al. 2017). Consistent with this suggestion, the loss of the ncRNAs or HU correlated with nucleoid decompaction (Macavin et al. 2012). BIME elements are related to REP sequences, DNA elements found at the ends of a number of highly transcribed genes in operons in *E. coli,* including operons likely to be prone to transertion (Bachellier et al. 1997; Gilson et al. 1982; Stern et al. 1984). REPs are known to form complexes with DNA gyrase and HU (BIMEs bind the HU-like protein, IHF [Integration Host Factor]) (Espéli and Boccard 1997; Yang and Ames 1988) and these nucleoprotein complexes may eliminate the DNA topological consequences of high levels of transcription at the upstream genes. Taken together, the ncRNA-BIME/REP/HU/IHF complexes may help simultaneously to manage local DNA supercoiling generated by transcription and intranucleoid scaffolding. Doing this at operons that are subject to transertion (e.g. transport operons) places them at the interface between nucleoid compaction forces (high-level transcription) and nucleoid expansion forces (transertion).

5

Gene Control: Regulation at the Protein Level

5.1 Control Beyond DNA and RNA

The base sequence information in mRNA is transformed into protein by the process of translation and this provides additional opportunities for regulation. Control can be applied at the level of the supply of the translation apparatus components, especially ribosomes, charged tRNAs and translational cofactors, and at the levels of translation initiation, elongation, and termination. Once a protein emerges, further opportunities for control exist in determining its physical destination in the cell (cytoplasm, periplasm, external environment, inner of outer membrane, nucleoid, etc.). The protein may be modified post-translationally by, for example, phosphorylation, and it may be subject to proteolytic degradation. The binding of a ligand may control protein activity and, in rare cases, a protein may undergo splicing to generate its mature, active form.

5.2 Translation Machinery and Control: tRNA and rRNA

Transfer RNA (tRNA) and ribosomal RNA (rRNA) are chemically stable molecules relative to mRNA and are expressed in the form of precursors that are in need of maturation. The transcriptional control of genes encoding stable RNAs is complex and has been discussed elsewhere (Section 1.39). Transcription termination at stable RNA operons is typically due to intrinsic, i.e. Rho-independent, terminators. The transcripts are usually polycistronic and tRNA transcripts can include other tRNAs, rRNAs, and even mRNAs. The transcripts are processed by RNase cleavage, often involving RNase E, that separates the segments that will become individual stable RNAs and removes the stem-loop terminator structure. The 5′ ends of tRNA molecules are generated by RNase P (Altman 1989) whereas a variety of RNases contribute to maturation of the 3′ end (Kelly and Deutscher 1992).

Pre-rRNA transcripts are long and include the precursors of 5S, 16S, and 23S rRNAs and at least one tRNA. The portions containing the 16S and the 23S pre-rRNAs loop out from the main transcript, creating double-stranded RNA substrates for RNase III (King et al. 1984). Cleavage by RNase III releases separate precursors of the rRNA molecules and any tRNA(s) that may be present in the initial transcript. The pre-tRNA is processed by a succession of enzymes that includes RNase E, Rnase G, and the YbeY endonuclease, with possible contributions from RNase II, RNase PH, PNPase, and RNase R (Davies et al. 2010; Jacob et al.

Structure and Function of the Bacterial Genome, First Edition. Charles J. Dorman.

2013; Rasouly et al. 2009; Sulthana and Deutscher 2013). RNase E also excises the proto-5S RNA and its maturation is completed by RNase T. RNase T completes the processing of pre-23S rRNA, with possible contributions from PAP I, RNase II, and RNase PH (Li et al. 1991). Endonucleolytic cleavage, possibly by RNase III, may be required for the final trimming of 5′ extensions in pre-5S and pre-23S rRNAs. With so many enzymes involved (and this is not an exhaustive list) in the processing of the precursors of stable RNAs, one can appreciate that there exist multiple opportunities to impose regulation or to create bottlenecks in the system that generates tRNAs and rRNAs, that have consequences for global gene expression patterns in the cell. For example, the genes encoding key RNases undergo autoregulation at the level of their mRNA in response to environmental signals that are relevant to the very processes that these RNases modulate (Gao et al. 2006). The RraA and RraB global regulatory proteins control RNase E and RNase III dimerization is prevented during cold shock by the intervention of the YmdB protein (Gorna et al. 2010; Kim et al. 2008; Lee, K., et al. 2003). Growth phase and temperature control RNase R levels through its proteolytic degradation (Liang et al. 2011).

5.3 Translation Machinery and Control: The Ribosome

The bacterial ribosome is made up of a 30S and a 50S subunit, combining to give an assembled ribosome sedimenting at 70S (Yusupov et al. 2001). The 30S subunit is composed of 21 proteins named ribosomal proteins S1 to S21. It also contains the 1542-nucleotide 16S rRNA (Schluenzen et al. 2000; Wimberly et al. 2000). Although the large subunit contains 33 ribosomal proteins these are, somewhat counterintuitively, named L1 to L36: there is no L8, L12, or L26 (Ban et al. 2014). It also contains the 2904-nucleotide 23S rRNA and the 120-nucleotide 5S rRNA.

The 30S subunit binds to the mRNA at translation initiation and reads the sequence of codons that this contains (Yusupova et al. 2001). Peptide bonds between amino acids in the growing polypeptide chain are made in the 50S subunit within the peptidyl transferase centre (Ban et al. 2000). The ribosomal RNAs compose the active core, making the ribosome a ribozyme (Nissen et al. 2000; Steitz and Moore 2003).

The ribosome is assembled from its rRNA and protein components in a series of coordinated steps that follow, or occur simultaneously with, the expression and modification of these components. The process is assisted by ribosome assembly factors that are released at the end of ribosome biogenesis (Shajani et al. 2011; Williamson 2005). Among the co-factors of ribosome assembly are the DEAD-box proteins CsdA (or DeaD), DbpA, RhlE, and SrmB (Charollais et al. 2003, 2004; Elles et al. 2009; Jagessar and Jain 2010; Jain 2008), representing four of the five known DEAD-box proteins in *Escherichia coli* (Section 4.15). A DbpA derivative with an active site mutation is associated with defective ribosome assembly, with cells having fewer 70S ribosomes and more 30S and 50S subunits (Elles and Uhlenbeck 2008; Elles et al. 2009). Mutations in the genes encoding CsdA, RhlE, and SrmB interfere with ribosome assembly in bacteria growing at low temperatures (Charollais et al. 2003; Jones et al. 1996; Peil et al. 2008; Toone et al. 1991; Trubetskoy et al. 2009).

The heat shock proteins DnaJ, DnaK, and GrpE contribute to ribosome assembly as protein chaperones; the chaperones GroEL-GroES also contribute (Alix and Guerin 1993;

Alix and Nierhaus 2003; El Hage et al. 2001; Maki et al. 2002, 2003). The cold shock protein, RbfA, is a ribosome-binding factor and is an essential protein for bacterial growth at low temperatures (Dammel and Noller 1995; Jones and Inouye 1996; Xia et al. 2003). Loss of RbfA can be compensated by over-expression of the Era GTPase (Inoue et al. 2003). Era is a Ras-like GTPase that is essential and binds to 30S subunits; a reduction in Era activity has a negative impact on ribosome assembly that can be compensated by over-expression of the KsgA 16S rRNA methyltransferase (Lu and Inouye 1998), yet another co-factor for ribosome maturation and assembly (Connolly et al. 2008). Another essential GTPase, Der, together with its partner protein YihI, is required for the maturation of the 50S subunit (Hwang and Inouye 2010). CgtA/ObgE (also known as YhbZ) is an essential GTPase that binds to both the 30S and the 50S ribosomes (Jiang et al. 2006; Sato et al. 2005). This protein is also an actor in the stringent response (Section 6.18). In addition to KsgA, other rRNA methyltransferases that contribute to ribosome maturation include RrmJ (Caldas et al. 2000) and RrmA (Gustafsson and Persson 1998). Ribonucleotide modification is also important to ribosome development and the activity of RluD, the pseudouridine synthase, is needed (Gutgsell et al. 2005).

The gene for the ribosome-binding factor RbfA is in an operon with the gene for the ribosome maturation factor P, or RimP. Loss of RimP interferes with the maturation of 30S subunits and the effect is exacerbated with rising temperature (Nord et al. 2009). RimP is just one of a series of ribosome maturation factors; others include RimJ, which associates with the 30S subunit at an early stage in its maturation (Roy-Chaudhuri et al. 2008), and RimM, which works with RbfA to process 16S rRNA and to ensure maturation of the 30S subunit (Bylund et al. 1998; Lovgren et al. 2004).

Ribosome assembly depends on cations to neutralise the negative charge on rRNA, allowing it to fold appropriately as the ribosome matures (Klein et al. 2004). The presence of magnesium cations is essential both for the structural integrity of ribosomes and for their ability to maintain peptidyl-transferase activity (Weiss and Morris 1973; Weiss et al. 1973). It follows that starvation for Mg^{2+} leads to a cessation of translation and that prolonged starvation for this cation results in a depletion of the mature ribosome population in the bacterium (McCarthy 1962) and in mammalian cells (Rubin et al. 1979; Terasaki and Rubin 1985). Manipulating the magnesium content of *Bacillus subtilis* by over-expression of an Mg^{2+} uptake system (MgtE) or elimination of an efflux protein (YhpD) for the same cation allowed the bacterium to overcome a genetic defect in the expression of 50S protein L34, allowing functioning ribosomes to be assembled and translation rates to be restored to wild type ($L34^{+}$) levels. The intracellular concentration of magnesium cations is linked to the number of 70S ribosomes and to the number of operons encoding ribosomal RNA (Akanuma et al. 2014), indicating a supply–demand association between Mg^{2+} and the translational capacity of the cell. *S*. Typhimurium can adapt to the macrophage vacuole, where it experiences starvation for Mg^{2+}. Here, the organism reduces its 70S ribosome population and its overall level of translation to live within its means as defined by the available magnesium in its cytosol. This is achieved through reduced transcription of the operons that encode ribosomal RNA by reducing ATP synthesis by the F_1F_0 ATPase and by activation of the stringent response. The strategy of making fewer ribosomes when magnesium is in short supply is likely to be employed widely by bacteria (Pontes et al. 2016). The downregulation of ATP production and its hydrolysis to ADP and inorganic

phosphate (Pi) that accompanies Mg^{2+} starvation induces a transient shortage of Pi in the cell. The activation of the PhoB-dependent Pi uptake system restores intracellular phosphate levels (Pontes and Groisman 2018).

Ribosomes are essential, complex machines and their assembly is a complicated process involving multiple steps, pathways, and actors. It happens much more quickly *in vivo* than *in vitro* with impressive precision, recalling the delicate nature of a finely crafted Swiss watch. Yet, ribosomes, and the process that produces them, are robust, with their maturation pathway showing evidence of redundancy and a tolerance for error. Given the demands imposed by the need to survive in an unpredictable and often hostile environment, perhaps this is not surprising.

5.4 Translation Initiation

Translation of the information in mRNA into protein begins with the recruitment of the 30S ribosomal subunit to the translation initiation region of the message. This consists of the purine-rich ribosome binding (or Shine-Dalgarno) site and the initiation codon, typically AUG (Ringquist et al. 1992; Shine and Dalgarno 1974). A pre-initiation complex is formed by the 30S subunit with the help of initiation factors (IFs) 1, 2, and 3 and the aminoacylated initiator tRNA, fMet-$tRNA^{fMet}$ (Hartz et al. 1989; Simonetti et al. 2009) (Figure 5.1).

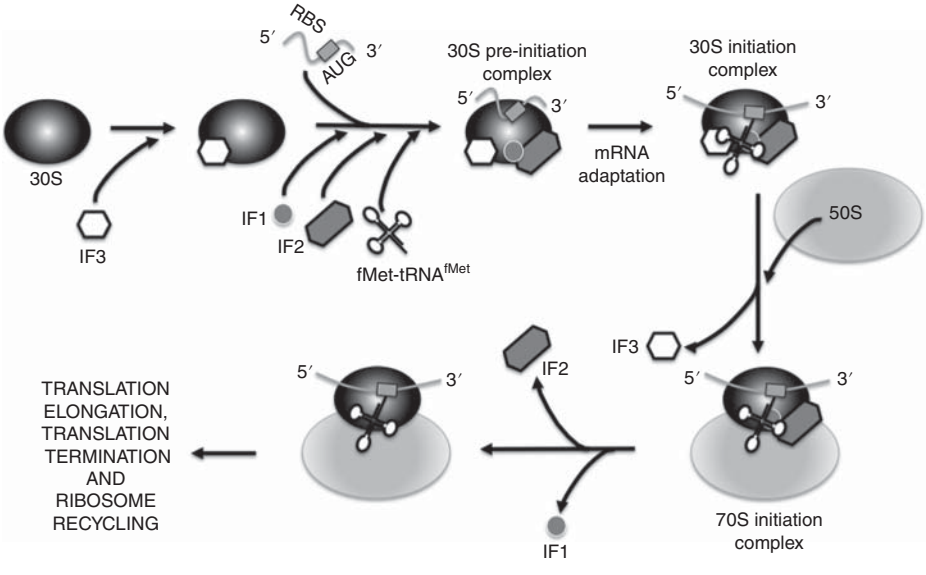

Figure 5.1 Translation initiation. Clockwise, from top left: Initiation factor 3 (IF3) binds to the 30S ribosomal subunit, followed by IF1, IF2, fMet-$tRNA^{fMet}$, and the 5′ end of the message containing the translation initiation signals, to produce the 30S pre-initiation complex (AUG, translation initiation codon; RBS, ribosome-binding site). Movement of the mRNA into its correct position for translation start generates the initiation complex proper. The addition of the 50S ribosomal subunit and the ejection of IF3 create the 70S initiation complex, which matures by the removal of IF1 and IF2, allowing the transition to translation elongation followed by its eventual termination and the recycling of the ribosome subunits.

Docking of the charged initiator tRNA with the start codon of the open reading frame engages it with the P-site on the 30S subunit. The 50S subunit is now recruited to the complex and the IF2 GTPase hydrolyses GTP, the IFs are released and the assembled 70S ribosome is launched into the elongation phase of translation (Lockwood et al. 1971; Simonetti et al. 2009; Subramanian and Davies 1970; Wintermeyer and Gualerzi 1983). Varying the degree of 'fit' between the Shine-Dalgarno sequence in the 5′ end of the mRNA and the anti-Shine-Dalgarno sequence in tRNA is an effective way to modulate the efficiency of translation initiation (Schurr et al. 1993; Marzi et al. 2008). Other important variables include the distance between the Shine-Dalgarno sequence and the translation initiation codon (Chen et al. 1994; Ringquist et al. 1992) and the presence of secondary structures in the vicinity of these translation initiation signals (de Smit and van Duin 1990; Schauder and McCarthy 1989; Studer and Joseph 2006).

Environmental signals influence the efficiency of translational initiation. The process is sensitive to magnesium concentration (Cromie et al. 2006), pH (Nechoostan et al. 2009), temperature (Narberhaus et al. 2006), nutrients (Wang et al. 2008), and feedback controls (Boni et al. 2000; Mandal et al. 2004) that operate in parallel with inputs from sRNA (Storz et al. 2011; Urban and Vogel 2007) and RNA-binding regulatory proteins (Park et al. 2010). For a description of riboswitches, see Section 4.11. Translational control by sRNAs is discussed in Sections 4.2–4.4.

Bacteria such as *E. coli* impose tight gene expression control at the level of translation initiation to match protein production to need (Li et al. 2014). This avoids wasteful production of surplus protein copies, failures to meet demand due to insufficient supply, and the delivery in the correct stoichiometric amounts of proteins involved in common activities (Li et al. 2014). The final point shows that the tuning of individual translation start signals for different genes along a common polycistronic mRNA is crucial to the running of a balanced and orderly cellular economy (Vazquez-Laslop and Mankin 2014). For example, in two-component systems translated from a common transcript, the response regulator (RR) protein is produced in higher numbers than the sensor-kinase partner and in co-transcribed toxin/antitoxin systems, the antitoxin is produced in higher amounts than the toxin. In the first case, one sensor-kinase can communicate with several RRs that, in turn, may regulate several transcription units within their regulon. In the second case, the chemically unstable antitoxin is needed to guarantee that the toxin is maintained in a neutral state until its toxic properties are required, hence the imbalance in the levels of the two factors in favour of the antitoxin. In the case of ABC transporter systems, the periplasmic binding protein, which patrols the periplasm and detects the cargo, is produced in higher quantities than the cytoplasmic-membrane-located uptake components. The rationalisation is that more periplasmic binding proteins increase the probability of the successful detection of the cargo and its delivery to the membrane-embedded ATP-dependent trafficking partners. The *atpIBEFHAGDC* operon encodes the F_1F_0 ATP synthase, which is made up from the protein products in the ratio AtpB (1): AtpE (10): AtpF (2): AtpH (1): AtpA (3): AtpG (1): AtpD (3): AtpC (1). Ribosome density measurements show that the polycistronic *atp* operon transcript has the highest density associated with the *atpE* segment of the transcript followed by the *atpA* and *atpD* segments, then *atpF* with the other segments being equal and having the lowest ribosome densities (Li et al. 2014). Under the growth conditions used by Li et al. (2014) the *atpI* gene was weakly transcribed and had almost no ribosome association.

The MukBEF condensin complex has the stoichiometry MukB(2)MukE(2)MukF(1) and ribosome density determinations show that the open reading frames in the tri-cistronic *mukBEF* operon are translated at rates that correspond to this ratio (Li et al. 2014). In keeping with the importance of translation as the primary determinant of physiologically appropriate levels of gene expression, far more of the *E. coli* proteome is devoted to translation than to other major processes such as transcription or DNA replication (Li et al. 2014). Translational tuning within polycistronic operons will work in tandem with mRNA processing and factors that influence it, such as the presence of secondary structures (some associated with repeat units such as REP elements) and cleavage sites for endoribonucleases (Sections 4.4 and 4.8).

5.5 Translation Elongation

The process of translation elongation is characterised by peptide bond formation between the most recently arrived amino acid and its immediate predecessor (Figure 5.2). Once the new bond is formed, the mRNA threads through the ribosome so that the next codon in the

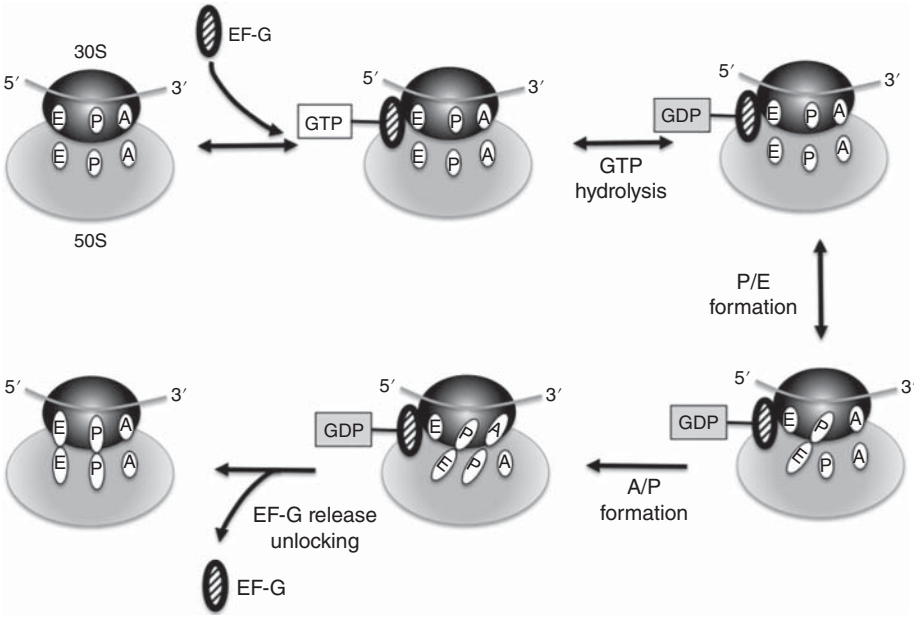

Figure 5.2 Translation elongation. Clockwise, from the top: the 70S ribosome with its 30S and 50S subunits represented by dark- and light-grey filled ovals, respectively. A labelled oval indicates the relative positions of the aminoacyl-tRNA-binding site (A site), peptidyl-tRNA-binding site (P site), and Exit site (E site) on each subunit. Translocation requires the binding to the ribosome of elongation factor G (EF-G) and the hydrolysis of GTP to GDP and Pi. The P site in the 30S subunit engages the E site in 50S and then the A site in 30S engages the P site in 50S, promoting the attachment of the amino acid from a newly arrived charged tRNA to the nascent peptide chain. The release of EF-G unlocks the ribosome, allowing the movement of mRNA and tRNA and repetition of the translocation cycle.

reading frame is presented for matching with the correct charged tRNA. This ratchet-like mechanism is called translocation and involves structural adjustments within the ribosome (Frank and Agrawal 2000; Frank et al. 2007). These include a rotation of the small ribosomal subunit relative to the large one that moves tRNAs along and ejects them (Horan and Noller 2007). Another rotation within the 30S subunit structure involving movement of its 'head' domain relative to the main body involves a helicase activity that suppresses secondary structures in mRNA that would otherwise interfere with the smooth action of the translocation process (Takyar et al. 2005). Transfer RNAs take up positions in the aminoacyl-tRNA binding site (A site), the peptidyl-tRNA binding site (P site), and the Exit site (E site) (Figure 5.2). The EF-Tu GTPase catalyses the binding of the aminoacyl-tRNA to the ribosome's A site. EF-Tu surveys ribosomes that have peptidyl-tRNA in the P site and hydrolyses GTP when the anticodon of its aminoacyl-tRNA pairs with its matching codon in the mRNA in the 30S subunit's A site (Loveland et al. 2017; Satpati et al. 2014; Voorhees et al. 2010). The aminoacyl-tRNA is now released from EF-Tu and can dock with the A/A site in the 70S ribosome, becoming a candidate for peptide bonding to the nascent peptide chain.

Each site lies partly in the 30S and partly in the 50S subunit and the energy for translocation comes from GTP hydrolysis. The GTPase is elongation factor G, EF-G, and this enzyme associates with and dissociates from the ribosome cyclically as elongation proceeds (Belardinelli et al. 2016; Gao et al. 2009; Shoji et al. 2009; Wasserman et al. 2016). The ratcheting action of translocation creates hybrids of the 30S-based and 50S-based A, P, and E sites: A/P and P/E (Figure 5.2). The ribosome has a dauntingly dynamic structure and is capable of adopting a multitude of states due to its many degrees of freedom of movement and the influences of co-factor binding (Dunkle and Cate 2010; Munro et al. 2009). *E. coli* has an elongation factor GTPase, LepA (or elongation factor 4), that can reverse the translocation cycle (Evans et al. 2008; Qin et al. 2006). A reverse translocation capability may be useful in maintaining the fidelity of the translation process. However, LepA, together with the BipA GTPase, are also likely to be involved in ribosome biogenesis (Gibbs and Fredrick 2018).

5.6 Elongation Factor P (EF-P)

Tracts of proline codons in mRNA present a challenge for the ribosome in maintaining its momentum during translation elongation. Proline is structurally an inflexible amino acid that has the ability to impede translocation of $tRNA^{Pro}$. The elongation factor EF-P resembles a tRNA, an example of molecular mimicry that allows it to insert into the ribosome between the exit and peptidyl sites during $tRNA^{Pro}$ translocation, where it is thought to guide proline into a conformation suitable for forming peptide bonds (Doerfel et al. 2015; Rajkovic and Ibba 2017). EF-P must be modified post-translationally on a region that interacts with the peptidyl-transferase centre of the ribosome; the nature of this modification varies across bacterial species. In *E. coli* and *Salmonella,* this involves β-lysylation of Lys34 of EF-P by the PoxA protein, a tRNA synthetase paralogue (Roy et al. 2011). The intervention of EF-P not only rescues translation elongation from pausing, it also has a beneficial effect on transcription by reducing the probability of RNA polymerase backtracking (Sections 3.12 and 3.13).

5.7 Translation Termination

Translation is terminated when a stop codon is detected in the A site by release factor 1 (RF1) or release factor 2 (RF2) (Youngman et al. 2008). RF1 recognises 5′-UAA-3′ and 5′-UAG-3′ while RF2 recognises 5′-UAA-3′ and 5′-UGA-3′ (Scolnick et al. 1968). The RF, which mimics a tRNA, induces the hydrolysis of the completed protein from the tRNA currently occupying the P site, terminating translation by releasing the protein from the ribosome (Laurberg et al. 2008). The GTPase and Class 2 release factor RF3, which clears Class 1 RF1 and RF2 from the ribosome by inducing conformational change in the ribosome (Gao et al. 2007). Release factors play a role in polypeptide proof reading, helping to eliminate polypeptides that contain erroneous peptide bonds from the ribosome (Zaher and Green 2009a, 2009b). This shows that RFs are capable of acting even with a sense codon in the ribosomal A site.

Errors that cause ribosomes to stall prematurely on mRNAs lack a stop codon in the ribosome A site and do not attract Class 1 release factors. These ribosomes are rescued by the transfer-messenger-RNA-(tmRNA)-small protein B (SmpB) trans-translation rescue system that reroutes the ribosome to conventional elongation and translation termination (Huter et al. 2017). TmRNA is made up of an RNA molecule that has a tRNA-like domain (TLD) resembling the acceptor stem of an alanyl-tRNA and a messenger-like domain (MLD) that encodes a short peptide of between 8 and 15 amino acids (Cheng et al. 2010). The domains are connected by a series of pseudoknots. Alanine is added to the TLD by the cellular alanine tRNA synthetase and the reaction is enhanced by SmpB. EF-Tu delivers the TLD charged with alanine to the vacant A site of a non-stop ribosome. The tmRNA-SmpB complex can distinguish translating ribosomes from stalled ones. The alanine on the TLD forms a peptide bond with the nascent polypeptide and EF-G translocates the TLD to the P site (Ramrath et al. 2012). Translation now continues along the MLD, which has a stop codon that results in standard translation termination with the recruitment of Class 1 release factors RF1 or RF2. The ribosome can now be recycled.

The ArfB (YaeJ) protein targets ribosomes that have inappropriately dissociated into 30S and 50S subunits during translation elongation. Such dissociation is typically a result of stress, such as heat shock, and it incapacitates the 50S subunit because a tRNA is covalently linked to the unfinished polypeptide in the exit tunnel of the ribosome. The Hsp15 heat shock protein rescues such complexes by stabilising the tRNA in the P site, liberating the A site so that a stop-codon-independent release factor, ArfB, can enter and excise the tRNA (Jiang et al. 2009).

In a third rescue system for stalled ribosomes, the ArfA (YhdL) protein works with the Class 1 release factor RF2 to alter RF2 conformationally to allow it to use a surrogate stop codon strategy to terminate translation and release the ribosome (Demo et al. 2017; Ma et al. 2017). ArfA binds to the vacant A site, possibly assisted by a tRNA, and then recruits RF2 (Chadani et al. 2012; Shimizu 2012).

Ribosomes translating proteins that are destined for insertion in the cytoplasmic membrane interact with the Signal Recognition Particle (SRP) system so that secretion occurs co-translationally. This avoids the extrusion from the ribosome of proteins with highly hydrophobic domains that would become unstable in the cytoplasm. SRP consists of the GTPase Ffh (‘54 homologue’, named after the eukaryotic SRP-54) protein and the 4.5S RNA

and it engages the hydrophobic transmembrane domains of the protein as these emerge from the ribosome (Doudna and Batey 2004; Luirink and Sinning 2004; Sijbrandi et al. 2003) (Section 5.9).

5.8 Protein Secretion

Bacterial proteins may take up their true biological functions only when they are delivered to the appropriate site in, on, or outside the cell. The protein secretion machinery of the cell ensures that polypeptides that are destined for locations outside the cytoplasm reach those destinations. This machinery is classified into seven types plus the general secretion (Sec) and the twin-arginine transport (Tat) systems. Sec and Tat are highly conserved across all domains of life, and in Gram-negative bacteria they deliver their protein cargo to the cytoplasmic membrane or to the periplasm. The translocated proteins may be destined to remain in those locations, or they may be transported further: to the outer membrane or to the external environment.

5.9 Protein Secretion: The Sec Pathway

The heterotrimeric SecYEG translocon transports proteins across the cytoplasmic member in their unfolded state (Figure 5.3). The cargo protein can be translocated as it comes off the ribosome (co-translational translocation) or post-translationally. The co-translation option is the one usually adopted by proteins that are to be inserted into the lipid bilayer; secreted proteins that are still in possession of their signal sequences use the post-translational pathway. Post-translational transport involves a complex containing SecY and the SecA motor protein. SecA has to compete with ribosomes for sites on SecY that overlap, indicating an intimate association between the co- and post-translational pathways (Denks et al. 2014; Junne et al. 2007; Kuhn et al. 2011). SecY passes through a series of states during protein trafficking: resting (just SecA and closed to all traffic, even water), primed (with the ribosome or SecA), and engaged (in a complex with the protein substrate). So-called constriction zones in SecY regulate the trafficking process. These are (i) a hydrophobic ring for secretory proteins, (ii) a lateral gate for transmembrane proteins, and (iii) a plug domain (Saparov et al. 2007). SecY is voltage gated and this is crucial for the maintenance of the cytoplasmic membrane barrier to the movement of cations and protons and, therefore, to the maintenance of membrane potential and proton motive force (PMF) (Knyazev et al. 2014). The insertion of the first segment of the nascent polypeptide relies on GTP hydrolysis as a source of energy (Ataide et al. 2011); the feeding in of the rest of the protein does not rely on SRP-SRP-receptor and is not thought to arise from pushing by the ribosome of the extruding polypeptide (Park et al. 2013). Instead, the driving force of the hydrophobic effect and PMF may supply the required energy (Ismail et al. 2015; Knyazev et al. 2018).

Co-translational translocation requires contact between the ribosome and SecY and that the SRP guides the nascent polypeptide chain to the translocon, having bound to the signal sequence or a transmembrane helix assisted by the SRP-receptor (Saraogi and Shan 2011). SRP delivers the nascent polypeptide and ribosome to its receptor, the FtsY protein, which

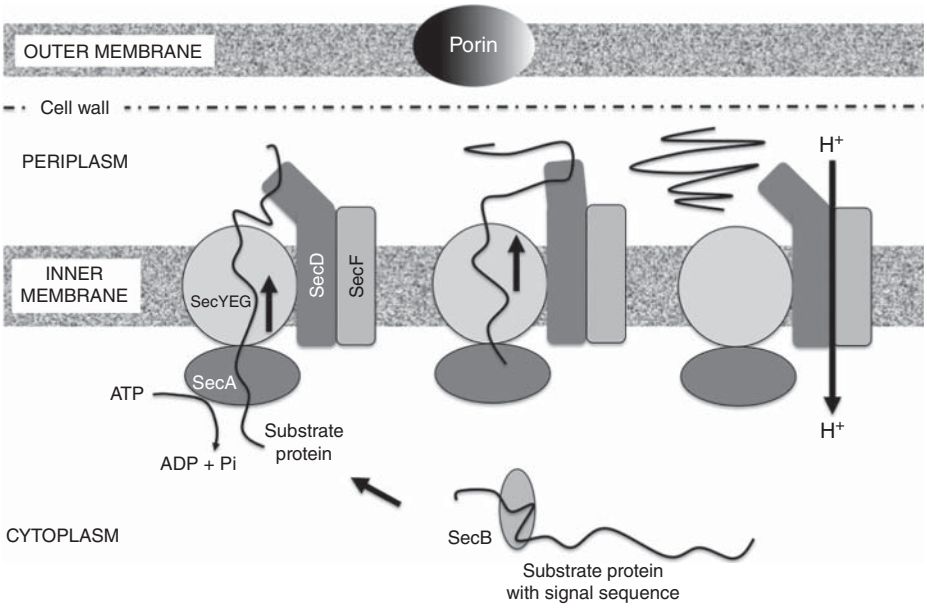

Figure 5.3 SecA-SecYEG-dependent protein secretion. The SecYEG complex in the cytoplasm is represented by a single oval. The cytoplasmic SecA ATPase protein is delivering the substrate protein (wavy black line) to SecYEG, which is in contact with the SecDF complex (left). SecDF 'pulls' the emerging substrate protein out of SecYEG and into the periplasm through a conformational change in the protein-binding domain of SecD (centre). This conformational change is reversed by inward traffic of a proton through the SecDF complex (right). Proteins with signal sequences are recognised and bound in the cytoplasm by the SecB chaperone protein and then delivered to SecA for translocation through the SecYEG translocase.

is found in a complex at the cytoplasmic membrane with SecYEG (Figure 5.4). In addition to SecDF interaction, the SecYEG-FtsY complex may interact with the YidC insertase protein. YidC assists with the lateral release of transmembrane domains from SecYEG but is unable to participate in the translocation of proteins with large periplasmic loops. Its main purpose is to guide the insertion of cytoplasmic membrane proteins into their final cellular destination (Facey et al. 2007; Serdiuk et al. 2016; Welte et al. 2012). The signal sequence is cleaved and removed by signal peptidases during translocation (Paetzel et al. 2002).

In post-translational translocation, the ATPase SecA binds to the SecYEG translocon, widening the lumen of the complex while leaving the sealing plug in position. Thus, SecA is thought to regulate the conformational state of SecY by ATP hydrolysis, driving secretion of the substrate protein and preventing backsliding in a process called Brownian ratcheting (Allen et al. 2016). A competing model, called the power stroke hypothesis, sees SecA using the energy from ATP hydrolysis to push bulky segments of the substrate protein through the translocon (Bauer and Rapoport 2009; Bauer et al. 2014; Zimmer et al. 2008). Backsliding may also be prevented by the intervention of periplasmic proteins (e.g. PpiD and Skp) that bind the emerging substrate polypeptide (Antonoaea et al. 2008; Schafer et al. 1999). The proton channel SecDF may be a partner for SecYEG in the performance of the translocation function (Arkowitz and Wickner 1994; Tsukazaki et al. 2011) (Figure 5.3).

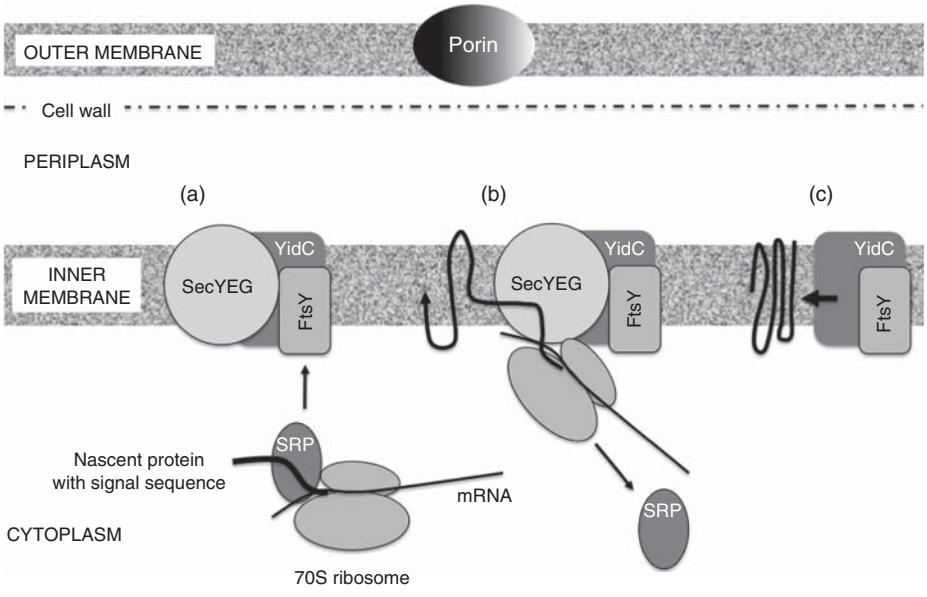

Figure 5.4 SRP-SecYEG-dependent protein secretion. (a) The signal recognition particle, SRP, monitors nascent polypeptide production at 70S ribosomes. When a protein with a signal sequence is detected, SRP interacts with its receptor protein FtsY at the cytoplasmic membrane, delivering the ribosome-nascent polypeptide to the SecYEG translocon. (b) GTP is hydrolyzed by SRP and FtsY and SRP is released back to the cytoplasm. SecYEG interaction with the YidC protein assists with the insertion of translocated proteins into the cytoplasmic membrane. (c) FtsY can also be in a complex with the insertase protein YidC but the details of how it inserts substrate proteins into the membrane without SecYEG are unclear (see Steinberg et al. 2018). Signal peptidases cleave off the signal sequence during protein translocation.

5.10 The Twin Arginine Translocation (Tat) Pathway of Protein Secretion

Once proteins leave the cytoplasm, they are beyond the reach of the cellular machinery that introduces many important post-translational modifications that may be essential for their biological function. These have to be carried out prior to secretion and the protein will be in a folded state as a result. The twin arginine translocation (Tat) system is used to secrete proteins that are folded and which may be bound to their co-factors (Figure 5.5). It identifies its cargo by the presence of two arginine amino acids in the signal sequence within a SRR sequence motif. The TatA and TatB proteins perform this task in Gram-negative bacteria; in Gram-positives, the two are fused to form one multifunctional polypeptide (Goosens and van Dijl 2017). TatA is the protein that forms the channel in the cytoplasmic membrane through which the protein cargo carried by TatA and TatB is secreted. TatB and TatC recruit TatA to the membrane, where it oligomerises (Berks et al. 2005; Müller 2005) (Figure 5.5). TatB and TatC form multimers that deliver the cargo protein to the oligomeric TatA pore-former in the membrane. The system is capable of transporting not just individual folded proteins but also heteromeric protein complexes. In such complexes, only

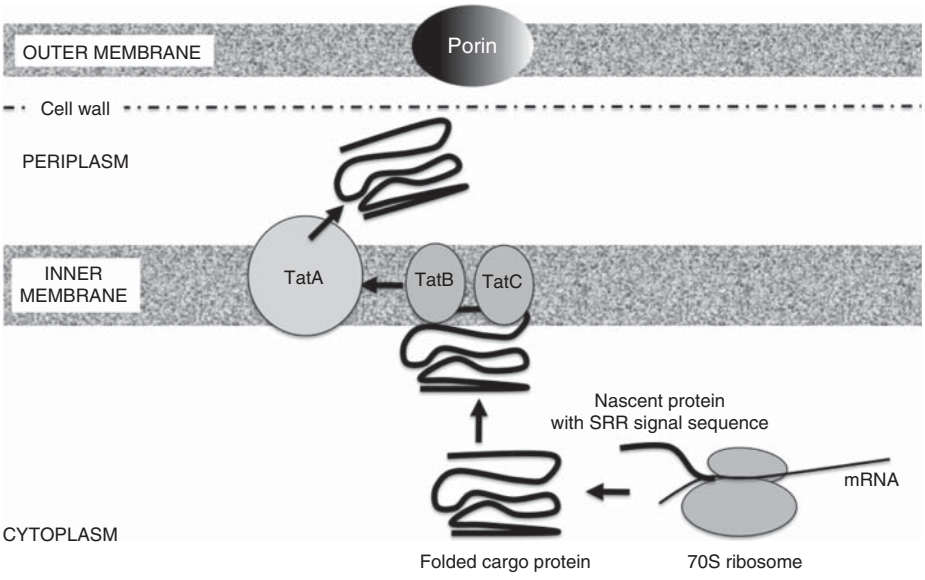

Figure 5.5 The twin arginine translocation (Tat) pathway. The Gram-negative version of the Tat system is illustrated. Multimers of the TatB and TatC proteins bind to a fully folded and post-translationally modified protein that has bound any required co-factor(s). They recruit the TatA protein to the cytoplasmic membrane where its oligomers form a pore through which the folded cargo protein is exported to the periplasm. Depending on the nature of the protein and its biological role, it may remain there or be exported through the outer membrane by yet another transport system.

one partner needs to have the signal sequence for recognition by TatB TatC, the others are simply co-transported (Cline 2015). The Tat system operates cyclically. Once the TatBC multimeric complex has delivered its cargo protein to the TatA oligomer and TatA has exported the protein, the TatABC heteromeric complex dissociates. It re-assembles when the next cargo protein arrives. The energy to drive the export process is derived from PMF.

5.11 Type 1 Secretion Systems (T1SS)

Type 1 secretion systems consist of a tripartite translocation apparatus made up of an inner membrane ATP-binding cassette, a membrane fusion protein and an outer membrane pore protein, TolC (Hantke et al. 2011; Wagner et al. 1983; Wandersman and Delepelaire 1990) (Figure 5.6). Formation of the secretion apparatus is induced by the presence of the substrate, an unfolded polypeptide, and this is exported in one step from the cytoplasm to the exterior of the cell (Thanabalu et al. 1998). Substrate-apparatus interaction depends on the presence in the cargo of a specific signal sequence at either its N-terminus (for bacteriocins) or C-terminus (all other T1SS substrates). The transport process is powered by ATP hydrolysis (Higgins et al. 1986). A derivative of T1SS in *Pseudomonas* has a periplasmic intermediate, with secretion happening via a two-step process; single-step secretion is the general rule for T1SS (Smith et al. 2018). Among the cargo proteins secreted via T1SS are

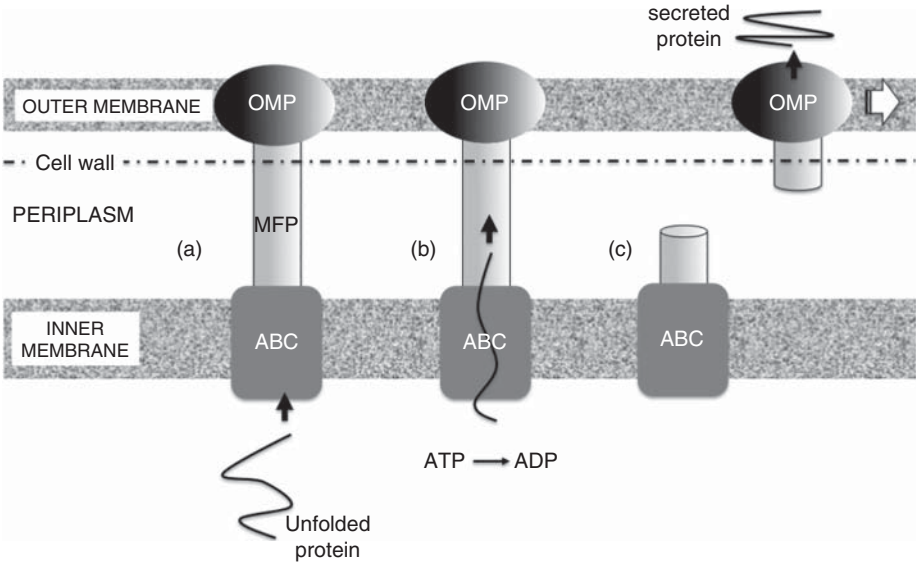

Figure 5.6 Type 1 secretion system (T1SS). The system has three components: an ATP-binding cassette (ABC) associated with the cytoplasmic membrane, a membrane fusion protein (MFP) and an outer membrane protein (OMP), usually TolC. An unfolded cargo protein with a suitable signal sequence is bound by the ABC (a) and translocated through the MFP to the OMP, using ATP hydrolysis to power the process (b). Once the cargo protein has been exported, the T1SS disassembles (c).

colicins and 'repeats-in-toxins' (RTX) toxins such as HlyA from *E. coli* (Mackman and Holland 1984; Welch 1991) and the MARTX toxin from *Vibrio cholerae* (Dolores et al. 2015).

5.12 Type 2 Secretion Systems (T2SS)

The type 2 systems are confined to the outer membrane in Gram-negative bacteria and are concerned with the secretion of folded proteins from the periplasm (Figure 5.7). These proteins have to be delivered from the cytoplasm to the periplasm either by a Sec-dependent pathway or the Tat pathway (Voulhoux et al. 2001). To qualify for export by these pathways, the cargo proteins must be synthesised with signal sequences that are Sec- or Tat-compatible. If they have come via a Sec pathway, they must fold in the periplasm because T2SS can only handle folded polypeptides. Cargoes are varied in nature, but extracellular enzymes are frequently carried, including enzymes that assist pathogens during infection, as seen for example, in *V. cholerae* and *Pseudomonas aeruginosa* (Lu and Lory 1996; Sandkvist et al. 1997).

T2SS, the general secretion pathway, consists of an outer membrane complex that provides the exit pore for the cargo. It is composed of multiple copies of the secretin protein (Korotkov et al. 2011, 2015). Altogether, about 70 proteins are involved in the assembly of the T2SS. Although secretin is located in the outer membrane, it is in contact with T2SS

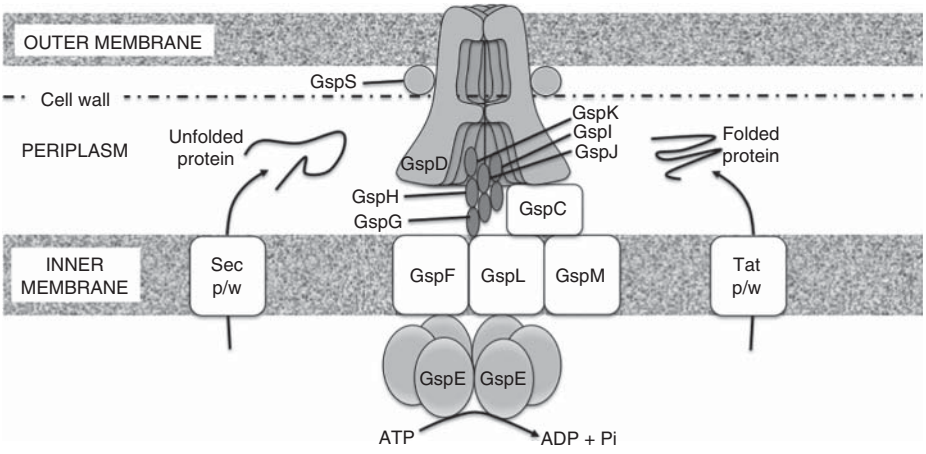

Figure 5.7 Type 2 secretion system (T2SS). The Gsp protein names refer to the General Secretory Pathway. GspE provides the ATPase function to power the system. GspH, GspI, GspJ, GspG, GspK, are pseudopilins, based on amino acid sequence similarity to components of type 4 pili and their relative positions in the T2SS and those of their counterparts in the type 4 pilus. GspD is a secretin and GspS is a pilotin lipoprotein. The inner membrane platform proteins are GspC, GspF, GspL, and GspM. Folded cargo proteins for export through the T2SS are provided via the Tat pathway while unfolded proteins that must now be folded in the periplasm prior to T2SS-mediated export are provided through the Sec pathway, shown arbitrarily right and left of the Gsp system, respectively.

components in the inner membrane. This portion of the system is called the inner membrane platform, is composed of several proteins and extends into the periplasm where it meets secretin. Energy for transport is provided by ATP hydrolysis, with the T2SS ATPase being located in the cytoplasm (Figure 5.7). The system possesses a pseudopilus, located on the cell surface and built from subunit proteins related to those found in type IV pili, including those involved in DNA uptake and competence (Hobbs and Mattick 1993). In the piston model of T2SS function, the pseudopilus retracts to expel the folded cargo protein from the exit pore (Sauvonnet et al. 2000). The protein–protein linkages extending to the periplasm activate the ATPase for generate the energy needed to operate the expulsion system.

5.13 Type 3 Secretion Systems (T3SS)

Type 3 secretion systems are also known as injectisomes because they have a syringe-and-needle structure (Figure 5.8) (Edgren et al. 2012). They are found in Gram-negative bacteria and form core components of the virulence systems of several important pathogens of humans, animals, and plants; they also contribute to symbiotic relationships (Abrusci et al. 2014; Burkinshaw and Strynadka 2014; Buttner 2012; Kubori et al. 1998; Radics et al. 2014). The genes that encode them have been acquired by horizontal transfer, they have a high A + T content in their DNA compared with the average A + T content of the core genome of the Gram-negative host, and they are present in clusters that make up pathogenicity or

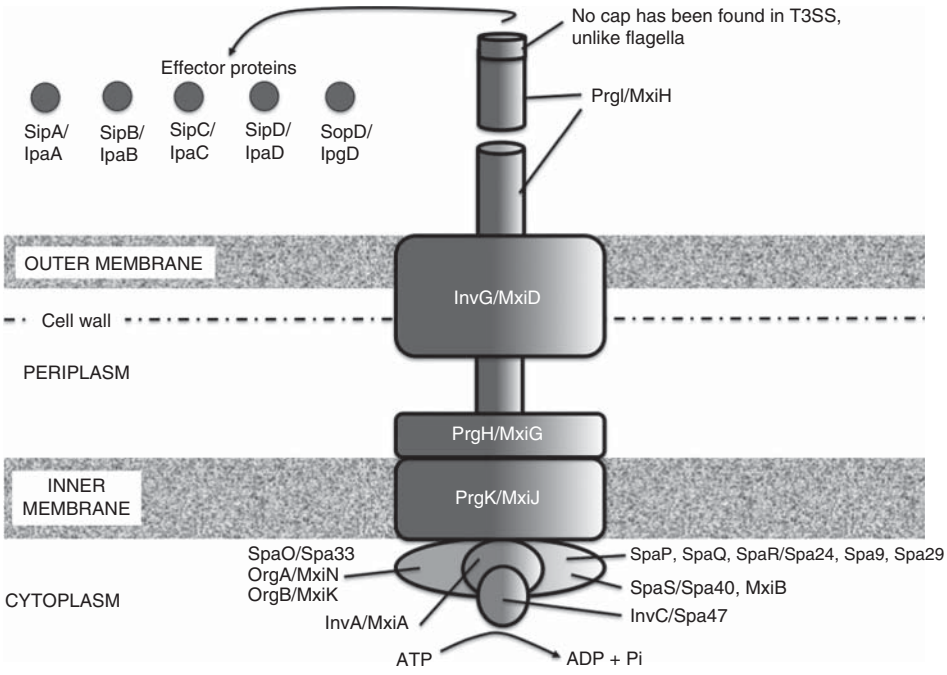

Figure 5.8 Type 3 secretion system. The proteins are given the names of counterparts from *Salmonella* (left)/*Shigella* (right) thus: SipB/IpaA. The T3SS consists of a cytoplasmic membrane complex, the needle and the translocon at the needle tip. Energy is supplied by ATP hydrolysis in the cytoplasm by InvC/Spa47. The needle is composed of PrgI/MxiH subunits proteins and the secreted proteins that promote invasion of mammalian epithelial cells are SipA/IpaA, SipB/IpaB, SipC/IpaC, and SipD/IpaD. Like the flagellar system, the T3SS have ring structures embedded in the inner and outer membranes and in the peptidoglycan layer.

symbiosis islands on the chromosome or on plasmids. Their role in infection and the regulation of their expression is described for the *Shigella flexneri* and the *S*. Typhimurium systems in Sections 7.10 and 7.16.

T3SS consist of three main sub-complexes: the basal body (or base complex), the needle, and the translocon (Figure 5.8). ATP hydrolysis at the basal body provides the power to drive the system. There are nine core proteins that are present in all T3SS and eight of these are held in common with the flagellar apparatus, which, technically, is also a T3SS in terms of the mechanism by which it assembles the flagellum and hook region. Injectisomes differ from the flagellar system in having a needle instead of a flagellum (Blocker et al. 2003). This structure is used to pierce the membrane of a host cell and to inject unfolded effector proteins that alter the cytoskeleton to facilitate invasion by the bacterium or otherwise modify the biology of the host cell to the advantage of the microbe. The needle tip complex senses contact with the host cell and controls effector protein secretion and the insertion of translocons into the host membrane, where they form a pore through which effectors are delivered. Thus, cargo proteins translocated by T3SS traverse three membranes, two in the Gram-negative bacterium and one in the host. This property is shared with type 4 secretion systems, T4SS.

5.14 Type 4 Secretion Systems (T4SS)

Type 4 systems target a wide variety of cell types, both prokaryotic and eukaryotic, and translocate a variety of cargo molecules. In addition to individual proteins and protein complexes, they can transport DNA and nucleoprotein complexes. T4SS are related to bacterial conjugation systems and these have evolved to transfer mobile genetic elements from one bacterium to another. T4SS are highly conserved and regardless of their transport function, they operate in the same way (Cascales and Christie 2003; Waksman 2019). T4SS are used by bacterial pathogens such as *Brucella, Helicobacter,* and *Legionella* to transfer effector

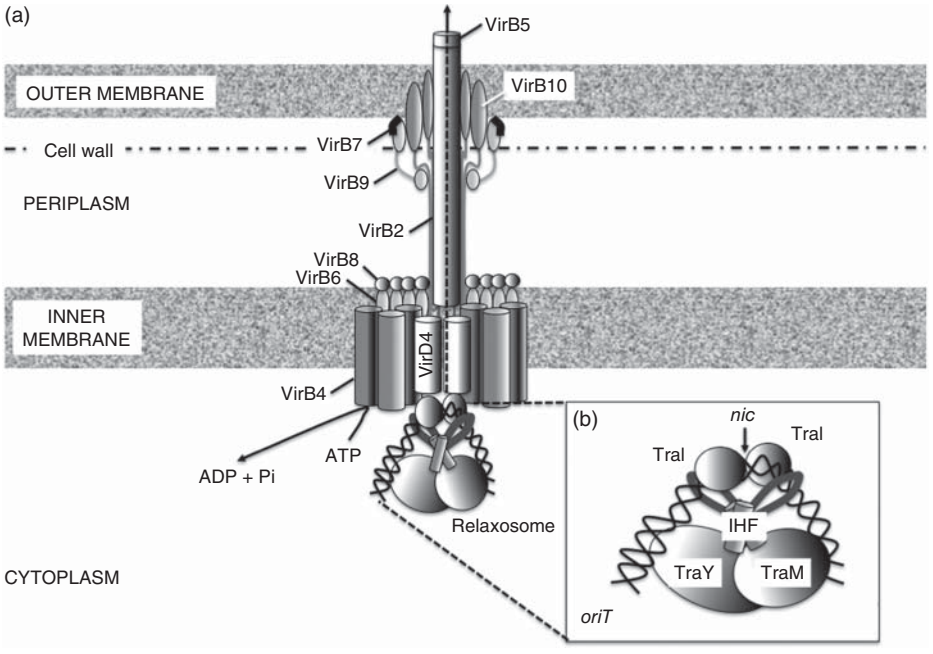

Figure 5.9 Type 4 secretion system. (a) A summary of the structure of the T4SS encoded by F-like self-transmissible plasmids. The F conjugation pilus is assembled from VirB2 subunits (the protein naming scheme from the *A. tumefaciens* T4SS is used here). This occurs when the VirB11 protein (not shown) interacts with the VirB4 complex, switching it to VirB2 recruitment. This has been postulated to occur via the lateral gate mechanism used by SecYEG to move proteins sideways through the membrane (Section 5.9). Here, the secretion system is engaged with the F plasmid relaxosome, which feeds single-stranded DNA through the apparatus, crossing the cell envelopes of the donor and recipient bacteria. Led by the relaxase, which is recruited to the secretion system by the VirD4 coupling protein, the DNA passes through the bore of the conjugation pilus (dotted line with arrowhead). ATP hydrolysis by the VirB4 and VirD4 ATPases generates the power to operate the system. (b) The relaxosome consists of the origin of transfer of the F plasmid, *oriT,* bound by the plasmid encoded TraI (2 copies), TraM, and TraY. IHF, provided by the bacterial host, binds and bends the *traT* DNA. It is represented here by two loops to represent the beta sheets that insert into the minor groove of the DNA to generate the sharp bend and two cylinders to represent the alpha helical bodies of the two IHF subunits. The TraI protein has a DNA relaxase function and it nicks the dsDNA at the *nic* site. This relaxes DNA supercoils in the *oriT* region and provides a 5′ DNA end for covalent attachment to the relaxase. The resulting nucleoprotein complex becomes the substrate for secretion through the type 4 system.

proteins to host cells that promote infection (Backert and Meyer 2006; Isberg et al. 2009). *Neisseria gonorrhoeae* uses T4SS to promote DNA uptake (Hamilton and Dillard 2006).

T4SS are typically encoded by plasmids or other mobile genetic elements that are self-transmissible. They play important roles in unidirectional horizontal DNA transfer and bacterial evolution (Frost et al. 2005). Each system involved in DNA transfer consists of a pilus, the associated secretion apparatus, and a nucleoprotein complex called the relaxosome (Figure 5.9). The relaxosome is assembled at the origin of transfer, *oriT,* on the plasmid and consists of a relaxase and accessory proteins. Relaxase cuts the *oriT* DNA at the *nic* site and forms a covalent bond to the 5′ end of the cleaved DNA. Relaxase has a helicase activity that allows it to unwind the dsDNA of the plasmid (Ilangovan et al. 2017; Zechner et al. 2017), generating the cargo for transfer to the recipient cell. It then guides the attached DNA strand through the T4SS by interacting with a coupling protein, VirD4 (Redzej et al. 2017) (Figure 5.9). This nomenclature is taken from the *Agrobacterium tumefaciens* T4SS which has been dissected in minute detail, not least because it facilitates horizontal DNA transfer between members of two domains of life: bacteria and plants (Waksman and Fronzes 2010). Accessory proteins assist the activities of the relaxase at *oriT*: in the case of the F-plasmid-encoded TraI relaxase these are TraM and TraY (both F-encoded) and the IHF protein encoded by the bacterium (Figure 5.9). The relaxase has a secretion signal in its mid-section that is detected by VirD4, leading to recruitment of the relaxase-ssDNA complex to the secretion apparatus (Alperi et al. 2013; Redzej et al. 2013). This apparatus is composed of multi-protein complexes in the outer and the inner membranes that are linked. It possesses at least two ATPases that provide power to operate the system: VirB4 and the VirD4 coupling protein (Pena et al. 2012; Wallden et al. 2012). The conjugation pilus is composed predominantly of a major subunit protein, VirB2, together with the VirB5 minor subunit at the pilus tip (Aly and Baron 2007) (Figure 5.9).

5.15 Type 5 Secretion Systems (T5SS): The Autotransporters

Autotransport describes a mode of protein secretion in which the cargo protein secretes itself. The autotransport step occurs at the outer membrane and involves passage of the cargo through a beta-barrel structure that is internal to the protein. In order to autotransport through the outer membrane, the protein must first reach the periplasm and it does this in an unfolded state via the Sec pathway using a signal sequence that is subsequently removed by signal peptidases (Leyton et al. 2012; van Ulsen et al. 2014). Its journey through the cytoplasmic membrane may be assisted by the Bam system or the Skp chaperone (Ruiz-Perez, et al. 2009; Wagner et al. 2009). The protein typically will possess a translocator domain with which to form a pore in the outer membrane, a passenger domain that is the functional component of the cargo, and a linker that attaches the passenger domain to the translocator (Figure 5.10). It may also possess its own protease to separate the passenger from the rest of the protein once it is exported (Leyton et al. 2012). Many autotransported proteins assist bacterial pathogens during infection, such as the secreted protease used by *N. gonorrhoeae* to cleave immunoglobulin A, undermining host antibody-based defenses (Pohlner et al. 1987), and the IcsA virulence factor used by *S. flexneri* to recruit host actin as a propulsion system inside human epithelia (Brandon et al. 2003) (Section 7.11). A division of labour can arise

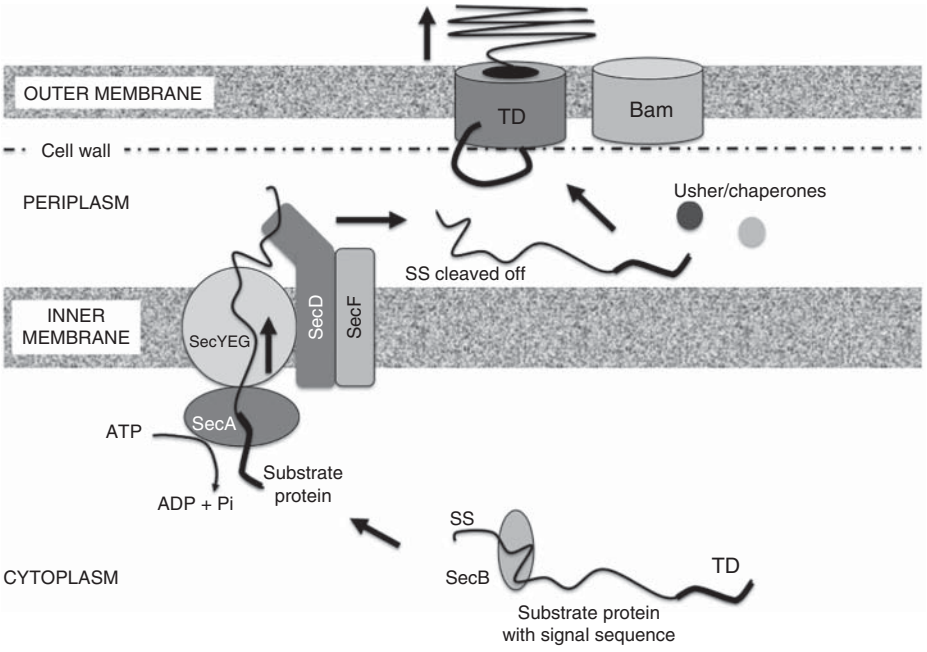

Figure 5.10 Type 5 secretion system (T5SS). The unfolded autotransporter protein enters the periplasm via the SecYEG pathway, recruited by its signal sequence, SS. Assisted by the Bam complex, its translocator domain (TD) folds into a 12-beta-barrel channel in the outer membrane, possibly assisted by usher/chaperone proteins in the periplasm. The passenger domain then exits the periplasm via the TD channel, perhaps driven by energy from the folding of the exported protein. In some autotransporters, an endogenous protease may cleave the passenger domain from the linker attaching it to the TD, liberating the exported protein from the cell surface.

in the case of T5SS that have a partner protein containing the beta-barrel through which the (usually quite large) passenger protein passes (Lambert-Buisine et al. 1998; McCann and St Geme 2014). The partnership may be further expanded to include an usher protein to assist the passenger protein through the outer membrane channel, as happens in some pilus subunit secretion pathways (Waksman and Hultgren 2009).

5.16 Type 6 Secretion Systems (T6SS)

These systems are especially common among the *Enterobacteriaceae* where they are regarded as assisting the bacteria in their intra- and inter-community relationships (Bingle et al. 2008; Cascales 2008). In parallel with quorum-sensing-based communications, bacteria have evolved methods for interacting with other microbes that can involve rather more aggressive strategies. T6SS can deliver toxins that target other bacteria, eukaryotes, or even both (Alcoforado Diniz et al. 2015; Bleves et al. 2014; Jiang et al. 2014; MacIntyre et al. 2010; Pukatzki et al. 2007; Sana et al. 2017). Pathogens such as Enteroaggregative *E. coli, Salmonella* (Sana et al. 2016), *Shigella* (Anderson et al. 2017), and *V. cholerae* (Fu et al. 2013) use T6SS to displace competitors and to establish themselves in the host gut, as do symbiotic gut bacteria (Hecht et al. 2016; Wexler et al. 2016).

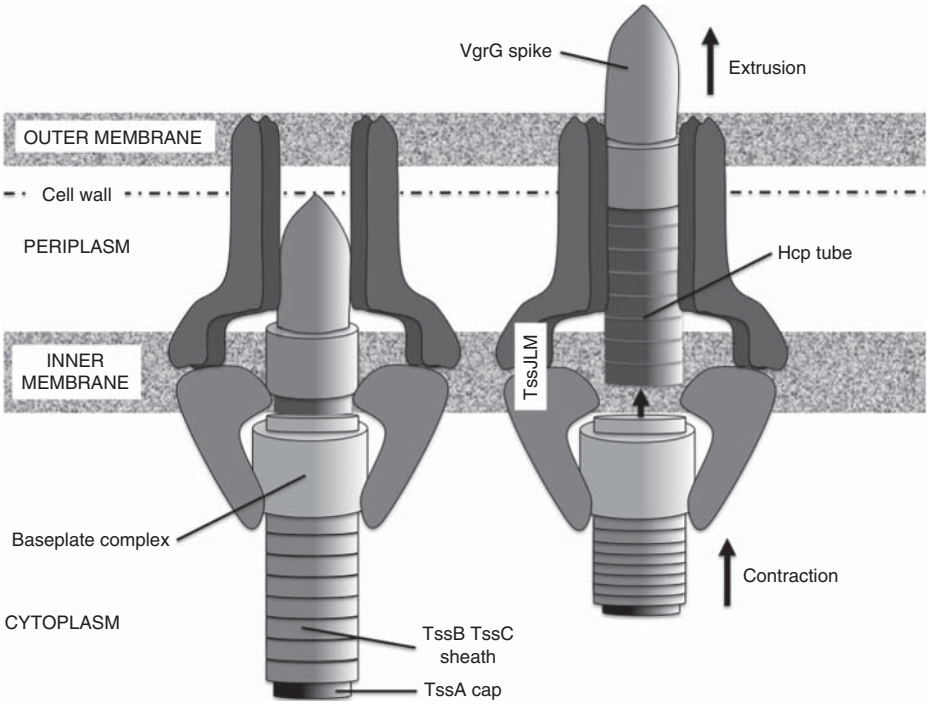

Figure 5.11 Type 6 secretion system (T6SS). The system consists of a contractile sheath composed of proteins TssB and TssC (called VipA and VipB, respectively, in *V. cholerae*) surrounding the Hcp internal tube with the VrgR spike structure on top, mounted on the baseplate. The TssA cap protein is at the bottom of the mature structure. Contraction of the sheath launches the VgrG-Hcp complex through the envelope-spanning TssJLM trans-membrane complex to the exterior of the cell where VgrG can make contact with its target.

T6SS is a complex nanomachine that traverses the different layers of the cell envelope (Figure 5.11). It is made up of a contractile unit with an inner tube fabricated from a stack of Hcp protein hexamers that is surrounded by a sheath of TssB and TssC proteins and tipped with a VgrG spike (Basler et al. 2012; Durand et al. 2015; Laiman et al. 2009; Silverman et al. 2013). The contractile tail is related functionally and evolutionarily to the contractile tails of bacteriophage and the TssA cap protein plays a key role in its biogenesis (Zoued et al. 2017). The sheath contracts, providing the energy needed for the injection of the toxins located in the Hcp tube or attached to VgrG. The ClpV ATPase recycles the contractile sheath after use, allowing a new round of assembly and injection to proceed (Silverman et al. 2013).

5.17 Protein Secretion in Gram-Positive Bacteria: SecA1, SecA2, and SrtA

Protein secretion systems in Gram-positive bacteria do not have an outer membrane with which to contend. Gram-positives possess the Sec and Tat pathways, already described for Gram-negatives, to translocate proteins across the cytoplasmic membrane.

Gram-positives also have two SecA counterparts: an essential SecA1 and a non-essential SecA2. Some, such as *Streptococcus* spp. and *Staphylococcus* spp. have an additional Sec pathway: aSec/SecA2-SecY2 that transports heavily modified cell wall anchor proteins with serine-rich repeats. In the absence of an outer membrane, surface expressed proteins have to be attached covalently to the cell wall. Sortase proteins carry out this function. SrtA is a generalist among sortases, handling proteins that possess a characteristic sorting signal, LPXTG. SrtA cleaves the protein between the T and G amino acids, covalently linking the T to a C amino acid in SrtA, shortening the cargo protein at its C-terminus (Hendrickx et al. 2011). The protein is next attached to lipid II, a cell wall precursor, for incorporation into peptidoglycan.

5.18 Type 7 Secretion Systems (T7SS)

The existence of the mycomembrane at the outer surface of the cell envelope of *Mycobacterium* spp. and other Gram-positive organisms poses a problem for protein export (Bansal-Mutalik and Nikaido 2014; Jankute et al. 2015). ESX protein secretion systems have been proposed as important contributors to solving this problem. Their genes are co-regulated with those involved in cell wall synthesis and their protein components are associated with the cell envelope where they form an ESX complex with the MycP protein, a subtilisin-like protease (Ates et al. 2016; Groschel et al. 2016; Shah and Briken 2016). ESX systems have relatives in a wide variety of Gram-positive bacteria, including *B. subtilis, Listeria monocytogenes,* and *Staphylococcus aureus* (Groschel et al. 2016). The ESAT-6 system form *S. aureus* is a virulence system that promotes persistence in the host and abscess formation, although it is not essential for growth. It shows low-level similarity to the mycobacterial counterparts but shares with them, and all ESX systems, proteins with similarities to ATPases related to FstK-SpoIIIE and small proteins with a WXG motif (Burts et al. 2005). Work to understand the structure-function relationships of all of the members of the ESX complex is still underway, as are efforts to explain the molecular details of the complex's communication with the outer layer of *Mycobacterium* spp. and protein translocation across this barrier (Green and Mecsas 2016).

5.19 Protein Modification: Acetylation

Acetylation of proteins is widespread in bacteria, affecting many cellular functions. The first bacterial protein found to be acetylated was CheY, a regulator of flagella rotation in the chemotactic pathway in *E. coli* (Barak et al. 1992; Wolfe et al. 1988). Acetylation occurs on N^{ϵ}-lysine and can be carried out enzymatically or nonenzymatically. Acetylation of lysine is carried out by lysine acetyltransferases, KATs, typically using acetyl-CoA as the source of the acetyl group. Nonenzymatic acetylation is linked to the intracellular concentration of acetylphosphate (Weinert et al. 2013; Wolfe 2016). Acetyl groups are removed from proteins called deacetylases and these come from the family of zinc-dependent deacetylases or from the NAD^{+}-dependent sirtuins (Carabetta and Cristea 2017).

Protein acetylation has consequences for broad areas of cellular activity in bacteria. Cell morphology and motility are sensitive to protein acetylation altering the MreB protein's function (and hence cell shape) and modulating the interaction of CheY with the flagellar motor to change the tumble-swim frequency of the cell. In *M. tuberculosis*, acetylation of HU alters the activity of this nucleoid-associated protein (NAP) and so has nucleoid architectural and gene regulatory consequences for the bacterium (Ghosh et al. 2016). DnaA is a target for acetylation, making the initiation of chromosome replication sensitive to this protein modification (Zhang, Q., et al. 2016). The Ku protein is reactivated by acetylation and this reduces the efficiency of non-homologous end-joining at double-stranded DNA breaks (Zhou et al. 2015). Acetylation of transcription factors (e.g. HilD, LRP, RcsB, PhoP, etc.) has consequences for the expression of their target genes and operons (Hu et al. 2013; Qin et al. 2016; Ren et al. 2016; Sang et al. 2016; Thao et al. 2010), including those involved in virulence in the case of pathogens (Ren et al. 2017); the acetylation of the alpha subunit of RNA polymerase has consequences for the transcription of all genes (Lima et al. 2011). Other processes that are sensitive to protein acetylation include RNA turnover during exponential growth (Liang et al. 2011; Song et al. 2016) and central metabolism through influences on the size of the acetyl-CoA pool size.

5.20 Protein Modification: Glycosylation

Bacteria and archaea perform both *N*- and *O*-linked glycosylation of proteins (Calo et al. 2010; Lu et al. 2015; Nothaft and Szymanski 2013). In the *N*-linked process, glycans are attached to the side-chain nitrogen of an asparagine within an N-X-S/T motif while in O-linked glycosylation the addition is made on the –OH group of an S or T. These are especially important modifications in pathogens such as *Acinetobacter* (Iwashkiw et al. 2012), *Burkholderia* (Scott et al. 2011), *Campylobacter* (Szymanski et al. 1999), diffusely adhering *E. coli* (Benz and Schmidt 2001), enterotoxigenic *E. coli* (Lindenthal and Elsinghorst 1999), *Francisella* (Egge-Jacobsen et al. 2011), *Haemophilus* (Grass et al. 2003), *Mycobacterium* (Dobos et al. 1996), *Neisseria* (Stimson et al. 1995), *Pseudomonas* (Brimer and Montie 1998; Castric 1995), and *Streptococcus* (Wu et al. 1998). Glycosylation of exported and surface linked proteins from/on the bacterium can assist with adhesion and the interaction of the microbe with the host defenses during infection. In addition, gylcosyltransferases produced and secreted by bacteria can target structural and regulatory processes in the host, modifying its cellular activity to the benefit of the bacterium (Belyi et al. 2013; Li et al. 2013; Pearson et al. 2017).

5.21 Protein Modification: Phosphorylation

Protein phosphorylation in bacteria is carried out predominantly by histidine protein kinases (HPKs), typically phosphorylating a RR protein on an aspartate amino acid in response to a signal from the external or internal environment of the cell (Goulian 2010). For this reason, HPKs are also known as sensor kinases and many of them, but not

all, are cytoplasmic membrane proteins. Although called kinases, many HPKs are also phosphatases and the balance between these conflicting activities is set by environmental input. They become autophosphorylated on a histidine amino acid and then transfer the phosphate to an aspartic acid amino acid in a receiver domain, usually in a RR. In more complex phospho-relays, the receiver may be in the same polypeptide as the autokinase activity or in a separate protein. Phosphorelays allow several layers of control to be built into the information flow pathway (Goulian 2010). RRs are DNA-binding proteins in many, but not all cases and they tune gene expression in response to information about the changing composition of the external environment or the metabolic status of the cell. When the RR is a DNA-binding protein, it may feed back positively onto the expression of its own gene and that of the partner HPK (Miyashiro and Goulian 2008; Shin et al. 2006). Negative feedback is less common and when it occurs it seems to generate oscillating outputs (Biondi et al. 2006; Holtzendorff et al. 2004; Mitrophanov et al. 2007).

Branching of signal transmission through HPK-RR systems may involve many signals being focused at one RR or one signal may be distributed to several RR recipients. The former scenario is illustrated by the information flow in the bacterial chemotaxis system from CheA to CheB and CheY (Kirby 2009). The latter is exemplified by the quorum-sensing system of *Vibrio* (Section 7.8) (Ng and Bassler 2009). Although a common chemistry is used to transmit information through 2-component HPK-RR systems, crosstalk between partnerships is rare arising from the high fidelity of HPK-RR interactions (Groban et al. 2009; Laub and Goulian 2007; Siryaporn and Goulian 2008; Szurmant and Hoch 2010). Cross-regulation via connector proteins has been reported. The PrmD protein of *Salmonella* inhibits the dephosphorylation of the PmrA RR by its HPK PmrB. The system is connected to the PhoQ-PhoP HPK-RR two-component system because phosphorylated PhoP is a transcription activator of *prmD* (Kato and Groisman 2004; Lippa and Goulian 2009; Mitrophanov et al. 2008). Other examples of connector proteins in HPK-RR networks are the SafA small membrane protein in *E. coli* that connects the PhoQ-PhoP and EvgSA 2-component systems: EvgSA regulates *safA* transcription and SafA stimulates PhoQ autophosphorylation by direct interaction (Eguchi et al. 2011; Ishii et al. 2013). Yet another illustration of this principle is provided by the small membrane protein MzrA which stimulates the EnvZ HPK directly, controlling the activity of the OmpR RR; the CpxRA 2-component system regulates *mzrA* transcription (Gerken and Misra 2010). The small membrane protein LetE connects 2-component systems CpxRA and LetAS in *Legionella pneumophila* (Feldheim et al. 2016) and LerC connects LetAS and PmrAB in the same organism: PmrAB activates its gene while LerC deactivates LesAS by acting as a phosphate sink for the LetS HPK (Feldheim et al. 2018). These examples show how individual HPK-RR partnerships can become nodes in wider networks. Those networks include protein phosphorylation events using serine/threonine chemistry, previously thought of as occurring only in eukaryotes (Dworkin 2015).

The historical link between Ser/Thr phosphorylation and eukaryotes has led to the bacterial kinases that use this chemistry being defined as eukaryotic-like Ser/Thr kinases, eSTKs. These are now recognised as the evolutionary precursors of the eukaryotic enzymes. As one might predict, bacteria are now known to possess their own counterparts to the phosphatases that reverse the Ser/Thr phosphorylation process: the eukaryotic-like Ser/Thr phosphatases, eSTPs (Pereira et al. 2011). Examples of protein targets for Ser/Thr

phosphorylation include cell-cycle players such as FtsZ, the cell-pole-targeting protein DivIVA, the translation elongation GTPase EF-Tu (Mijakovic and Macek 2012), transcription factors of the RR class (Lin et al. 2009), proteins involved in bacterial virulence (Misra et al. 2011), and HipA, a protein of importance in the emergence of persisters in antibiotic-treated populations (Germain et al. 2013; Kaspy et al. 2013).

Bacterial Ser/Thr kinases can influence gene expression by modulating the activities of HPK-RR partnerships. The CovS HKP phosphorylates its CovR RR partner in Group A and B streptococci and the Stk eSTK can also target CovR, inhibiting its dimerization and DNA-binding activities (Horstmann et al. 2014). The eSTK1 kinase of *S. aureus* inactivates the DNA-binding activities of the RRs GraR and VraR. GraR is the RR partner in the GraSR two-component regulatory system and it controls cell wall metabolism and resistance to Vancomycin and cationic peptides (Falford et al. 2011; Fridman et al. 2013). VraR is an RR partner in a three-component signal transduction system, VraTSR, that controls the expression of genes involved in the response to cell wall stress and to antibiotics that target the wall (Canova et al. 2014). Several eSTKs contribute to cell wall metabolism, cell division, central metabolism, and (in the case of pathogens) the expression of virulence genes: these enzymes have a characteristic motif known as PASTA (Peptidoglycan and Ser/Thr kinase associated) (Pensinger et al. 2018). PASTA kinases are associated with the cell envelope, have a single transmembrane domain, and bind muropeptides on the extracellular side of the membrane. The signals that they transmit into the cell affect bacterial virulence, biofilm formation, and antibiotic resistance. For example, the *S. aureus* PASTA kinase Stk1 exerts its multiple roles through influencing the expression of global regulators such as the *agr* locus (a master control system for virulence gene expression), the Sigma-B stress-and-stationary-phase sigma factor of RNA polymerase and the SarA transcription factor and regulator of virulence gene expression (Pensinger et al. 2018). The PASTA kinase PrkA in *L. monocytogenes* is similarly required for the expression of a fully virulent phenotype in this pathogen (Pensinger et al. 2016). PASTA group members also control the uptake of carbohydrates and important aspects of central metabolism such as the activities of enzymes involved in glycolysis and gluconeogenesis (Pensinger et al. 2018).

5.22 Protein Splicing

Like some RNA molecules, certain proteins undergo splicing and, by analogy with introns and exons in RNA splicing, the protein segment that is removed is called an intein and flanking polypeptides are exteins (Lennon and Belfort 2017). Exteins have been detected in about 25% of sequenced bacterial genomes as well as in bacteriophage. They are most often (80%) found in proteins involved in DNA metabolism and frequently (70%) in the ATPase domain of proteins that possess that activity. Inteins are removed seamlessly from proteins in a series of nucleophilic attacks that sever peptide bonds, with new bond formation leaving a normal peptide bond at the site in the protein where the exteins join and where the intein had once been (Mills et al. 2014). Excision may be triggered by environmental signals, indicating that at least some protein splicing may be programmed to meet the needs to the bacterium (inteins had been regarded for some time as examples of selfish molecules that provided no benefit to the host) (Reitter et al. 2016; Topolina et al. 2015).

5.23 Small Proteins

Proteins of 50 amino acids or fewer in length are emerging as important contributors to bacterial physiology. Overshadowed by their larger counterparts, small proteins have been overlooked for decades but are now attracting attention due to discoveries made during the mining of data from whole genome sequences or from functional studies (Storz et al. 2014). Small proteins can exert big effects in systems of fundamental importance to the bacterium, as a few examples will show. Cell division is an example of a fundamental process where small proteins make significant contributions. The SOS-induced inhibitor of cell division, SidA, is produced in *Caulobacter crescentus* in response to DNA damage. Its gene has a LexA box, indicating membership of the SOS response system, and the SidA protein is predicted to have a transmembrane helix (Modell et al. 2011). The protein seems to inhibit FtsW, responsible for moving precursors of peptidoglycan from the cytosol to the periplasm but which may have a membrane remodelling role at cell division, a role with which SidA can interfere. In *E. coli,* the small protein Blr can interact with divisome members FtsI, FtsK, FtsN, FtsQ, and FtsW (the latter is the counterpart of the protein in *C. crescentus* with which the SidA small protein interacts) (Karimova et al. 2012). Consistent with its interaction partners, Blr locates to the cell division plane and mutants that fail to express it have a filamentation phenotype that is dependent on growth conditions. The MciZ small protein is an inhibitor of FtsZ in *B. subtilis*. It is produced during sporulation and may act by interfering with its GTPase activity (Handler et al. 2008).

Small proteins also serve as chaperones, such as the Fbp proteins of *B. subtilis* whose expression is repressed by the Fur regulator under iron replete growth conditions, have been proposed to assist the activities of sRNAs (Baichoo et al. 2002; Gaballa et al. 2008). They are also active at the cell envelope: PmrR, a small protein in *S*. Typhimurium that is associated with the cytoplasmic membrane, modulates LPS homeostasis (Kato et al. 2012) and the small (37-amino-acid) CydX protein in *E. coli* is required for full function of the cytochrome *bd* oxidase in the respiratory chain (Vanorsdel et al. 2013). The small MgtR protein controls the stability of the MgtC cytoplasmic protein of *S*. Typhimurium, a regulator of ATP synthesis by the F_0F_1 ATP synthase, perhaps by influencing MgtC turnover by the FtsH membrane-linked protease (Alix and Blanc-Potard 2008).

It is evident that small proteins are often linked to the cytoplasmic membrane, making them candidates for interaction with cytoplasmic-membrane-based signalling of regulatory proteins, such as MgtC. MgbR is a small protein that regulates the PhoQ HPK by protein–protein interaction, attenuating its phosphotransfer activity to the PhoP RR (Lippa and Goulian 2009). Other examples of small proteins with physiologically meaningful influence include Sda (sporulation in *B. subtilis*) (Burkholder et al. 2001), MntS (manganese metabolism in *E. coli*) (Waters et al. 2011), FbpB and FbpC (iron metabolism in *B. subtilis*) (Gaballa et al. 2008), KdpF (K+ uptake in *E. coli*) (Gassel et al. 1999), AcrZ (exclusion of toxic compounds) (Hobbs et al. 2012), and SgrT (translated from a long sRNA transcript, modulates the Hfq regulon by binding the Hfq chaperone) (Kosfeld and Jahreis 2012; Vanderpool and Gottesman 2004; Wadler and Vanderpool 2007). Questions related to small proteins currently under investigation concern their modes of action in the cell, how they are inserted into the cytoplasmic membrane and their orientations once inserted, and how these proteins are turned over.

5.24 Selenocysteine and Pyrrolysine: The 21st and 22nd Amino Acids

The 21st and 22nd amino acids are, respectively, selenocysteine and pyrrolysine. They are encoded by the UGA and UAG codons, respectively, and these normally serve as translation stop codons. Selenocysteine is found in a small number of selenoproteins and is co-translationally inserted through the recoding of in-frame UGA codon (Böck et al. 1991; Stadtman 1996). The presence of selenium rather than sulphur is required for the optimal activities of selenoproteins and reflects the differing redox potentials of the two elements (Yoshizawa and Böck 2009). In *E. coli,* the translation of a selenoproteins mRNA requires a stem-loop structure located immediately downstream of the UGA codon together with factors that act *in trans* to insert selenocysteine (Driscoll and Copeland 2003; Liu et al. 1998; Thanbichler and Böck 2002). SelB is a specialist translation elongation factor that is required for the cotranslational incorporation of selenocysteine; it is found in all three kingdoms of life (Leibundgut et al. 2005). SelB works with selenocysteine (Sec) tRNA ($tRNA^{Sec}$) to deliver Sec to the ribosome, triggering the GTPase activity of the SelB translation elongation factor (Fischer et al. 2016).

All methanogenic methylamine methyltransferases contain an in-frame UAG stop codon that is decoded as I-pyrrolysine (Hao et al. 2002). Pyrrolysine is the 22nd amino acid and its presence is required for the conversion of methylamines to methane (Gaston et al. 2011). Close to the gene encoding the methylamine methyltransferase is the *pylT* gene, encoding a tRNA with a CUA anticodon, $tRNA^{Pyl}$ (Srinivasan et al. 2002). Adjacent to this is *pylRS,* encoding a LysRS-like aminoacyl-tRNA synthetase capable of charging the *pylT*-specified tRNA with lysine, the initial step in translating the UAG amber codon as pyrrolysine (Tharp et al. 2018). The tRNA is initially charged with lysine and this is subsequently modified to pyrrolysine (Gaston et al. 2011). This activity, detected initially in archaea, is also present in eubacteria (Atkins and Gesteland 2002; Srinivasan et al. 2002), and *E. coli* expressing the archaeal *pylS* and *pylT* genes can produce the pyrrolysine-containing methylamine methyltransferase (Blight et al. 2004).

6

Gene Control and Bacterial Physiology

6.1 The Bacterial Growth Cycle

In a laboratory setting, a bacterial cell in a liquid culture is an individual within a community of genetically identical siblings. If the culture has not been synchronised by the investigator through the use of genetic or physiological tricks, the cells in the population will be at different stages of the cell cycle and will present individual (though broadly similar) physiological states. Following inoculation of a rich and supportive growth medium, the entire culture will pass through a *growth cycle* (which is distinct from a *cell cycle*) consisting of a lag phase, a period of exponential growth and then a stationary phase that may be followed by a death phase or by an extended stationary phase (Figure 6.1).

The lag phase is a period of adjustment to the new environment in which bacterial cells alter their gene expression programme in order to exploit the opportunities that this environment offers (Pin et al. 2009; Rolfe et al. 2012). In a typical experiment, the bacteria will have grown for many hours (perhaps overnight) to create the starter culture needed to inoculate the fresh liquid medium. Once the cells have adjusted to their new circumstances, they will begin to divide, a process that starts randomly across the population. Here the bacteria replicate themselves, using the resources in the environment to support this enterprise. Self-replication will continue until some essential component in the medium is exhausted or until an inhibitor of growth reaches a threshold value that terminates further replication. At this point the rate at which cells die either matches or exceeds the rate at which new cells are generated and no expansion in the numbers of viable bacteria can be detected. The culture is said to have reached the stationary phase of growth. A death phase may follow in which viable cell numbers decline and there may be a so-called long-term stationary phase in which some bacteria survive indefinitely. The fate of the population will depend on the nature of the growth medium and other environmental factors, and on the genetic and physiological capacity of the bacteria to recycle resources, to cannibalise themselves, or to enter successfully into a state of dormancy to await an improvement in their circumstances.

Investigators monitor the development of the bacterial culture by counting the total number of cells or, more accurately, the numbers of living cells and plotting the logarithm of the total, or the number of living cells, against time. This plot (Figure 6.1) reveals the beginning and end of each of the three major phases of the growth cycle: lag, log (or exponential),

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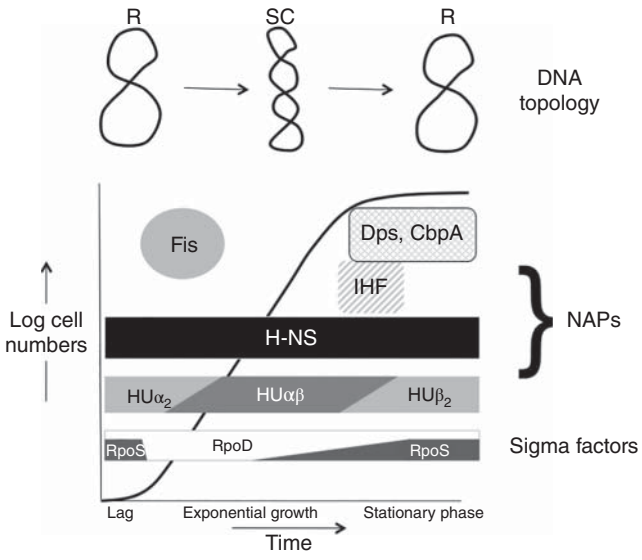

Figure 6.1 The bacterial growth cycle. The graph shows an idealised growth curve for *Escherichia coli* K-12 in rich medium at 37°C. The three principal phases of the growth cycle are listed along the x-axis, with increasing cell numbers shown on the y-axis. The period when particular NAPs appear in the cell, together with the fluctuation in the population of the RpoD and RpoS sigma factors, is represented by filled shapes that have been superimposed on the growth curve. At the top of the figure, the changing topology of cellular DNA through the growth cycle is represented by the relaxed (R) to negatively supercoiled (SC) to relaxed (R) transition.

and stationary phase within the time limits of the experiment. The same data can be used to compute the growth rate of the culture. Many factors will modulate the growth rate, including (inter alia) the chemical composition of the growth medium, whether the culture was aerated, the nature of the growth vessel, the growth temperature, etc.

6.2 Physiology Changes Throughout the Growth Cycle

As time passes and the bacteria move through the growth cycle, their composition and physiology will change. A bacterial cell in mid-exponential growth will present a distinct physiological profile to one that has entered stationary phase. For example, bacteria on the point of entering stationary phase will display enhanced resistance to stress, including osmotic and thermal stress, and they will express scavenging systems to win scarce resources from the environment. In the case of rod-shaped *Escherichia coli* cells in stationary phase, their cell walls will thicken and the cells will adopt a more rounded shape (Freire et al. 2009). The periplasm will be modified to enhance resistance to water loss and the bacteria will become less susceptible to antibiotics. As more time passes, the bacteria will express special proteins such as Dps that protect the genetic material from damage by oxidative stress, helping the organism to adapt successfully to dormancy and to be capable of emerging ready to exploit a more supportive environment if circumstances improve in the future. Specific control proteins are used to alter the gene expression profile of the microbe as it undergoes the

transition from log to stationary phase. The RpoS sigma factor of RNA polymerase has an especially important role in this process (Schellhorn 2014) (Section 3.4). Exiting lag phase involves, to some extent, an undoing of the steps that the bacterium has taken to protect itself during the stationary phase.

A bacterium in the exponential phase of growth that is having all of its growth requirements met is set up to support a rapid rate of growth and environmental exploitation. Rapid and accurate copying of the genetic material is an important feature of fast-growing bacteria, as is the provision of the cellular machinery required for optimal rates of transcription of those genes that support exponential growth and for the translation of those proteins that support this activity. In particular, the production of ribosomes and other components of the translation machinery of the cell is a priority. Matching the supply of these items to the growth rate that can actually be supported by the environment is an important problem to be solved: oversupply, or continuing to supply machinery that is no longer in demand, can undermine the competitive fitness of the bacterium. For these reasons, the organism needs to monitor both the external environment and its own internal environment to avoid miscalculation. The success of bacteria in managing these tasks reveals that they possess the means to sense and to interpret signals concerning environmental composition and to regulate their own behaviour in response to those signals. The processes of sensing and responding appropriately impose on bacteria the need to constantly remake themselves to match the demands of the environment and to do this within the constraints of their own genetic and physiological capacity for adaptation.

A model organism such as *E. coli,* growing in a supportive liquid medium, with aeration to maintain a good oxygen supply, and at its optimal temperature for growth (37 °C) will grow and divide as quickly as it can (dividing approximately every 20 minutes), showing no signs of attempting to live in a sustainable way. Once the environment can no longer support growth or a growth inhibitor has reached a critical threshold concentration, the culture will stop growing. Mutant derivatives may emerge that can continue to grow because they have acquired a new physiological trait that was not expressed in the immediate ancestor. This new feature may not support growth quite as well if the mutant is transferred to a fresh batch of the original medium in its pristine form, making it now less fit than its immediate ancestor. Similarly, the ancestor will not thrive if it is placed in the spent medium without having acquired the enabling mutation. The ancestor has evolved but there is a trade-off: it can outperform the unevolved ancestor, but only in spent medium.

Experimental evolution experiments (of the sort illustrated by the very simple example in the previous paragraph) give useful information about the genes that are most often found to have mutated in the process of adapting a bacterium to its environment over time. Perhaps not surprisingly, many genes on this list encode regulators that affect the expression of more than one target gene or operon (Cooper et al. 2008; Stoebel et al. 2009). In the external environment, the process of adaptation by mutation is made much more complex by the fact of horizontal DNA transfer acting through the mechanisms of conjugation, transformation, and transduction. Even in pure cultures growing in lab conditions, the release of free DNA by dying cells can complicate genetic studies by providing a means to repair mutated genes or to propagate mutations horizontally as well as vertically.

6.3 Generating Physiological Variety from Genetic Homogeneity

Outside the lab, the environment is often unpredictable and bacteria that live in niches that are prone to sudden change may be at risk of local extinction if they do not possess the capacity of respond to unpredictable change. An example of such change is the exposure of a population of sensitive bacteria to a beta-lactam antibiotic such as penicillin. This antibiotic makes bacteria susceptible to killing by inhibiting an enzyme that is essential for cross-linking the peptidoglycan cell wall during wall synthesis, allowing the turgor pressure in the cytoplasm to inflate the cytoplasmic membrane, bursting the bacterium, with lethal consequences (Waxman and Strominger 1983). To work, penicillin must act on bacteria that are actively synthesising cell walls. Dormant bacteria do not do this and are insensitive to penicillin, even if they are penicillin-sensitive when growing. By generating a small sub-population of dormant bacteria stochastically, bacteria can create a reserve of penicillin-insensitive members that can survive the antibiotic catastrophe that carries away their growing (and sensitive) siblings. These survivors are called 'persisters' (Bigger 1944) and their protective, dormant state can be reached by a variety of molecular mechanisms. The key feature that is shared by these mechanisms for becoming dormant is the randomly acting nature of their modes of action (Harms et al. 2016; Page and Peti 2016). By being as unpredictable as the external environment, bacteria can survive and prevail in that environment.

A second example of bacterial exploitation of stochastic processes to promote bacterial survival concerns the means by which some pathogenic bacterial populations produce members that can avoid or evade the host defences during infection. Interaction with a host frequently involves the expression by bacteria of specialist surface features that adhere the microbes to the host or allow the microbe to invade the cells of the host. These surface components are often proteins and they are immunogenic, causing the bacterium to be targeted by host antibodies. By randomly switching between different antigenic forms of the same surface protein, or by randomly switching the expression of the protein on and off, or by combining the two strategies, the bacterial population can create antigenic variety that may be capable of outwitting the host's defences. Classic examples include (i) flagellar phase variation in *Salmonella* in which two antigenically distinct forms of the principal flagellar structural protein are expressed in a population due to the random inversion of a regulatory DNA element in the chromosome (Johnson et al. 1986); (ii) the on/off switch that governs expression of Pap pili in uropathogenic strains of *E. coli* that infect the urinary tract and kidneys: this mechanism involves stochastic methylation of adenine residues in the *pap* regulatory region and the negative effects of this methylation on the binding of regulatory proteins, and the effects of prior binding by the regulatory proteins on methylation (van der Woude et al. 1992); and (iii) the creation of *opa* frame-shift mutations that post-transcriptionally alter the expression of opacity proteins on the surface of the pathogen *Neisseria* species (Sadarangani et al. 2011).

A third example of a bacterial survival strategy based on stochasticity concerns the advantage to the bacterium of being able to utilise as many sources of carbon as possible, even if some carbon sources, when metabolised, produce lethal products. Beta-glucosides are in this category of useful, but sometimes lethal, carbon sources and bacteria such as *E. coli* have

transport and utilisation systems (*bgl*) for β-glucosides that are normally cryptic (because the *bgl* genes are maintained in a transcriptionally silenced state). At random, individual cells in an *E. coli* population activate the cryptic beta-glucoside utilisation genes and gain a competitive advantage if a suitable, harmless beta-glucoside is available (Defez and de Felice 1981; Reynolds et al. 1981). If the available sugar does produce a lethal product on metabolism, only this individual *E. coli* cell is killed; the remainder of the population, with its still cryptic *bgl* genes, is secure.

A lesson from analyses of stochastic processes in bacterial populations is that stereotypical responses in which all members of the population act in unison represent a risky life strategy in an unpredictable environment. Variety across populations can be achieved by genetic means, relying on mutation. Examples include the activation of the *bgl* genes by insertion of a mobile DNA element (Humayun et al. 2017; Madan et al. 2005) or the creation of variety in bacterial surface protein expression through the operation of randomly-acting regulatory switches that utilise site-specific recombination (Dorman and Bogue 2016). However, physiological variety can also arise in bacterial populations where cell cycles are not in synchrony due to the firing of key genes at different times in different cells of that population. Indeed some genes may not fire at all in particular cells due to unpredictable relationships among populations of gene regulatory proteins, RNA polymerase, its sigma factors, and the transcriptional proficiency of that gene caused by random events in its genomic neighbourhood arising from the transcriptional activity at nearby genes or the passage of a replication fork (to give a non-exhaustive list of possible generators of stochasticity at the level of gene expression).

Some studies of the bacterial cell cycle require synchronisation of cell division in all members of the population. Typically, synchrony lasts for just a few rounds of cell division before cell-to-cell variation reintroduces asynchrony. Even during the brief period during which the cells replicate their chromosomes in step with each other, it is unlikely that all other cellular functions are proceeding in unison. This is a source of physiological variety between cells and can be useful to the population when facing an unpredictable environment. The population members may differ in the numbers of ribosomes, copies of DNA or RNA polymerases, or other molecular machines that are essential for growth and reproduction (Vendeville et al. 2010). When faced with an environmental signal, such as the arrival of a potentially useful carbon source, not all of the cells may express the transport and utilisation systems needed to exploit this windfall. In the face of a threat, such as the onset of osmotic stress, the individual members of the population may express the defence systems required for survival with different degrees of success.

6.4 Bacterial Economics – Some Basic Principles

The operations of a national economy provide a useful metaphor, within certain limits, for the functioning of the bacterial cell, and help us to understand why bacteria do the things that we observe. Survival and replication are important goals for the bacterium, as they are for all living things. To succeed in these activities, the organism must have the raw materials to build new copies of itself and the energy to operate the machinery that this process requires.

Raw materials can be manufactured internally or they can be imported. The more of these that the bacterium can make for itself, the more self-sufficient it is, reducing its dependency on unpredictable external sources of supply. In some organisms, a reliable external source of raw materials selects for a reduction in the number of genes required to underpin self-sufficiency. This is seen in the case of some obligate parasites that have evolved a strategy for survival that regards the provision by the host of essential nutrients as a given. The strategy succeeds until the organism finds itself separated from the host. The complexity of a genome is approximately related to the degree to which a bacterium is self-sufficient. The self-replicating organisms with the smallest known genomes are members of the species *Mycoplasma,* and these are obligate parasites (Gibson et al. 2008a). Not only is the *Mycoplasma* genome small, so is the number of proteins involved in gene regulation (Fraser et al. 1995). Organisms of this type combine genetic simplicity with a degree of specialisation in terms of the environments in which they can survive. Regardless of genome size, the bacterial chromosome typically has little DNA that is not used either to encode information or to control the expression of that information, or both.

A dependency on external sources of raw materials (or on fully assembled items) implies that the bacterium possesses the means to detect these and to import them. Transport is a vital function in most cells and it tends to be somewhat specialised. Transporters have evolved to handle either one specific molecule or several related members of one class of cargo. There may also be a link between a type of transporter and when it is expressed. For example, those systems that handle less-preferred cargoes may be used for scavenging and only deployed when the bacterium is in poor metabolic health, possibly upon entering stationary phase.

6.5 Carbon Sources and Metabolism

In the model organism *E. coli,* glucose is a preferred carbohydrate and its presence in the environment can cause the organism to shut down the expression of transporters of other types of carbohydrate. The classic example is the preferential usage by *E. coli* of glucose over lactose when both carbohydrates are present in the growth medium. Here, glucose is consumed first and its exhaustion leads to a period of growth arrest. Then the culture begins to grow again at a lower rate using lactose as a carbon source. This two-stage growth rate phenomenon is known as diauxic growth and it arises from catabolite repression: the shutting down by a preferred carbohydrate of the transport and utilisation system for a second, less-preferred sugar (Görke and Stülke 2008; Stülke and Hillen 1999).

Glucose produces high yields of energy when it is oxidised completely to CO_2 and water in the presence of oxygen: this process has a pervasive influence on the operation of the cellular economy. When O_2 is present, the energy from glucose is generated in the form of adenosine triphosphate (ATP) and heat through a process known as oxidative phosphorylation, and the carbon becomes available for the manufacture of cell constituents. Oxidative phosphorylation involves the transmission of reducing equivalents in the form of electrons along a transport chain within the cytoplasmic membrane, a chain that ends with the reduction of oxygen to water. As the electrons are shuttled along the chain, protons are extruded from the cell into the periplasm, creating a proton gradient (Figure 6.2). This is available to

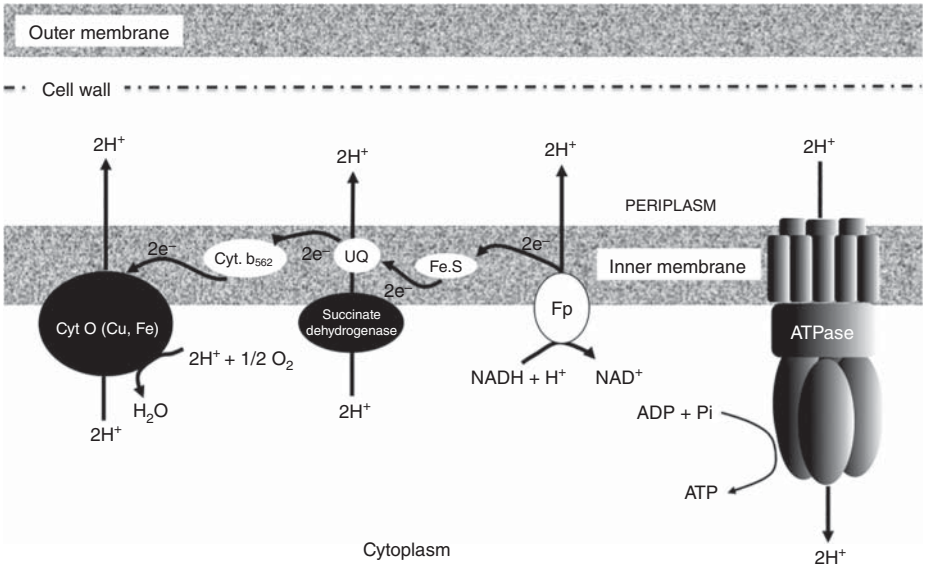

Figure 6.2 Generation of proton motive force and the operation of the F_1F_0 ATPase. During aerobic and anaerobic respiration, a respiratory chain is established in the cytoplasmic membrane of facultative anaerobes such as *Escherichia coli*. An aerobic respiration chain is shown here. It is composed of alternating electron and hydrogen carriers. Electrons are passed down the chain to cytochrome O via carriers with intermediate redox potential: oxygen has the highest positive redox potential and is used as the terminal electron acceptor. Protons are ejected to the periplasm by the flavoprotein (FP), ubiquinone (UB), and cytochrome O (cyt O), creating a net positive charge. These protons can be readmitted to the cell via the F_1F_0 ATPase, with proton translocation providing the energy to convert ADP and P_i to ATP. Protons can also re-enter through the TonB-ExbB complex (Figure 6.5) and the flagellar motor, in each case providing the power needed to perform work. The flavoprotein regenerates oxidised nicotine adenine dinucleotide (NAD) from its reduced form ($NADH_2$) by removing a reducing equivalent (an electron and an associated proton). The proton is ejected to the periplasm while the electron is transferred to an iron-sulphur protein, the next component in the chain. Succinate dehydrogenase is a component of the TCA cycle and it feeds reduced flavin adenine ($FADH_2$) dinucleotide to the respiratory chain, regenerating FAD (flavin adenine dinucleotide). The operation of the respiratory chain powers oxidative phosphorylation, the generation of ATP using oxygen to draw reducing equivalents through the chain. In this way, it produces energy for the cell and some heat is also generated as a by-product. Switching to terminal oxygen acceptors other than oxygen, such as nitrate, during anaerobic respiration involves rewiring the respiratory chain with new components, a process that begins at the level of transcription regulation and involves such global regulators as the FNR protein.

drive protons back into the cytoplasm through suitable portals, of which ATP synthase is one. This multi-protein complex generates ATP from ADP and inorganic phosphate using the flow of protons as an energy source (proton motive force, PMF). This proton flow creates a chemiosmotic circuit (Figure 6.3). The entire system works because the protons are unable to cross the membrane unaided and will remain in their compartments, separated by the cytoplasmic membrane, unless a system to facilitate proton movement is provided. ATP synthase is just one example of many systems that can translocate protons along a gradient: the motor that powers bacterial flagellar rotation is another (Minamino and Imada 2015).

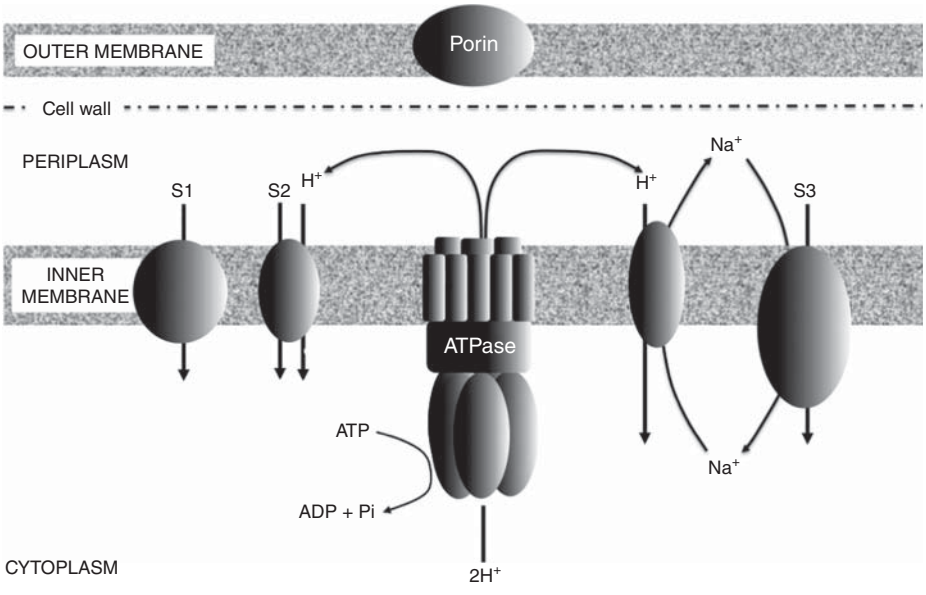

Figure 6.3 A simple chemiosmotic circuit. The cytoplasmic membrane is impervious to protons and these enter and leave through membrane proteins and molecular machines such as the F_1F_0 ATPase, shown here at the centre of the diagram. ATP hydrolysis drives protons from the cytoplasm to the periplasm. Some can re-enter via antiport of symport transport systems. A simple permease is shown at left that facilitates the uptake of a specific substrate (S1). Substrate S2 travels from the periplasm to the cytoplasm via a symport system in which a proton accompanies S2, probably to neutralise a negative charge at a salt bridge in the symport protein. The Lac permease, LacY, encoded by the *lacZYA* operon in *Escherichia coli,* functions in this way. Substrate S3 is also trafficked through a symport system (right) but the co-transported ion is sodium and not a proton. The sodium cation was excreted from the cytoplasm though an antiport system that used a proton as a counterion.

ATP has been described as the energy currency of the cellular economy. In addition to being a precursor for nucleic acid synthesis, ATP is consumed by molecular machines involved in molecular rearrangements that contribute, inter alia, to transport processes and the organisation of the DNA in the cell. The ratio of the intracellular concentrations of ATP and ADP is a useful indication of metabolic flux, with high ratios being associated with periods of rapid growth (Koebmann et al. 2002).

Central to the exploitation of glucose as an ideal carbon source is the system that transports it into the cell, the metabolic pathways that rearrange and simplify its molecular structure, and the signalling system that shuts down the operation of other systems for the transport and utilisation of alternative, less favoured, carbon sources. In *E. coli,* cyclic-AMP (cAMP) plays a key role as a signalling molecule whose task is to rebalance the cellular economy in favour of glucose utilisation. This second messenger is produced by adenylate cyclase, an enzyme that is inactivated when in a complex with a phosphorylated component of the glucose uptake system (protein IIA^{glc}) (Deutscher 2008; Postma et al. 1993). When glucose enters the cell, becoming phosphorylated in the process, it causes dephosphorylation of phospho-IIA^{glc}, resulting

in inactivation of adenylate cyclase and loss of cAMP production (Deutscher et al. 2006). For this reason, the concentration of cAMP in the cell is an indication that a high-growth-rate-yielding carbohydrate is being imported and consumed in preference to other, less-preferred, carbon sources. Overall, the higher the intracellular cAMP concentration, the lower the position of the available carbon source in the hierarchy (Aidelberg et al. 2014).

6.6 Gene Control and Carbon Source Utilisation

Switching between glucose utilisation and exploitation of alternative carbon sources involves, in part, changes to the pattern of gene expression in the bacterium. The cAMP Receptor Protein, CRP, sometimes called CAP, the Catabolite Activator Protein, is a DNA-binding protein that is required for full expression of a number of genes that encode uptake and utilisation systems for carbohydrates other than glucose (Lewis 2013; Savery et al. 1996). CRP gains DNA-binding activity only when it forms a complex with cAMP, making the formation of this active complex conditional on the absence of glucose from the cell. A great deal of our understanding of CRP molecular biology came historically from studying its role in the positive regulation of the *lac* operon in *E. coli,* a group of co-transcribed genes required for the transport and utilisation of lactose (Jacob and Monod 1961). The CRP protein has relatively strict requirements for the base sequence of the DNA to which it binds and the location of these DNA sequences with respect to transcription promoters allows cAMP-CRP to affect the activity of RNA polymerase positively or negatively (Grainger et al. 2005; O'Byrne and Dorman 1994). A hierarchy of carbon sources also exists among non-glucose sugars and cAMP also plays a role in the preferential expression of the transport systems for these carbohydrates (Aidelberg et al. 2014; Ammar et al. 2018).

Glucose utilisation by *E. coli* is most efficient in terms of ATP synthesis when the bacterium uses oxygen as the terminal electron acceptor in its respiratory chain. The bacterium operates across a wide range of oxygen concentrations, respiring aerobically at O_2 levels above 5 mbar, anaerobically between 1 and 5 mbar, and switching to fermentation below 1 mbar (Unden 1998). Fermentation does not involve a respiratory chain and the carriers of reducing equivalents are reduced and re-oxidised within the fermentation pathway itself. ATP synthesis occurs at the level of the pathway intermediates in a process known as substrate-level phosphorylation and the yields of ATP are very poor compared with oxidative phosphorylation with glucose. Paradoxically, rapidly growing bacteria respiring with glucose may switch to fermentation to generate ATP if the cytoplasmic membrane becomes overloaded with respiratory chain components. This phenomenon is known as overflow metabolism and may represent a solution to protein crowding in the membrane (Szenk et al. 2017).

6.7 Anaerobic Respiration

The respiratory chain can be rewired to cope with a reduced concentration of oxygen in so-called micro-aerobic environments and further rewiring allows the organism to function

at even lower oxygen concentrations, using an alternative electron acceptor, such as nitrate in place of the scarce O_2. This is anaerobic respiration. Regulators that respond to the presence or absence of oxygen control the expression of the genes that encode alternative components of the respiratory chain. One of these regulators, FNR, is a close relative of the CRP protein (Green et al. 2009). FNR has a redox-sensitive iron-sulphur domain in its amino terminus that allows it to become proficient for DNA binding under anaerobic conditions. Like CRP, FNR has relatively strict requirements in the base sequences of the DNA sites where it binds (Green et al. 2001). The ArcAB two-component system is involved in transcriptional regulation under both aerobic and anaerobic conditions. As oxygen levels decline, the terminal oxidase of the respiratory chain changes from the cytochrome bd oxidase to cytochrome o (cyt O) complex. ArcA is an activator of the *cydAB* genes encoding the high-oxygen-affinity cytochrome bd oxidase. It is a repressor of genes encoding components of the Krebs (tricarboxylic acid/citric acid) cycle and the *cyoABCDE* genes encoding the cytochrome o low affinity terminal oxidase of the respiratory chain (Bai et al. 2014). The *cydAB* operon is under dual control by ArcA (activation) and FNR (repression) (Cotter et al. 1997). While FNR senses oxygen directly, ArcA relies on its cytoplasmic-membrane-associated partner ArcB, a sensor of the redox levels of the quinone pool in the cell (van Beilen and Hellingwerf 2016). Communication between ArcB and ArcA involves phosphorylation, with the phosphorylated form of ArcA being proficient for DNA binding (Iuchi and Lin 1992).

6.8 ArcA, Mobile Genetic Elements, and HGT

ArcA is not restricted to transcription control of genes with a direct role in metabolism: it is also involved in plasmid replication and horizontal gene transfer by conjugation. In addition, it has been reported to inhibit chromosome replication in *E. coli* (Lee, Y.S., et al. 2001). The low copy number plasmid pSC101 relies on site-specific recombination at the *psi* site catalysed by the chromosomally encoded XerCD tyrosine integrases to maintain pSC101 as a monomeric plasmid. For low copy number plasmids, multimerization poses a threat to their successful segregation into daughter cells at cell division (Pinto et al. 2012; Thomas 2000). This multimer resolution process requires the ArcA protein, acting in an architectural mode and linking plasmid replication to the aerobic/anaerobic status of the bacterium (Colloms et al. 1998). Conjugative transfer of the large, self-transmissible multidrug-resistant R1 and R100 plasmids also requires ArcA, which is a transcription activator at the main promoter of these plasmids' transfer operons (Strohmaier et al. 1998; Taki et al. 1998). This finding indicates that plasmid transfer from cell to cell is optimal under low oxygen conditions, a finding that has implications for the efficiency of horizontal gene transfer and the rate of evolution of bacterial species. This is also an important issue in the evolution of pathogens: the *Salmonella* virulence plasmid pSLT, which is related to the F plasmid, undergoes conjugative transfer that is dependent on the ArcA protein, especially under microaerobic growth conditions (Serna et al. 2010). For enteric pathogens, the microaerobic environment at the surface of the gut epithelium may be an important arena for horizontal gene transfer and for the oxygen-dependent control of virulence gene expression (Aviv et al. 2016; Marteyn et al. 2010).

6.9 Stress and Stress Survival in Bacterial Life

Changes to the chemical and/or physical environment can impose stress on a bacterium. The bacterium can also experience stress that is generated internally, for example following the gain or loss of a gene. Some bacteria inhabit environments that are relatively stable, while others have lifestyles that expose them to a constantly shifting scene that would prove challenging to a specialist that usually experiences unchanging surroundings.

Work with model organisms has taught us a great deal about the mechanisms used by bacteria to interpret their environment and to respond appropriately. Meeting environmental challenges correctly is essential if the microbe is to survive and to continue to replicate. To be survivable, the environmental challenge must fall within a range where the organism is capable of responding in time and of mounting an adequate response. Autoclaving (treatment at high temperature and pressure) represents a challenge that is generally unanswerable by bacteria, so they don't survive the experience. However, there are very many stresses that, while capable of causing harm, fall short of being instantly lethal and bacteria have evolved the ability to respond. The diversity of microbial life represents a spectrum of abilities to inhabit different environments, allowing some organisms to adapt to niches where others would perish. Thus, what is stressful to one bacterium may be routine and not stressful to another. This diversity of ability to deal with stress is underpinned by genetic diversity. Some stress-resistance structures or pathways in the cell may be expressed constitutively as part of the bacterium's specialisation for life in its niche. Others may be inducible and only expressed when circumstances dictate that they are needed. Much of the response to stress, but not all, will involve changes to gene expression. Non-genetic responses include alterations in the motility of the bacterium (Colin and Sourjik 2017), protein secretion (Tsirigotaki et al. 2017), protein turnover (Alexopoulos et al. 2017; Jonas 2014), and changes to the composition of the cell envelope (Ekiert et al. 2017; Rassam et al. 2015).

6.10 Oxygen Stress

The links between efficient growth, glucose consumption, and the exploitation of oxygen as a terminal electron acceptor have been outlined in Section 6.5. In addition to playing a constructive role in respiration, oxygen can be a source of stress due to the production of reactive oxygen species (ROS) and their ability to oxidise cellular components. Bacteria possess stress response systems to defend them from oxidative damage. With SoxRS and RpoS, the OxyR protein is a central component of the bacterial response to ROS (Chiang and Schellhorn 2012). OxyR is a LysR-like protein that forms homo-tetramers and plays an important role in hydrogen peroxide detoxification (Kullik et al. 1995). Among the genes that are controlled by OxyR is *dps,* encoding a nucleoid-associated protein, Dps (Section 1.41) that protects the genetic material in the cell from ROS. Dps is ferritin-like in structure and is an iron storage protein (Chiancone and Ceci 2010). OxyR also positively regulates the *fur* gene whose product, Fur, is a master regulator of genes involved in iron transport and metabolism (Varghese et al. 2007; Zheng et al. 1999). SoxR also affects *fur* transcription

(Zheng et al. 1999). A link between iron homeostasis and the oxidative stress response is important because free iron in the bacterium is easily oxidised via the Fenton reaction, generating potentially dangerous reactive hydroxyl radicals (Imlay et al. 1988):

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + OH$$

The KatG catalase breaks down hydrogen peroxide and its expression is under positive control by OxyR (Tartaglia et al. 1989). OxyR auto-represses the transcription of its own gene, *oxyR,* and the gene encoding the biofilm-associated Agn43 autotransporter, also known as Flu (Waldron et al. 2002). It is also involved in controlling the life cycle of bacteriophage Mu through the transcriptional repression of the *mom* gene (Hattman and Sun 1997). OxyR can bind to DNA both in the presence or the absence of ROS. However, the nature of the binding differs between the two states. In the oxidised state the protein makes contact with a more extended region of DNA than it does in its reduced state (Toledano et al. 1994). OxyR senses ROS directly through the reversible oxidation of disulphide bonds between critical cysteine amino acids (Zheng et al. 1998).

SoxR is a DNA-binding protein that is related to the mercuric-ion-sensitive MerR transcription repressor (Amábile-Cuevas and Demple 1991). SoxR uses two redox-sensitive iron-sulphur clusters to respond to oxidative stress (Watanabe et al. 2008). Like MerR, it binds to DNA both in the presence and the absence of its inducing signal and it is thought to alter DNA twist at its binding site in the target promoter spacer region to facilitate transcription initiation (Hidalgo and Demple 1997). In this way, SoxR induces the transcription of the *soxS* gene in response to oxidative stress (Nunoshiba et al. 1992). SoxS modulates the expression of scores of genes; prominent among these are genes encoding manganese-dependent superoxide dismutase and DNA repair proteins (Blanchard et al. 2007).

RpoS is a third regulatory component of the oxygen stress response. It is a sigma factor (Section 3.4; Table 3.2) and is part of several stress responses. RpoS seems to be called into action whenever a stressful experience results in growth arrest. It controls the expression of genes that help the bacterium to survive the effects of stress, including growth arrest. RpoS is a very good example of an agent of regulatory networking because it ties together so many different aspects of bacterial physiology.

6.11 Iron Starvation

Iron is essential for bacterial life, and is the most abundant metal on earth, yet bacteria routinely struggle to meet their requirements for this element. Iron is an essential component of the aerobic and the anaerobic respiratory chains and expression of the genes that encode these chain members responds to iron limitation (Cotter et al. 1992); it is also essential for the functioning of enzymes involved in DNA repair and central metabolism (Cornelis et al. 2011). Its utility arises from the fact that it is a redox-sensitive metal and it is found in proteins within heme or iron-sulphur prosthetic groups (Waldron et al. 2009). Iron is also found in environmentally responsive transcription factors such as FNR (response to anaerobic growth; Green et al. 1991), Fur (the presence of iron; Bagg and Neilands 1987), IscR (anaerobic growth and control of iron-sulphur cluster assembly; Schwartz et al. 2001), NorR (nitric

oxide response; D'Autreaux et al. 2005), NsrR (nitric oxide response; Tucker et al. 2008), and SoxR (oxidative stress; Hidago and Demple 1994). Although the metal is plentiful, available iron is a scarce commodity because it is found in insoluble complexes. The oxidation state of free iron is a determining factor in its availability to bacteria. While Fe^{2+} (ferrous) can enter bacterial cells, the more oxidised Fe^{3+} (ferric) must be transported into the microbe. A hazardous by-product of Fe^{2+} acquisition is the creation of ROS through the Fenton reaction (Imlay et al. 1988) (Section 6.10). This makes it imperative that bacteria regulate iron uptake tightly. It is likely to be for this reason that OxyR and SoxRS control the expression of the *fur* gene, which encodes the master regulator of iron transport system genes (Zheng et al. 1999). Fur controls the expression of the iron-dependent superoxide dismutase, SodB, via the RyhB sRNA (Masse and Gottesman 2002). Assisted by the Hfq RNA chaperone, RyhB interacts with the *sodB* transcript to make it a target for RNase E and RNase III cleavage, downregulating SodB levels in iron-starved cells (Figure 6.4) (Afonyushkin et al. 2005; Urban and Vogel 2007). The *ryhB* gene is repressed by Fur under iron-replete conditions (Vassinova and Kozyrev 2000) and this eliminates RyhB interference with SodB mRNA translation, allowing the iron-dependent superoxide dismutase to accumulate in the cell, protecting it from oxidative damage. The same Fur-RyhB-Hfq partnership controls the expression of the succinate dehydrogenase operon, *sdhCDAB,* connecting iron metabolism to the operation of the tricarboxylic acid cycle (Masse and Gottesman 2002).

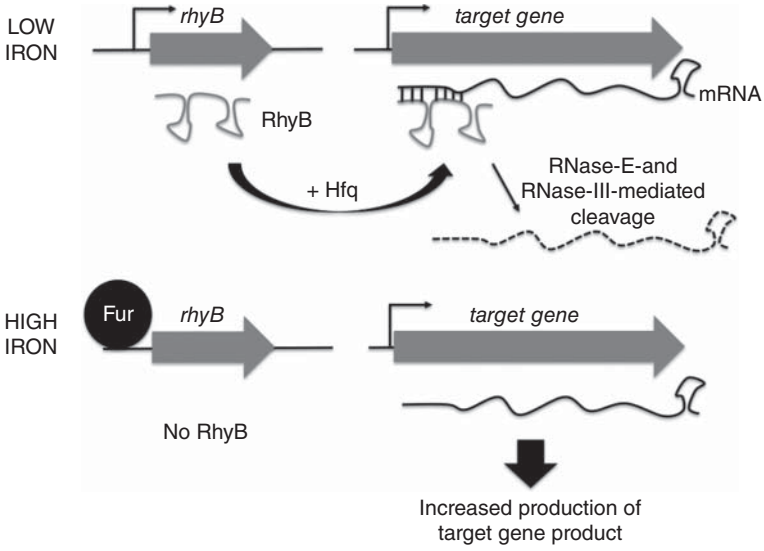

Figure 6.4 Iron-mediated gene regulation via the RyhB sRNA. In iron-restricted growth conditions, the *ryhB* gene is transcribed free from interference by the Fur repressor. The RyhB, in association with the Hfq RNA chaperone protein, interacts with the mRNA expressed by a target gene in the RyhB regulon. The sRNA-mRNA-Hfq complex is then targeted for destruction, with RNase E and RNase III beginning the process by cleaving the mRNA. As a result, the target gene's protein product is not expressed. In iron-replete growth conditions, the Fur repressor binds iron and binds to a Fur box sequence at the *ryhB* gene, repressing its transcription. This prevents RyhB sRNA production, leaving the mRNA expressed by the target gene free to be translated and boosting the production of the target gene protein product in the cell.

Although Fe^{2+} is usually regarded as the form responsible for promoting cellular damage, Fe^{3+} is also toxic, at least in Gram-negative bacteria that lack the PmrA regulatory protein. The damaging effects of Fe^{3+} are independent of Fur and oxygen free radicals. In *pmrA* mutants, Fe^{3+} permeabilizes the outer membrane, making the bacterium susceptible to vancomycin, a drug that does not normally inhibit Gram-negative organisms (Chamnongpol et al. 2002).

6.12 Siderophores and Iron Capture

Bacteria scavenge for iron using iron chelators, iron-carrying molecules called siderophores, such as aerobactin, enterochelin, and ferrichrome. These must compete with iron carriers found in the environment, including those produced by host organisms that try to limit microbial growth by imposing iron starvation (Cassat and Skaar 2013; Schaible and Kaufmann 2005). Humans and other mammals withhold iron from microbes by using molecules such as transferrin and lactoferrin (Theurl et al. 2005; Weiss and Schett 2013) and an ability to prevail in the struggle for iron can be an important virulence determinant in the case of pathogenic bacteria. Gram-negative bacteria such as *E. coli* produce, release, and then recover a variety of siderophores, bringing them and their iron cargo across the cell envelope to be disassembled in the cytoplasm where the iron is used (Figure 6.5).

6.13 TonB-Dependent Transporters

Export of siderophores from the bacterium is achieved via the AcrAB-TolC system (Newton et al. 2010) (Figure 6.5). Once iron has been captured by the extracellular siderophore, the iron-siderophore complex enters the bacterium by crossing the outer membrane using specialised receptors such as FhuA, a ligand-gated porin, whose activity is controlled by the TonB-ExbBD complex. This sophisticated complex is rooted in the cytoplasmic membrane and crosses the periplasm to make physical contact with the outer membrane receptor (Figure 6.5). It is powered by PMF and it sets and resets the receptors, energising them for iron-siderophore uptake. The TonB-ExbBD complex has been proposed to rotate and to move laterally through the fluid inner membrane, finding and energising different peptidoglycan- and outer-membrane-associated receptors by imparting kinetic energy (Klebba 2016). Specificity of communication between receptors and TonB is assured when the receptors possess a 'TonB box' motif for contact with the rigid, rotating, periplasm-spanning arm of the TonB protein (Evans et al. 1986; Larsen et al. 1993; Peacock et al. 2005; Seliger et al. 2001). Transport from the periplasm to the cytoplasm is achieved using ATP-binding cassette (ABC) complexes working in cooperation with periplasmic binding proteins (Figure 6.5). The system is modular and its components reflect the iron carrier that is in use. For example, when *E. coli* uses enterochelin to chelate iron, the outer membrane receptor for iron-enterochelin recovery is FepA and the inner membrane ABC transporter complex is composed of FepCDG (with FepB as the periplasmic binding protein). The system is versatile and is not restricted exclusively to iron uptake: it can also

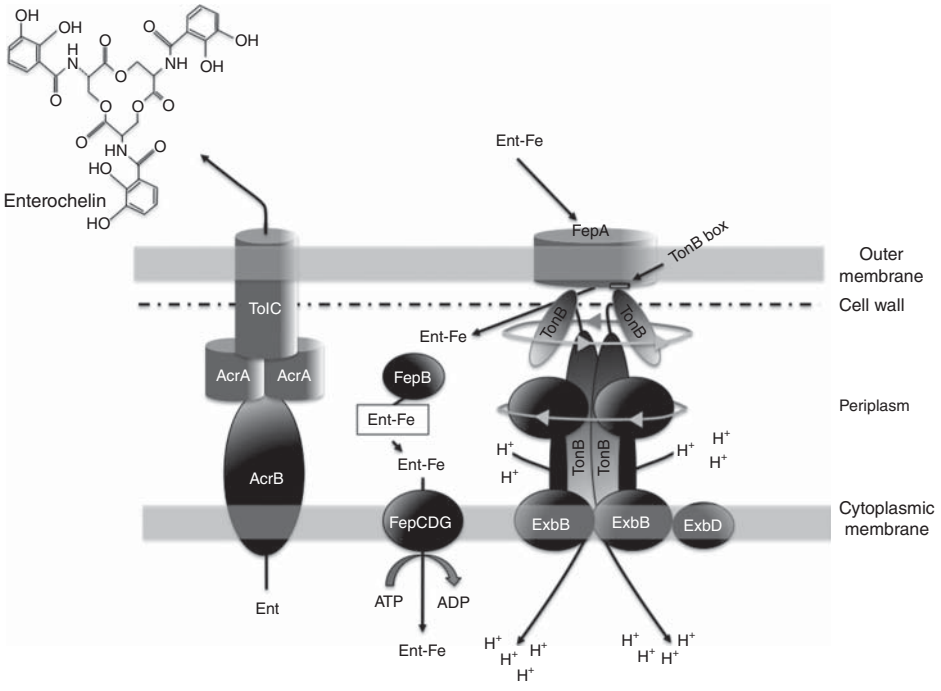

Figure 6.5 TonB-dependent transport systems. The TonB dimer contra-rotates within the rotating ExbB complex with rotation being powered by proton motive force. The curved arrows represent the flow of protons from the periplasm through the complex to the cytoplasm. The rotary movement is thought to move the complex laterally through the cytoplasmic membrane, allowing TonB to conduct a survey of the inner surface of the outer membrane, searching for proteins with copies of the TonB box. In this example, the carboxyl-terminal domain of the rotating TonB protein contacts the TonB box of the FepA ligand-gated porin in the outer membrane. This contact provides the energy needed to move the enterochelin-iron (Ent-Fe) complex into the periplasm, where the FepB periplasmic binding protein captures it and delivers it to the FepCDG ABC transport system in the cytoplasmic membrane. Following transport of the Ent-Fe cargo from the periplasm to the cytoplasm, ATP hydrolysis resets the FepCDG transporter for its next Ent-Fe uptake task. Newly synthesised Ent is exported via the AcrAB-TolC complex. The structure of enterochelin (Ent, also known as enterobactin) is shown at top left.

be used for the acquisition of vitamin B12 (cyanocobalamine, a source of cobalt), using the BtuB protein as the outer membrane receptor, BtuCD as the ABC transporter (DeVeaux and Kadner 1985), and BtuE as the periplasmic binding protein (Ferguson and Deisenhofer 2002). In addition to FepA, other TonB-dependent receptors for iron-siderophores are FhuA (the ABC transporter is FhuBC; the periplasmic binding protein is FhuD) and FecA (the ABC transporter is FecCDE and the periplasmic binding protein in FecB) (Ferguson and Deisenhofer 2002). FecA is the receptor for citrate-chelated iron: it is not utilised by *Salmonella* but is used by other organisms, including *E. coli* (Mahren et al. 2005; Wagegg and Braun 1981). The pathogen *Vibrio cholerae* has an additional layer of sophistication in having two TonB systems with overlapping and distinct siderophore-iron preferences: TonB1 and TonB2. Both transport haemin, and the siderophores ferrichrome

and vibriobactin; TonB1 is a specialist for schizokenin while TonB2 translocates enterobactin (Seliger et al. 2001). *Salmonella* produces a siderophore known as salmochelin that is essential for virulence, exported through IroC/TolC and taken up following iron chelation by the FepA and IroN outer membrane proteins (Crouch et al. 2008; Hantke et al. 2003).

6.14 Gene Regulation and Iron Transport

While the iron uptake ensemble is vital to the health of the bacterium, its outer surface components pose a threat because bacteriophage and colicins have evolved to exploit them to gain entry to the microbe (Breyton et al. 2013; Cao and Klebba 2002). Regulating the expression of the iron scavenging and transport apparatus is important not only because its production imposes a cost on the cell, but also because it may represent an existential threat to the bacterium if an appropriate phage or colicin is in the environment. The expression of colicins and their cognate colicin immunity functions is also intimately integrated with iron metabolism, with colicin gene transcription coming under direct Fur control (Spriewald et al. 2015) and some colicin/bacteriocin genes being co-located on self-transmissible plasmids with operons for iron uptake systems (Waters and Crosa 1991).

The genes for iron metabolism are often co-located and co-regulated within operons. Regulation is imposed transcriptionally and post-transcriptionally, with the Fur DNA-binding protein being a prominent regulator. Binding sites for the Fur protein are found along the whole length of the *E. coli* chromosome, in both replichores, with 81 genes in 42 transcription units coming directly under Fur control (Seo et al. 2014). Fur is an Fe^{2+}-responsive DNA-binding protein (Bagg and Neilands 1987; Hantke 1981, 1984) whose action is potentiated by anaerobic conditions, conditions favourable to the conversion of Fe^{3+} (ferric iron) to Fe^{2+} (ferrous iron) (Beauchene et al. 2017). In the absence of oxygen, Fe^{2+} enters the cell under iron-replete conditions through the Fur-regulated *feoABC* iron uptake system, bypassing the TonB-dependent transporters used by Fe^{3+}-siderophores under aerobic conditions. Fur represses the ferric transporters in anaerobically grown bacteria: at the same time, negative regulation of the *feoABC* operon by Fur is offset under anaerobic conditions by positive control by FNR and ArcA (Beauchene et al. 2017). The overall effect is to enhance ferrous iron acquisition via the anaerobically expressed *feo* system while downregulating the aerobically favoured ferric iron transporters. Fur acts as a conventional repressor at target promoters where it exerts negative control, binding in a metal-dependent way such that RNA polymerase is excluded (de Lorenzo et al. 1988; Griggs and Konisky 1989). Fur binds to an operator site with a conserved, inverted-repeat DNA sequence whose position determines the mode of action of the protein on transcription initiation (Baichoo and Helmann 2002; Calderwood and Mekalanos 1988). In addition to being a repressor of transcription, Fur can act as a positive regulator of gene expression in at least three ways: indirectly by controlling sRNA expression (Masse and Gottesman 2002; Ellermeier and Slauch 2008); directly by acting as a conventional transcription factor that recruits RNA polymerase, as at *norB* in *Helicobacter pylori* (Delany et al. 2004) or *hilD* in the SPI1 pathogenicity island of *Salmonella* Typhimurium (Teixido et al.

2011), and by performing the role of an anti-repressor at H-NS-silenced genes such as the *fntA* gene in *E. coli* (Troxell and Hassan 2013). In the *S.* Typhimurium hilD case, it is interesting to observe that H-NS is a repressor of *hilD* transcription and that Fur is a repressor of *hns* transcription in *S.* Typhimurium (Troxell et al. 2011). Iron metabolism and bacterial virulence are intimately linked (Frawley and Fang 2014) and in this context it is interesting to note that the SitABCD transporter for ferrous iron and magnesium is located within the SPI1 pathogenicity island of *S.* Typhimurium (Boyer et al. 2002; Zhou et al. 1991). Fur is also capable of gene regulation in the absence of iron binding (Seo et al. 2014).

6.15 Iron Storage and Homeostasis

Iron is not simply taken up and put to work in bacterial cells; iron can also be stored by ferritin-like proteins. Two prominent examples are FtnA and Bfr, whose expression is stimulated by Fur via the sRNA gene *ryhB* (Figure 6.4) (Masse and Gottesman 2002). Under high-iron growth conditions, the Fur DNA-binding protein acts as an anti-repressor at *ftnA,* displacing the H-NS transcription silencer (Masse and Gottesman 2002; Nandal et al. 2010; Velayudhan et al. 2007). The Dps nucleoid-associated protein (NAP) is also a ferritin-like molecule, albeit one with a role in protecting DNA from oxidative stress damage (see Section 1.41). In *Salmonella, dps* transcription is repressed by Fur in iron-replete conditions (Velayudhan et al. 2007). When required, and presumably to reduce the risk of oxidative damage arising from the Fenton reaction, iron can be exported from bacteria. In *Salmonella,* this efflux involves the proteins STM3944 (iron) and IceT (iron-citrate) (Frawley et al. 2013; Velayudhan et al. 2014).

Iron homeostasis in *Salmonella* also involves the PmrA-PmrB signal transduction system, where the PmrB sensor protein binds ferric iron in the periplasm. Iron enhances the transcription of PmrA-regulated genes and induces polymyxin resistance (Wösten et al. 2000). This observation ties the iron response to the PhoQ-PhoP-dependent magnesium starvation and low pH responses that are elaborated by *Salmonella* during adaptation to the macrophage vacuole (Kox et al. 2000; Perez and Groisman 2007). This theme of regulatory integration is developed further in Section 6.27. It should be noted, however, that the detail of the PmrA-PrmB/PhoQ-PhoP regulatory connection differs between *Salmonella* and *E. coli,* reflecting differences in genome evolution between these closely related bacteria (Winfield and Groisman 2004).

6.16 Osmotic Stress and Water Relations in Bacteria

Water can move freely across the bacterial cytoplasmic membrane in either direction, following a water concentration gradient. If the bacterium encounters an environment that is dry, water leaves the cytosol, creating a crisis for the organism because the removal of the water can result quickly in damage to macromolecules and processes that are essential for life. Loss of turgor pressure causes the cytoplasmic membrane to become flaccid, altering the structure of the bacterial cell envelope, and changes to cell shape

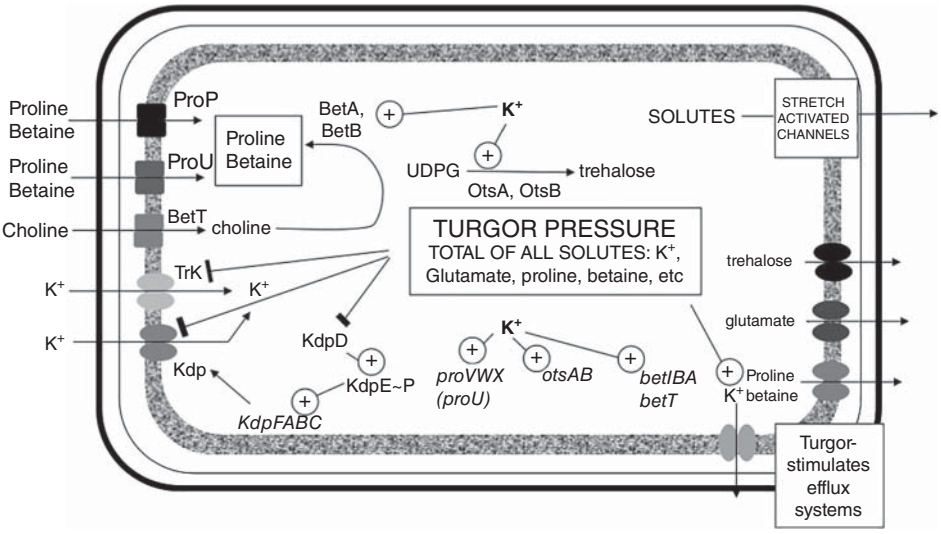

Figure 6.6 Operation of the osmotic stress response during upshock. The bacterium loses water from the cytoplasm and can no longer maintain turgor pressure. The cytoplasmic membrane becomes flaccid and growth stops. This induces the expression of the RpoS sigma factor (Figures 1.20 and 6.1). The bacterium responds in the first instance by accumulating potassium ions and secondly by accumulating glutamate (to balance the internal charge in the cell). K^+ acts as a second messenger and activates the expression of genes involved in the production of transport systems for compatible solutes (e.g. betaine), biosynthetic pathways for the production of trehalose, and the conversion of choline to betaine. High levels of K^+ feed back negatively onto the potassium uptake systems (e.g. Trk and Kdp) as zwitterionic-compatible solutes replace the lost water and restore cellular function. In the event of downshock, water floods the cell and cellular solutes are jettisoned in response via stretch-activated channels and specialist export systems.

will follow. The growth arrest that accompanies osmotic shock expands the number of copies of RpoS sigma factor proteins in the cell, reprogramming its transcriptional profile. However, the first step in responding and recovering from the shock seems to involve importing potassium ions using specialist transport systems that are either already in place (Epstein 2003) or are inducible (Epstein 2015). With the amino acid glutamate, these potassium ions play an important second messenger role in the cytosol, preparing the next phase of the stress response: the accumulation of zwitterionic-compatible solutes that can take the place of the lost water without harming macromolecules. Some of the compatible solutes are manufactured by the bacterium itself, others are imported using transport systems whose expression is induced in response to potassium and other signals (Figure 6.6) (Epstein 2015). The *proU*-encoded transporter of glycine-betaine is a prominent example of an osmotically inducible system that aids adaptation to water loss (Figure 6.7) (Sutherland et al. 1986). It uses a periplasmic binding protein to accumulate its substrate and the cytoplasmic-membrane-associated transporter proteins import the cargo, hydrolysing ATP in the process. In the absence of osmotic stress, the H-NS NAP normally silences transcription of the *proU* operon (Section 1.42), with the silencing being relieved when the osmotic signal is detected (Gowrishankar and Manna 1996; Lucht and Bremer 1994).

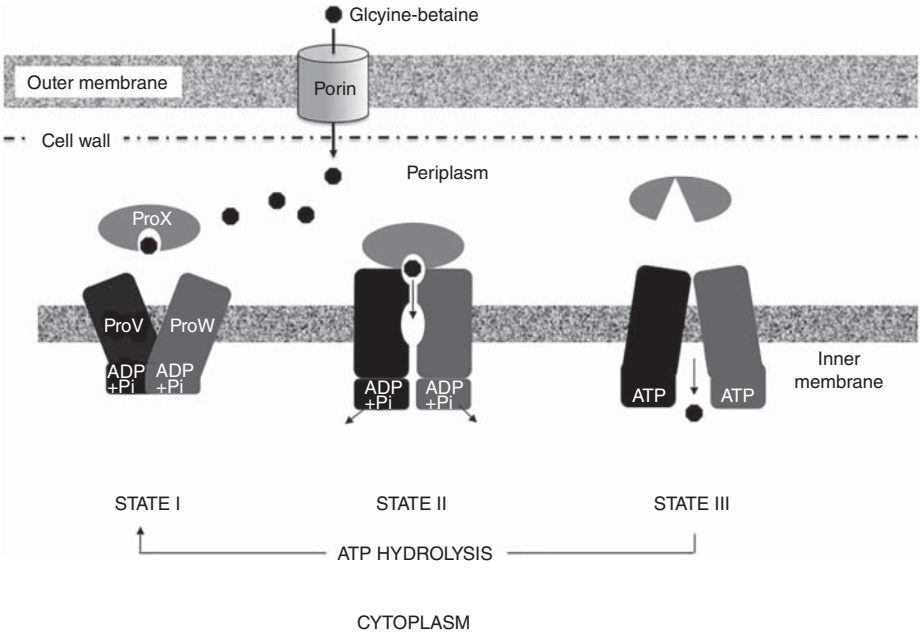

Figure 6.7 Operation of the ProU uptake system in osmotically stressed bacteria. The glycine-betaine osmoprotectant enters the periplasm via porins in the outer membrane. Here, it is captured by the periplasmic protein, ProX, and delivered to the cytoplasmic-membrane-associated ProV-ProW complex, in conformational state I. Transfer of the cargo from ProX to ProV-ProW induces a transition to conformation state II, and a binding pocket in ProV-ProX accepts glycine-betaine from ProX. The cytoplasmic complex binds ATP and adopts conformation state III, passing the cargo to the cytoplasm. Hydrolysis of the bound ATP resets ProV-ProW to conformation state I. This process is repeated each time a molecule of glycine-betaine is transported into the cytoplasm.

6.17 Signal Molecules and Stress

The principal stress-associated signal molecules in bacteria are cAMP, cyclic di-GMP (c-di-GMP), guanosine tetraphosphate (ppGpp), and guanosine pentaphosphate (pppGpp): the last two are known collectively as (p)ppGpp. Others include c-di-AMP and cGMP-AMP (also called cGAMP). These small molecules are known as second messengers and their accumulation in the bacterium is indicative of a specific stress and triggers a set of responses to help the organism to survive the emergency.

Adenylate cyclase produces cAMP in bacteria growing in the absence of glucose, the preferred carbon source. The control of cAMP production and its role in regulating the expression of genes involved in the transport of alternative carbon sources were described in Sections 6.5 and 6.6. C-di-GMP is produced by diguanylate cyclases using two GTP molecules to manufacture c-di-GMP. Turnover of c-di-GMP is carried out by phosphodiesterases, with the countervailing activities of phosphodiesterases and diguanylate cyclases setting the cellular concentration of the second messenger. This in turn interacts with effectors, which can be proteins or RNAs, to carry out a wide range of functions such as controlling motility, biofilm formation, virulence phenotype expression, development

and morphogenesis, modulation of gene transcription, etc. (Dahlstrom and O'Toole 2017; Hengge 2009; Jenal et al. 2017; Srivastava et al. 2013). The chief second messengers participate in crosstalk between their respective networks, illustrating the highly integrated nature of the sensing/signalling/response systems in bacteria (Jenal et al. 2017).

6.18 The Stringent Response

When bacteria experience starvation for amino acids, fatty acids, iron, phosphate, or carbon, they adjust their capacity for protein synthesis through negative regulation of genes coding for components of the translational machinery using a process known as the stringent response (Battesti and Bouveret 2006; Bougdour and Gottesman 2007; Liu et al. 2015; Potrykus and Cashel 2008; Seyfzadeh et al. 1993; Vinella et al. 2005). The trigger for the response in *E. coli* is a build-up of uncharged tRNA and this leads to the production and accumulation of the alarmone (p)ppGpp, with ppGpp, rather than pppGpp, being the more potent effector (Mechold et al. 2013). The alarmone is synthesised by the enzymes SpoT (Seyfzadeh et al. 1993; Vinella et al. 2005) and RelA (Haseltine et al. 1972; Gallant et al. 1977).

SpoT is bifunctional, combining (p)ppGpp synthetase and (p)ppGpp hydrolase activities, with (p)ppGpp hydrolysis in the cell being uniquely the responsibility of SpoT. This hydrolytic activity is stimulated through the direct interaction of the σ^{70} anti-sigma-factor, Rsd with SpoT. The dephosphorylated form of the Hpr protein from the phosphoenolpyruvate:sugar phosphotransferase system (PTS) antagonises the anti-sigma activity of Rsd, inhibiting the formation of an Rsd-SpoT complex. In this way, unphosphorylated Hpr interferes with the (p)ppGpp hydrolysis activity of SpoT, allowing (p)ppGpp to accumulate when the bacterium shifts from a preferred to a less-preferred carbon source (Lee, J.-W., et al. 2018).

RelA associates with ribosomes and monitors translational activity by sensing the accumulation at those ribosomes of uncharged tRNAs (Haseltine and Block 1973). Although the sensing by RelA occurs while it is associated with the ribosome, the synthesis of (p)ppGpp occurs once RelA has dissociated (English et al. 2011; Wendrich et al. 2002). RelA only has (p)ppGpp synthetic activity whereas SpoT can both synthesise and hydrolyse it (Figure 6.8) (Xiao et al. 1991). In *E. coli* and related bacteria, the (p)ppGpp alarmone cooperates with

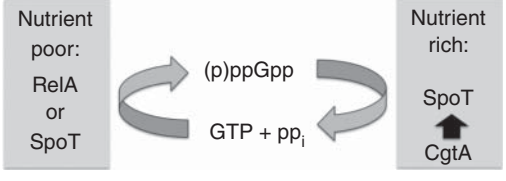

Figure 6.8 Synthesis of the alarmone (p)ppGpp. In nutrient-poor conditions, RelA and/or the bi-functional SpoT synthesis (p)ppGpp from GTP. In nutrient-rich growth conditions, the G-protein CgtA (also known as ObgE or YhbZ) modulates the activity of SpoT, a ppGpp synthetase/hydrolase, such that it degrades (p)ppGpp to GTP and inorganic pyrophosphate. CgtA is a 50S ribosome assembly factor that is thought to promote (p)ppGpp degradation on the ribosome by SpoT. For further information, see Jiang et al. (2007) and Wout et al. (2004).

a protein known as DksA to modify RNA polymerase activity, leading to a shutting down of transcription at these stringently regulated promoters (Hauryliuk et al. 2015; Paul et al. 2004) (Sections 3.6 and 3.9). These effectors bind on opposite surfaces of RNA polymerase and don't physically interact with one another: DksA is thought to amplify the effect of (p)ppGpp on RNA polymerase function (Lennon et al. 2012; Tagami et al. 2010). The modified RNA polymerase is inhibited in forming stable open complexes at stringently regulated promoters (see Section 3.6); typically these are the promoters of genes and operons that encode rRNA, tRNA, ribosomal proteins, and factors that promote translation. In contrast, the modified RNA polymerase is now more efficient at forming open complexes at promoters involved in amino acid synthesis (Paul et al. 2005). The *E. coli* paradigm is not applied universally: for example, in *Bacillus* (p)ppGpp acts indirectly by altering the concentration of initiator nucleotides for mRNA synthesis rather than by changing directly the behaviour of RNA polymerase (Krasny and Gourse 2004). In Gram-positive bacteria in general, (p)ppGpp feeds back negatively onto GTP synthesis, indirectly curtailing (p)ppGpp's own producton (Kriel et al. 2012; Liu et al. 2015) and downregulating ribosome assembly (Corrigan et al. 2016).

In addition to its influence on transcription initiation, the bacterial stringent response also inhibits translation of mRNA (Milon et al. 2006; Mitkevich et al. 2010), the initiation of chromosome replication (Kraemer et al. 2019), the elongation phase of chromosome replication (Wang et al. 2007), and chromosome segregation (Ferullo and Lovett 2008). It influences, inter alia, cell growth, survival, biofilm formation, motility, pathogenesis, competence, and persistence (Hauryliuk et al. 2015; Potrykus and Cashel 2008).

The alarmone (p)ppGpp inhibits the initiation of chromosome replication by inhibiting the transcription-driven negative supercoiling of *oriC* (Kraemer et al. 2019). DNA replication is also linked to the pppGpp/ppGpp ratio in *E. coli* by the GTP protein ObgE (also known as CgtA and YhbZ). ObgE binds ppGpp and during the stringent response *obgE* mutants expand the pool of pppGpp at the expense of ppGpp. This adjustment to the ratio of the two pools correlates with a delay in inhibiting chromosome replication following onset of the stringent response and a further delay in resuming it once the stringent response ends (Persky et al. 2009). It also concurs with the observation that ppGpp is the more effective of the two alarmone forms (Mechold et al. 2013). Obg/CgtA is a versatile protein with roles beyond arresting chromosome replication: it is also required for ribosome assembly and in association with ppGpp it can induce persistence. Here, a state of dormancy arises stochastically in individual cells in the population during periods of nutrient stress, rendering them temporarily insensitive to antibiotics that would kill or inhibit them if they were metabolically active (Gkekas et al. 2017; Verstraeten et al. 2015). In *Vibrio cholerae,* loss of (p)ppGpp and/or DksA results in an impairment in the expression of the cholera toxin (Oh et al. 2014). In addition to RelA and SpoT, *V. cholerae* also has the RelV enzyme that can synthesise (p)ppGpp in *relA spoT* double knockout mutants undergoing glucose or fatty acid starvation (Das et al. 2009). All three (p)ppGpp synthases are required for biofilm formation by *V. cholerae,* showing the link between stress starvation and the adoption of a sessile lifestyle by the bacterium (He et al. 2012). Biofilm production is also enhanced by high intracellular concentrations of c-di-GMP; at low concentrations of c-di-GMP the H-NS NAP silences transcription of the *V. cholerae* biofilm expression genes (Ayala et al. 2015a,b). The DksA protein, with the RpoS sigma factor and the HapR regulatory protein, controls

the expression of the *V. cholerae* hemagglutinin protease, an enzyme that plays a crucial part in the shedding phase of the cholera disease process (Basu et al. 2017) (Section 7.6). DksA also has a role in maintaining the expression of the RpoS sigma factor in stressed cells (Basu et al. 2017). As a further example of the integrated nature of bacterial global regulatory networks, DksA, and (p)ppGpp are necessary for the normal expression of the RNA chaperone protein Hfq, a molecule that plays a central part in RNA-based regulation (Sharma and Payne 2006).

6.19 Regulation of the Acid Stress Response

The mechanisms by which model bacteria such as *E. coli* and *Salmonella* respond to low pH stress have been studied intensively. Both organisms are exposed to pH values as low as 2 as they traverse the host stomach and *Salmonella* has evolved mechanisms for surviving low pH stress in the macrophage phagosomal vacuole. It is interesting to see how many of the global regulators that contribute to the acid stress response are also involved in responses to other forms of stress, emphasising the interconnected nature of the global regulatory processes used by these bacteria. Familiar players such as Fur, PhoP/Q, OmpR, H-NS, and RpoS, all identified in other stress responses, will also emerge as central to the low pH stress response. Here, emphasis will be on just a few model organisms. For a comprehensive description of acid stress response strategies employed across neutralophilic bacteria, readers should consult Lund et al. (2014).

In Gram-negative bacteria, the outer membrane presents a largely ineffective barrier to acid shortly after a reduction in the external pH; the pH of the periplasm falls to match the external value (Wilks and Slonczewski 2007). The periplasm has chaperone proteins, e.g. HdeA and HdeB, that work to refold proteins that have become denatured during acidification (Zhang, S. et al. 2016). It is the cytoplasmic membrane that represents the principal defence against low pH stress of external origin. While it is intact, this membrane is impervious to protons and the buffering capacity of the cytoplasm, together with inducible acid stress resistance mechanisms, help to protect the cytoplasmic contents by preserving an internal pH that is close to neutral. The composition of the membrane is important in determining its robustness to acid stress. In particular, the *cfa*-encoded production of cyclopropane fatty acids is significant (Chang and Cronan 1999). The transcription of *cfa* is under multifactorial control: it requires the RpoS sigma factor and is (p)ppGpp-sensitive, linking low pH stress to osmotic stress, the stringent response, hydrostatic pressure resistance, and the stationary phase of growth (Charoenwong et al. 2011; Eichel et al. 1999). The accumulation of weak acids, such as glutamate, by osmotically stressed bacteria is another link between the pH and osmotic stress responses (Rosenthal et al. 2006). Under some circumstances, the internal pH can be driven well below neutral and this represents a threat to the survival of the cell. Low internal pH can denature proteins, inhibiting enzyme function, and this can interfere with essential metabolic processes. Reduced internal pH is damaging to DNA (Jeong et al. 2008) and the expression of the Dps protein helps to prevent this damage. Dps is induced by oxidative stress and by the transition from exponential growth to stationary phase, providing yet another example of the integrated nature of the various stress responses that are exhibited by bacteria. DNA undergoes surveillance by repair

systems for damage by protonation, especially damage due to depurination. Mutants that are defective in DNA repair machinery do poorly when they undergo acid stress (Hanna et al. 2001). Acid stress that leads to a fall in internal pH inhibits the negative supercoiling activity of DNA gyrase, resulting in a general relaxation of DNA supercoiling (Colgan et al. 2018). This general shift in DNA topology forms an underlying component of the global response to acidic environments (Karem and Foster 1993), especially the acidified phagosomes of the macrophage (Colgan et al. 2018; Ó Cróinín et al. 2006; Quinn et al. 2014).

Neutraphilic bacteria like *E. coli* possess an acid tolerance response (ATR) and an amino-acid-dependent extreme acid resistance (XAR). The former provides protection against mild acid stress and prepares the cell to survive a more severe acid attack. ATR-associated protection operates down to pH 3.0. Some organisms can survive acid stress below pH 3.0 even without prior induction of an ATR by virtue of having an XAR system (Foster 2001, 2004). *V. cholerae* lacks an XAR system and this may explain its very high infectious dose (10^3–10^8 cells) in comparison with other enteric pathogens of humans, such as *Shigella* (10–100 cells): passage through the stomach, with its extremely low pH, may kill high numbers of vibrios, reducing the probability that an infection will be established in the gut unless the initial inoculum is very high (Lund et al. 2014).

Resistance to acid stress involves a variety of strategies. For example, the F_1F_0 ATPase can pump protons out of the cytoplasm by hydrolysing ATP as part of a strategy that involves altering proton traffic across the membrane. Similarly, the operation of the respiratory chain, with its proton-expelling activity, enhances acid stress tolerance (Sun et al. 2012) as does the secretion of protons during the operation of antiporter-based transport systems (Iyer et al. 2003). Protons can be mopped up by cytoplasmic enzymatic reactions that consume them, such as the decarboxylation of amino acids or the generation of ammonia (Iyer et al. 2003; Lu et al. 2013). The bacterium can also mobilise repair mechanisms to make good acid-induced damage (Hanna et al. 2001; Zhang et al. 2016).

The XAR response of *E. coli* is subdivided into a number of acid-responsive pathways: AR1 is glucose-repressed, operates in stationary phase in bacteria that are exposed to pH 2.5, grown aerobically (F_1F_0 ATP synthase activity is required), and it depends on the RpoS sigma factor (Richard and Foster 2004; Sun et al. 2012). AR2, 3, and 4 operate in *E. coli* when growing under fermentative conditions. AR2 is the glutamate-dependent system and is made up of the GadA and GadB enzymes (two isoforms of glutamate decarboxylase) and the GABA (glutamate/γ-amino butyric acid) antiporter, GadC (Diez-Gonzalez and Karaibrahimoglu 2004; Lin et al. 1996). The glutamate dependency in AR2 refers to external glutamate. If the intracellular glutamate supply has to be depleted, the cell suffers (i) because of the role played by glutamate as a counter-ion to K^+ accumulation during the initial stages of osmotic upshock (McLaggan et al. 1994), and (ii) because glutamate is needed as a key donor of nitrogen during the production of biomass (Bennett et al. 2009). *Salmonella* lacks AR2 and this should be borne in mind when considering the role of the acid stress response in the life of this facultative intracellular pathogen (Sayed and Foster 2009).

The arginine-dependent AR3 system is composed of AdiA (arginine decarboxylase) and the arginine/agmatine antiporter AdiC while the AR4 lysine-dependent system is composed of CadA (lysine decarboxylase) and CadB (the lysine/cadaverine antiporter) (Meng and Bennett 1992; Gong et al. 2003). CadC governs the expression of the AR4 system: it is a cytoplasmic-membrane-located winged helix-turn-helix transcription factor that activates

the *cadBA* operon when the pH drops and lysine is present (Watson et al. 1992). CadA converts lysine to cadaverine by decarboxylation and CadB excretes cadaverine from the cell, importing lysine in its place. The *cadA* gene is not present in *Shigella* or enteroinvasive *E. coli*, where CadA has been described as an anti-virulence factor because cadaverine is inhibitory to the enterotoxin activity expressed by these pathogens (Maurelli et al. 1998). In *V. cholerae,* the *cadC* gene is under the control of the LysR-like AphB master regulator of virulence gene expression when the bacterium is growing anaerobically, intimately connecting the acid stress response and the pathogenic phenotype (Kovacikova et al. 2010). CadC in *E. coli* undergoes intramembrane proteolytic cleavage (Lee et al. 2008) that is reminiscent of the cleavage events that control the stability of the membrane-associated winged helix-turn-helix (wHTH) virulence regulators ToxR and TcpP in *V. cholerae* (Teoh et al. 2015). During adaptation to the acidified phagosomes of the macrophage, *Salmonella* uses OmpR to block expression of the *cadBA* operon and the *cadC* gene, allowing the bacterial cytosol to acidify as part of its adaptation strategy (Chakraborty et al. 2015). In addition, CadC feeds back negatively onto OmpR by binding to the *ompR* gene during acid stress: CadC also inhibits the transcription of the phase-variable *fliC* gene, impairing bacterial motility during acid stress (Lee and Kim 2017).

The OmpR-dependent and osmotically regulated porin proteins OmpC and OmpF are also needed for the operation of AR3 and AR4 (Bekhit et al. 2011). Expression of arginine decarboxylase is derepressed in an *hns* mutant and this defect can be complemented by plasmids encoding the H-NS paralogue StpA and the RNA chaperone protein Hfq (Shi and Bennett 1994). StpA regulates OmpF expression at a post-transcriptional level (Deighan et al. 2000). These regulatory links highlight the interrelated nature of the acid and osmotic stress responses. Once again, the nucleoid-associated H-NS protein emerges as a central player in the control of an important physiological pathway. The OmpR protein was identified originally as a regulator of the genes encoding the outer membrane porin proteins OmpC and OmpF in response to osmotic stress (Taylor et al. 1981). OmpR is now firmly linked to the acid stress response too and plays a direct role, for example, in regulating the expression of virulence genes in *Salmonella* during adaptation to the acidified phagosomes in the macrophage (see Section 7.18).

The importance of glutamate in the *E. coli* AR2 system and its central role, with glutamine, in nitrogen regulation introduces a further example of physiological overlap. When nitrogen levels become low, the transcription factor NtrC is phosphorylated by the cytoplasmic sensor kinase NtrB in response to signals from the nitrogen assimilation pathway (Rombel et al. 1998). NtrC controls a regulon of genes that use the RpoN (σ^{54}) form of RNA polymerase sigma factor (Weiss et al. 2002) and among these is the gene that encodes the Nac transcription factor which is responsible for controlling a regulon of RpoD (σ^{70})-dependent genes (Muse and Bender 1998; Zimmer et al. 2000). Whole genome analysis has shown Nac to bind to *gadE,* the AR2 regulator gene, and to regulate its transcription positively (Aquino et al. 2017).

The AR2 GadE DNA-binding protein forms a heterodimer with the RcsB regulatory protein in order to be active. This provides a physical link between AR2 and the large Rcs regulon of stress-regulated genes (Wall et al. 2018). This regulon senses cell envelope damage through the RcsF lipoprotein in the outer membrane, communicating with the IgaA protein in the cytoplasmic membrane. IgaA is a negative regulator of the Rcs signalling system

that communicates with the membrane-associated sensor-kinase proteins RcsC and RscD, which in turn phosphorylate the RcsB protein. In its homodimeric form, phosphorylated RcsB controls the expression of the sRNA gene *rprA* (Majdalani et al. 2002), the osmotically induced gene *osmC* (Davalos-Garcia 2001), and the cell division gene *ftsZ* (Gervais et al. 1992). Phosphorylated RcsB can repress the transcription of the *flhDC* operon, inhibiting motility. When it forms a heterodimer with RcsA, phosphorylated RcsB downregulates motility by repressing *flhDC* even more severely and it upregulates the expression of colanic acid (*rcsA* is silenced by H-NS). The unphosphorylated form of RcsB heterodimerises with GadE to activate *gadA* transcription (*gadE* is silenced by H-NS) and it heterodimerises with BglJ to regulate β-glucoside metabolism (the *bgl* operon is silenced by H-NS). The *gadA* gene is directly silenced by H-NS (Giangrossi et al. 2005), indicating that part of the role of RcsB-GadE complex is to overcome the silenced state. These links have multiple ramifications for gene expression in the cell. For example, the *rprA* sRNA is a positive regulator of RpoS expression (Majdalani et al. 2001), so RcsB can influence indirectly the RpoS regulon and hence the cellular response to the stationary phase of growth, osmotic and acid stress, etc.

Counter-silencing of H-NS is a feature of the *E. coli* Acid Fitness Island (AFI) (Hommais et al. 2004) (Figure 6.9). This island contains the genes encoding the GadA and GadB glutamate decarboxylases, the GadC cytoplasmic-membrane-located glutamate/γ-aminobutyrate antiporter, the LuxR-like GadE protein, the GadW and GadX AraC/XylS-like transcription factors, the GadY sRNA, the HdeA, HdeB chaperone proteins, the 6H57 sRNA gene, the lipoprotein Slp, the putative membrane proteins HdeD and YhiD,

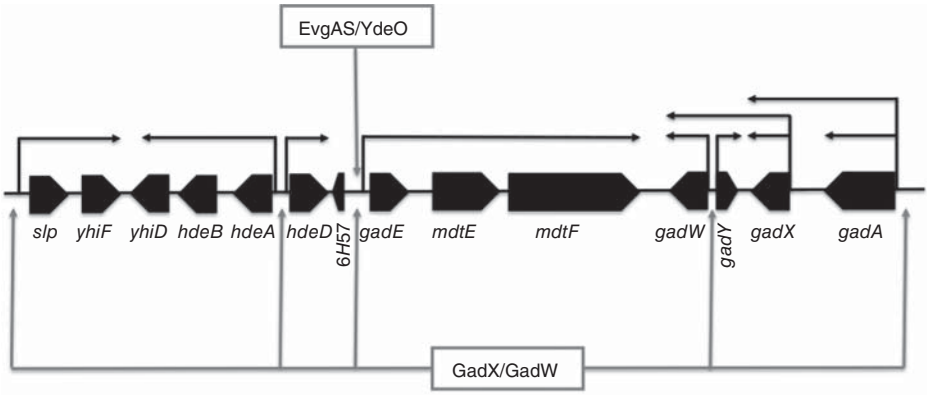

Figure 6.9 The Acid Fitness Island (AFI) of *Escherichia coli*. The locations, orientations, and transcriptional relationships of the genes in the AFI are shown. Each gene is represented by a solid arrow with the orientation of the arrow indicating the direction of transcription. Angled arrows represent the direction and extent of transcription units. The positive regulatory inputs of the GadW/GadX regulatory proteins are indicated by upward vertical grey arrows, each regulatory site is also subject to negative control by the H-NS transcription silencing NAP (see Tramonti et al. 2008). The EvgAS 2-component regulatory system and the YdeO protein also control the *gadE* regulatory gene positively (downward grey arrow). Regulatory inputs by the *gadY*- and *6H57*-encoded sRNAs have also been described, as have contributions by cAMP-CRP and RpoS. For further reading, see Aiso et al. (2011) and Tramonti et al. (2008).

the YhiF putative regulatory protein, and the multidrug efflux pump components MdtE and MdtF (Aiso et al. 2011; Mates et al. 2007; Nishino et al. 2008; Tramonti et al. 2008; Tree et al. 2011). The GadW and GadX transcription factors upregulate transcription by overcoming H-NS-mediated silencing at the promoters of the *gadA, gadW, gadE-mdtE-mdtF, hdeAB-yhiD*, and *slp* genes/operons (Figure 6.9) (Tramonti et al. 2008).

Gene regulation within the AFI is complex. The 2-component regulator EvgAS controls the *gadE* gene in AR2, with the EvgA response regulator binding directly to the *gadE* gene; EvgAS also regulates *gadE* expression indirectly through the YdeO AraC-like protein (Figure 6.9). Their inputs are, however, conditional and seem to be restricted to exponential growth in acidified minimal medium containing glucose (Ma et al. 2004). The *gadE* gene is regulated post-transcriptionally by the 6H57 antisense RNA, encoded in the AFI immediately upstream of the *gadE* open reading frame (Figure 6.9). GadE, in turn, regulates the 6H57 gene promoter positively in response to acid stress (Aiso et al. 2011). The cAMP-CRP complex is a transcription repressor within the AFI while expression of *gadA* and *gadBC* requires the RpoS sigma factor (Tramonti et al. 2008).

6.20 Alkaline pH Stress Response

Exposure of *E. coli* to alkaline pH induces the transcription of the *nhaA* gene, encoding a sodium/proton antiporter. This gene is under the positive control of the NhaR, a LysR-like transcription regulator. NhaR senses sodium concentrations directly and alters the pattern of its interactions with the regulatory domain of the *nhaA* gene in response to sodium (Carmel et al. 1997). The *nhaA* gene also responds negatively to the H-NS NAP (Dover et al. 1996), perhaps indicating that part of the role of NhaR is to overcome H-NS-mediated silencing of *nhaA* transcription. The NhaR regulon includes the horizontally acquired *pgaABCD* operon, which is required for the production of the biofilm adhesin poly-β-1,6-N-acetyl-D-glucosamine. NhaR promotes the transcription of this operon, and hence biofilm expression, in the presence of NaCl at alkaline pH (Goller et al. 2006). The RNA-binding protein CsrA (called RsmA in *Pseudomonas* spp) binds to the 5′ end of the NhaR mRNA and inhibits its translation by out-competing ribosomes for access, linking the alkaline stress response to the CsrA regulon (Potts et al. 2017; Vakulskas et al. 2015). In addition to its indirect negative effect on *pgaABCD* transcription achieved by removing the activator NhaR, CsrA also prevents *pgaABCD* translation directly by binding to the 5′ end of the operon's polycistronic transcript (Pannuri et al. 2012). While CsrA controls NhaR production post-transcriptionally, NhaR feeds back onto CsrA activity indirectly. The link is a complex one and involves NhaR acting through the UvrY/BarA 2-component system to upregulate *csrBC* transcription, with the sRNAs CsrB and CsrC inhibiting CsrA production (Król 2018). The *csrB* and *csrC* genes seem to be the principal targets for UvrY(SirA)/BarA (Zere et al. 2015). The acid-induced regulatory protein YdeO, an AraC-like transcription factor, has been reported to control the transcription of *nhaR* positively from a secondary promoter located inside the upstream *nhaA* open reading frame (Yamanaka et al. 2014). While it seems counterintuitive that the positive control of an alkaline-stress-response gene requires an acid-induced regulator, the wider involvement of NhaR in the expression of phenotypes such as biofilm production may point to a fine-tuning or modulatory role for

YdeO. Also, the action of YdeO seems to be restricted to limited physiological conditions (Ma et al. 2004).

6.21 Motility and Chemotaxis

Motility represents a practical response to an environment whose composition is complex and unpredictable because it allows the bacterium physically to relocate itself. Chemotaxis modulates motility by informing it in response to signals about environmental conditions. There are times in the life of a motile bacterium when a transition to a sessile lifestyle is indicated, so downregulation of motility, accompanied by expression of structures that support the new lifestyle (adhesins, biofilm, etc.), must be initiated. Similarly, bacteria that have formed a stable attachment to a surface may produce members that activate their motility and chemotaxis functions as they switch to a planktonic phase of life. Motility that involves swimming depends on the production and assembly of flagella, flagella motors, and the components that attach each flagellum to its motor and embed the motor in the cell envelope. Large collectives of genes are devoted to the expression of these structures and their regulation is complex. The motors operate using proton flow generated by PMF (as in the case of the F_1F_0 ATP synthase) so motility reflects PMF status, the composition of the external environment, and the physiology of the bacterium. In model bacteria such as *E. coli,* motors reverse their direction of rotation. Counter-clockwise rotation promotes flagellar rotation that is counter-clockwise, entwining the flagella in a bundle that promotes smooth swimming with the flagellar bundle performing the role of a propeller. Clockwise rotation causes the bundle to fly open and the bacterium to tumble suddenly, changing its direction of travel the next time the propulsive bundle forms. Alternating swimming (also called 'running') and tumbling causes the microbe to describe a random walk through its environment. This 'walk' can be made less random by suppressing the frequency of tumbling, something that is done in response to environmental signals that are detected by the chemotaxis system and transmitted to the motor through a phosphorelay. In general, signals that indicate an improving environment suppress tumbling, allowing the organism to move along a gradient of improving conditions. If threats, such as toxic substances, are detected, suppression of tumbling ceases and the bacterium alters its course in a random way.

The master regulatory genetic locus for flagellar component gene expression, secretion, and assembly is *flhDC,* encoding the transcription factors FlhC and FlhD (Chevance and Hughes 2008; Chilcott and Hughes 2000; Wang, S. et al. 2006). This operon is under complex control at the transcriptional and the post-transcriptional levels (Fahrner and Berg 2015). The cAMP-CRP complex is an essential positive regulator of *flhDC* transcription, linking its expression to levels of glucose and the cAMP second messenger (Soutourina et al. 1999; Yokota and Gots 1970). The QseB quorum-sensing regulator, when phosphorylated by sensor-kinase QseC, links flagellar expression to the presence of autoinducer 2 (AI-2) (Clarke and Sperandio 2005; Sperandio et al. 2002). The *flhDC* operon is linked into several of the major stress response networks of the cell. For example, the RNA-binding protein CsrA of *E. coli* makes an input here (Figure 4.2) (Wei et al. 2001); the sRNAs ArcZ, ImrA, OmrB, and OxyS affect *flhDC* mRNA negatively (De Lay and Gottesman 2012) while the sRNA McsA has a positive input (Thomason et al. 2012). RcsB, the regulator of colanic acid

biosynthesis, also binds there either alone or as a heterodimer with RcsA (Filippova et al. 2018; Wall et al. 2018). In avian pathogenic *E. coli,* the YjjQ transcription repressor binds to a site that overlaps the one used by RcsA-RcsB (Wiebe et al. 2015). YjjQ also regulates the *ompC* porin gene, the *gfc* locus that specifies the group 4 capsule (Peleg et al. 2005). It also controls the *yfiRNB* operon that is required for the production of cyclic-di-GMP (YfiN), a lipoprotein (YfiB), biofilm formation, swarming, swimming, and CsgD-dependent cellulose formation (all dependent on YfiR) (Hufnagel et al. 2014). OmpR is another negative regulator of *flhDC* transcription (Shin and Park 1995) and one of its binding sites is also targeted by the fimbrial regulator LrhA, linking motility and biofilm expression (Blumer et al. 2005; Lehnen et al. 2002). Transcription of *flhDC* is also reduced by the LysR-like regulator HdfR (Ko and Park 2000) and the fimbrial regulator MatA (Lehti et al. 2012).

The normal pattern of *flhDC* expression can be overridden by the insertion upstream of a variety of insertion sequences (IS) elements, altering the motility phenotype of *E. coli* (Fahrner and Berg 2015). The insertion sites correspond with regions that are predicted to experience DNA supercoiling-induced duplex destabilisation, which is consistent with a role for DNA topology in *flhDC* expression (Zhang et al. 2017). A requirement for the NAPs HU and H-NS for full motility may also be indicative of the importance of DNA architecture in the operation of the FlhDC regulon (Mangan et al. 2011; Nishida et al. 1997; Soutourina et al. 2002). The FlhDC regulon forms part of the stringent response and is regulated by the (p)ppGpp alarmone and the DksA protein (Lemke et al. 2009). This level of transcriptional and post-transcriptional control is consistent with the central role played by the FlhDC master regulators in the life of the bacterial cell and the importance to the cell of refining their expression and activity with precision in response to multiple inputs reporting the state of the cell and of the external environment.

The FlhC and FlhD proteins regulate just a few genes directly but the FliA protein, whose expression they control, regulates an extensive regulon (Fitzgerald et al. 2014). FliA is an alternative sigma factor that reprogrammes RNA polymerase to transcribe genes involved in the production of flagellar motor and other flagellar components and their assembly at the cell envelope (Arnosti 1990; Arnosti and Chamberlin 1989; Helmann and Chamberlin 1987). The activity of FliA (also known as σ^{28}, σ^{F}, or RpoF) is regulated by an anti-sigma factor protein called FlgM that is eventually secreted via the maturing flagellar structure, a structure that is a de facto type III secretion system (Ohnishi et al. 1992). The principal transcription units that are under FlhDC control are the seven flagellar operons: *flgAMN, flgBCDEFGHIJ, flhBAE, fliAZY, fliE, fliFGHIJK*, and *fliLMNOPQR,* with the genes encoding FliA and FlgM being among these (Fitzgerald et al. 2014). Once FlgM has been removed by the T3SS, FliA can initiate transcription of its regulon, which includes the one containing its own gene, *fliAZY*. The other members of the FliA regulon are *fliDST, flgKL, flgMN, fliC, tar-tap-cheRBZY*, and *motAB-cheAW*. These operons encode the final components of the motor, the flagellum (including flagellin, the main flagellar subunit protein, FliC) and the proteins from the chemotactic sensory and signal transduction pathway. The system has a hierarchical arrangement with the unique Class 1 operon *flhDC* at the top, the σ^{70}-dependent Class 2 operons next, and the σ^{28}-dependent Class 3 operons at the base. FliA is also involved in expressing the chemotaxis genes *aer, trg*, and *tsr,* genes involved in c-di-GMP control of motility (*yhjH, ycgR*) (Ko and Park 2000), and some Class 2 operons in addition to *fliAZY* (Chilcott and Hughes 2000; Liu and Matsumura 1996; Macnab 1992).

Motility is modulated by changes in temperature. At low cell densities, *E. coli* swims along a temperature gradient towards warmer sites (Maeda et al. 1976). Low temperatures correlate with increased rate of tumbling and tumbling is suppressed as the temperature goes up, encouraging smooth swimming in a chemotaxis-related process known as thermotaxis (Nara et al. 1996; Salman and Libchaber 2007). If the density of the culture increases and nutrients are depleted, *E. coli* will move to cooler locations, possibly as a way to downregulate its metabolism (Salman and Libchaber 2007). The Gram-negative pathogen *Yersinia* stops making flagella at 37 °C, rendering itself non-motile at mammalian host body temperature; it does this by suppressing expression of both the σ^{28} sigma factor (Table 3.2) and the FlgM anti-sigma factor (Kapatral et al. 1996), possibly by adjusting the topology of the DNA at the *fliA* and *flgM* genes (Rohde et al. 1994). In the food-poisoning pathogen *Campylobacter,* the interaction of the σ^{28} sigma factor with the FlgM anti-sigma factor is temperature dependent and the bacterium is more motile at 42 °C in its avian host than at 37 °C (Wösten et al. 2010). Motility in *Campylobacter* is also sensitive to factors that alter DNA supercoiling (Shortt et al. 2016).

6.22 Quorum Sensing

Bacteria manufacture signalling molecules that are used in quorum sensing, a process that allows microbes to control behaviours as a function of signal molecule concentration (Abisado et al. 2018; Fuqua et al. 1994; Papenfort and Bassler 2016). Looked at simplistically, an individual bacterium cannot make enough of the quorum-sensing molecule (called an autoinducer) to affect the behaviour in question, but a group of bacteria that pools its signalling molecules can. The important variable is the concentration of the signalling molecule as this acts as a proxy for the number of microbes within effective range. Interestingly, the autoinducer does not have to be provided by bacteria of the same species, allowing for complex community structures among the participating organisms. To participate, a bacterium must possess the means to detect the signals and to use these to alter some cellular function. It usually has the capacity to produce the signal too, but this is not essential so long as the other neighbouring organisms are producing enough of the signalling molecule to elicit a biological response.

Quorum sensing is important because it provides a molecular basis for collective action at the level of a population. It is also important because it provides a mechanism for coordination of activities among individual bacterial cells, allowing them to function as members of a community. In this way, a population of unicellular bacteria can become more than the sum of its parts. The bacteria do not always respond to the autoinducer in a stereotypical manner and this allows quorum sensing to introduce stochasticity into the response, creating the physiological diversity that is so important in survival in an unpredictable and potentially hostile environment (Carcamo-Oyarce et al. 2015). Quorum sensing within individual bacterial cells does not take place in isolation from other stimulus/response pathways in bacteria but is integrated with them. Analyses of bacterial responses to stress at the level of the whole genome, in combination with single gene and single protein studies, have revealed the extent of this regulatory integration. Small regulatory RNAs have a special role in the achievement of this integration (Papenfort and Vogel 2010).

The autoinducer molecules in use by Gram-negative bacteria are derived ultimately from s-adenosylmethionine (SAM) and are acyl homoserine lactones (AHLs). The lactone typically comes from SAM and the side chain is derived from fatty acid biosynthesis, with the AHL synthesis being performed by a LuxI enzyme (Case et al. 2008). Autoinducer nomenclature reflects the bacterial species producing the molecule. For example, CAI-1 is the autoinducer in *V. cholerae* and it is produced by the CqsA autoinducer synthase from SAM and decanoyl-CoA (Higgins et al. 2007; Kelly et al. 2009; Ng et al. 2011; Wei et al. 2011). The AI-2 autoinducer produced by the marine organism *Vibrio harveyi* is detectable by other bacterial species (Schauder et al. 2001; Winans 2002). This, and the discovery in both Gram-positive and Gram-negative bacteria of homologues of the *luxS* gene for AI-2 production, has led to the proposition that AI-2 can form the basis of a cross-species signalling-and-response system (Schauder et al. 2001; Surette et al. 1999). For example, AI-2 from *V. harveyi* can alter the expression of the Lsr ABC transporter system in *Salmonella* that imports AI-2 (Taga et al. 2001, 2003). Whiteley et al. (2017) have pointed out that AI-2 may act as a *cue* (where the molecule and the response have not co-evolved) in certain circumstances and as a *signal* (where molecule and the response have co-evolved) in others.

The AI-2 molecule plays a role in the resetting of the gut microbiome in the aftermath of antibiotic treatment, enlarging the population of Firmicutes at the expense of *Bacteroides* spp. (Thompson et al. 2015). In the guts of individuals infected with *V. cholerae,* the virulence of the pathogen is restricted when it takes up the AI-2 molecule that is produced by the gut commensal bacterium *Blautia obeum,* aiding the patient in recovery from the disease (Hsiao et al. 2014). AI-2 in the gut also induces the killing of rival bacteria by *V. cholerae* by means of its T6SS (Shao and Bassler 2014; Zheng et al. 2010). DNA released by the lysed rivals is available to *V. cholerae* for uptake, driving its evolution by horizontal gene transfer (Borgeaud et al. 2015). In the oral cavity, AI-2 contributes to the formation of mixed-community biofilms (Rickard et al. 2008). AI-2 in the gut is also a participant in bacterium–host communication: these molecules can induce inflammation and promote programmed cell death in mammalian cells (Shiner et al. 2006; Zargar et al. 2015).

Mammalian cells are active in the inter-kingdom conversation through the production of autoinducer mimicking molecules, with the bacterial AI-2 receptors LuxP and LsrB being able to bind them and use them to elicit a quorum sensing response (Ismail et al. 2016). Bacteria also have the ability to eavesdrop on host communications using receptors that can detect the presence of mammalian stress hormones. Adrenaline is detected by the bacterial sensor kinase QseC while QseE detects noradrenaline (Karavolos et al. 2013). Bacteriophage can exploit bacterial autoinducers to control decisions in the phage lifecycle. The vibriophage VP882 produces a receptor protein ($VqmA_{Phage}$) for the 3,5-dimethylpyrazin-2-ol (DPO) autoinducer and uses it to govern the lysis/lysogeny switch when VP882 is in DPO-producing *V. cholerae* (Silpe and Bassler 2019).

Autoinducer signalling molecules can traverse bacterial membranes and are bound by specific receptor proteins at the membrane or in the cytoplasm. The detection by these proteins of an autoinducer typically results in the production of more of that molecule and the associated amplification of the signal. These signalling events alter the expression of scores of genes with downstream effects that alter the behaviour by those bacteria that make up the affected population. LuxI and LuxR orthologues work in pairs in that the

LuxI-like enzyme in a given species will have a LuxR partner that binds its autoinducer product and elicits a response. However, LuxR orphans have been discovered in bacteria that do not possess a LuxI counterpart. *Salmonella* expresses a LuxR transcription factor called SdiA but not a LuxI homologue; *E. coli* has an SdiA protein too and this can detect signals from the host as well as autoinducers of bacterial origin (Hughes et al. 2010; Nguyen et al. 2015; Smith and Ahmer 2003; van Houdt et al. 2006). Orphan, or solo, LuxR homologues are common in Gram-negative bacteria and their numbers exceed those of LuxI proteins. The *Salmonella* SdiA protein responds to autoinducers produced by other bacteria (Smith and Ahmer 2003) indicating the extent of inter- and intra-species signalling and response in bacterial populations.

The behaviours that are affected by quorum sensing include virulence and biofilm formation (Laganenka et al. 2016; Rutherford and Bassler 2012; Whiteley et al. 2017). Both intersect with signalling pathways governed by second messengers such as (p)ppGpp, c-di-GMP, and cAMP, illustrating the very complicated nature of bacterial regulatory networks (Pesavento and Hengge 2009). These behaviours represent lifestyles that are chosen by bacteria with the genetic capacity to make the choice in response to the reception by the organism of information about the quality of the external environment (including information about local autoinducer concentration) and the condition of the organism's own physiology. Pathogens with mutations that make them defective in quorum sensing have reduced virulence, as in the case of the Gram-positive pathogen *Staphylococcus aureus* (Ji et al. 1995; Novick and Geisinger 2008), the Gram-negative pathogen *Pseudomonas aeruginosa* (Pearson et al. 2000), and the Gram-negative plant pathogen *Erwinia carotovora* (Pirhonen et al. 1993). These observations indicate that, for some pathogens, infection of a host is a community affair. In the case of *S. aureus,* the signalling molecule is a cyclic peptide of eight amino acids called AIP-1 and the regulated phenotype is the expression of exported toxins (Ji et al. 1995). Competence in the Gram-positive bacterium *Streptococcus pneumoniae* is also controlled by a peptide quorum-sensing signal, known as a pheromone (Håvarstein et al. 1995; Tomasz 1965). Bacteriophage of the SPBeta group use a peptide called arbitrium as the basis of a quorum-sensing system to make lysis/lysogeny decisions when infecting their Gram-positive *Bacillus* host (Erez et al. 2017). Quinolones are used as signalling molecules by *Ps. aeruginosa* to control biofilm formation and virulence factor expression (Heeb et al. 2011). This family of molecules includes inhibitors of type II topoisomerases such as DNA gyrase and topoisomerase IV (Sections 1.28 and 1.29). These examples help to illustrate the diversity of the molecules that are used as signals by bacteria engaging in quorum sensing. A much more comprehensive coverage of their structural diversity is available in Papenfort and Bassler (2016) and in Whiteley et al. (2017).

6.23 Biofilms

A biofilm consists of a high-density bacterial community encased in an extracellular polymeric matrix that is attached to a surface (Costerton et al. 1987). Biofilm is produced to provide a home for a static population of microbes either of one species or of several species. The communal effort involved in producing the extracellular components of

biofilm is rewarded by the protection that the biofilm affords the resident microbes, shielding them from stress, including the shear stress associated with the colonisation of a surface in an environment that is subject to fluid flow (Kim et al. 2016). Biofilm membership enhances bacterial resistance to antimicrobial agents (Parsek and Greenberg 2005) and has important clinical implications, making this topic a target for intense investigation. Biofilms also encourage horizontal gene transfer and so contribute to the acceleration of microbial evolution (Ghigo 2001). The cell-to-cell communication that contributes to biofilm formation by a community involves quorum sensing (Popat et al. 2012), and quorum sensing can promote biofilm assembly and, in some cases, biofilm disassembly (Ball et al. 2017; Whiteley et al. 2017). In the case of *V. cholerae,* biofilm formation is associated with low cell density, and is promoted by the AphA regulator and inhibited by HapR (Figure 7.6). In contrast, the behaviour known as aggregate formation is promoted by HapR and occurs at high cell density. Under aggregate-promoting conditions, HapR inhibits biofilm formation (Jemielita et al. 2018). Biofilm structure helps to exclude bacteria that 'cheat' by failing to participate fully in quorum-sensing-dependent processes (Nadell et al. 2016).

6.24 'Cheating' as a Lifestyle Strategy

An example of cheating can be seen in the case of quorum sensing that controls the production of secreted proteases in several bacterial species. These break down proteins in the environment to provide sources of nutrients. Bacteria that do not contribute a protease of their own, perhaps because they have a mutation in the protease gene or another component of its expression and secretion pathway, can benefit from the proteolytic activity of their more communautaire siblings (Diggle et al. 2007; Sandoz et al. 2007). Such non-contributing members of the population derive the nutritional benefits without paying the physiological costs and are therefore called 'cheaters'. Cheating is a sound survival strategy as long as it is confined to a minority of the population. If every cell becomes a cheater, there will be no protease producers left and the population is liable to collapse. The competitive fitness benefit enjoyed by a cheater will drive cheater numbers to expand in the population, risking eventual extinction of the cheater genotype. In this way, the appearance and disappearance of cheaters can be regarded as part of a self-regulating cycle. Cheater advantage is offset in microbial quorum-sensing systems in which one shared product (e.g. the protease) is co-regulated with a non-shared product by the same autoinducer. In this way, the advantage of not contributing the communal protease is offset for an autoinducer-deficient cheater by its inability to express a second product (e.g. a metabolic intermediate) that contributes at an individual level to its own survival (García-Contreras et al. 2015; Yan et al. 2018). The LasR elastase (a protease) and adenosine catabolism are linked in this way by quorum sensing in *Ps. aeruginosa* (Dandekar et al. 2012; Yan et al. 2018). Quorum sensing controls the expression of T6SS toxic factors and factors for toxin immunity. Mutants deficient in quorum sensing lose the ability to express both toxins and immunity factors, making them vulnerable to killing by wild type bacteria that continue to express both (Majerczyk et al. 2016). This helps to restrict the proliferation of mutants with a cheater phenotype.

6.25 Thermal Regulation

Regulating gene expression in response to changes in temperature is an important behaviour for bacteria. In the case of pathogens that infect mammals, including humans, being able to respond to a shift in temperature to 37 °C is an important part of the process of adapting to the host. Bacteria can also respond to temperature stress caused by sudden temperature shifts that cause heat shock or cold shock. Changing temperature affects the structure of DNA, RNA, and proteins, allowing thermal control to operate at multiple levels in the cell and allowing the biomolecules of the cell to function as thermosensors and thermometers (Eriksson et al. 2002).

The supercoiling of bacterial DNA changes with the growth temperature (Goldstein and Drlica 1984) and temperature-induced changes to DNA topology are associated with the activation of virulence genes when pathogens encounter mammalian hosts (Dorman et al. 1990; Rohde et al. 1994). Temperature influences pathogen motility and thermally induced changes in DNA topology have been proposed to form part of the regulatory mechanism (Rohde et al. 1994; Shortt et al. 2016). Thermal influences on DNA flexibility, curvature, bending, and the positioning of bend centres along the duplex can form parts of transcriptional switches (Prosseda et al. 2004). The TlpA transcription repressor and coiled-coil-protein that is encoded by the virulence plasmid in *Salmonella enterica* loses its repressor activity when the bacterium is shifted up to a temperature of 37 °C (Hurme et al. 1997). Many pathogens use AraC-like transcription factors to control gene expression in response to thermal signals. In the case of the VirF master regulator in *Shigella* spp., the mechanism seems to involve variable DNA topology working in concert with VirF binding rather than relying on the protein to perform the thermosensory role itself (see Section 7.11).

RNA makes an excellent biological thermometer because RNA folding is sensitive to changes in temperature. Folding patterns also determine the vulnerability of the RNA to degradation, so the half-life of the RNA is influenced by temperature. Several RNA-binding proteins and RNA chaperones are cold shock proteins, further emphasising the connection between RNA biology and thermal sensing. CspA is the major cold shock protein of *E. coli* and it is an RNA chaperone (Goldstein et al. 1990; Jiang et al. 1997; Rennella et al. 2017). Like its relative CspE, CspA can destabilise RNA duplexes, allowing it to interfere with transcription termination and mRNA translation (Phadtare et al. 2002, 2006). CspA regulates the transcription of the *hns* gene by binding to the promoter region (Brandi et al. 1994; La Teana et al. 1991) making H-NS a component of the cold shock response in *E. coli* (Dersch et al. 1994). The *V. cholerae* counterpart of H-NS, the VicH protein, has also been described as a cold shock protein (Tendeng et al. 2000). In contrast, the H-NS paralogue, StpA, is expressed in increased quantities in *E. coli* as the temperature rises (Free and Dorman 1997). The inhibitory role of the Hfq RNA chaperone on the expression of the VirB/InvE protein has been described elsewhere (Section 7.13). Hfq is temperature dependent (Beauregard et al. 2013) and at 30 °C it binds more efficiently to *invE* mRNA, destabilising it and down-regulating VirB/InvE expression with negative downstream effects on the expression of the T3SS in *Shigella* (Mitobe et al. 2008). Similarly, the SPI2 T3SS in *Salmonella* is subject to H-NS-mediated thermoregulation: gene expression is silenced by H-NS

at the *ssrB* master regulatory gene, with the Hha protein acting in concert with H-NS (Duong et al. 2007).

The H-NS protein plays a pervasive role in controlling transcription in response to temperature. The general pattern seen is one in which H-NS silences genes at low temperatures and the silencing is relieved by a variety of means at 37 °C (Stoebel et al. 2008b). In addition to external influences, such as other proteins or changes to DNA topology, that relieve transcription silencing by H-NS at low temperatures, it has been suggested that conformational changes internal to H-NS itself may operate as an autoinhibitory switch (Shahul Hameed et al. 2018). The negative influence of this NAP is seen particularly in the case of virulence genes in Gram-negative pathogens. In many cases the stimulatory effect of rising temperature is enhanced if this is combined with osmotic stress, and indeed, H-NS has a well-described role as a repressor of genes involved in the osmotic stress response (Higgins et al. 1988; Porter and Dorman 1994). The H-NS protein controls the composition of the Gram-negative outer membrane by affecting the expression of genes involved in lipopolysaccharide modification. This, in turn, influences the sensitivity of the bacterium to antimicrobial peptides (Bengoechea et al. 2002; Reines et al. 2012). These are part of the same pathway that is controlled by the PhoPQ and PmrAB regulatory systems in *Salmonella,* systems involved in iron stress resistance and resistance to polymyxin (Bevins and Salzman 2011; Choi and Groisman 2013; Nishino et al. 2006; Wösten et al. 2000). Iron scavenging by the ChuA (enteropathogenic *E. coli*) and ShuA (*Shigella dysenteriae*) systems within the mammalian host is thermally controlled. The mRNAs specified by the *chuA* and *shuA* genes contain an RNA thermosensor that folds to sequester translation initiation signals when the bacteria grow at temperatures below 30 °C (Kouse et al. 2013).

Heat shock induces the expression of a dedicated sigma factor, RpoH (σ^{32}, σ^{H}). The *rpoH* mRNA contains a thermometer sequence that operates to enhance *rpoH* translation when the bacterium is shifted to a temperature of 42 °C, i.e. when it experiences a heat shock (Morita et al. 1999). The heat shock chaperone proteins DnaK/DnaJ/GrpE and GroEL/GroES enhance the degradation of RpoH and the sigma factor is turned over proteolytically by the membrane-anchored FtsH protease (Bittner et al. 2017; Guisbert et al. 2004; Herman et al. 1995; Straus et al. 1990; Tomoyasu et al. 1998). RpoH is taken to FtsH at the cytoplasmic membrane by the signal recognition particle, SRP, part of a major pathway for protein export (Lim et al. 2013; Miyazaki et al. 2016). Once heat shock occurs, the RpoH-associated chaperones are redeployed to damaged proteins, leaving RpoH free to interact with core RNA polymerase (Gamer et al. 1992, 1996; Horikoshi et al. 2004). After the shock response is complete, DnaK/DnaJ can once again sequester RpoH, withholding it from RNA polymerase core enzyme (Blaszczak et al. 1995; Gamer et al. 1996). RpoH competes with other sigma factors for access to core RNA polymerase, so increasing its expression and stabilising it at high temperature increases the number of RpoH-programmed polymerases in the cell, from 50 copies in unstressed cells to around 1000 following heat shock (Straus et al. 1987). The RpoH regulon consists chiefly of genes that encode chaperones and proteases that deal with the consequences of thermal stress: misfolded and damaged proteins; all of the chaperones and proteases that contribute directly to RpoH sequestration and degradation are encoded by the RpoH regulon (Bittner et al. 2017).

6.26 Epigenomics and Phasevarions

Chemical modification of the bases in DNA by methylation can act as an epigenetic regulator in prokaryotes and eukaryotes. Prokaryotes are thought to lack the regulatory option afforded by histone modification because they don't have histones, but their NAPs may undergo post-translational modification to produce analogous effects (Dilweg and Dame 2018). The principal DNA methylation event in bacteria is the conversion of adenine to N6-methyladenine by a methyltransferase, using S-adenosyl-L-methionine as the source of the methyl group (Wion and Casadesús 2006). Other base methylation events in bacteria produce N4-methylcytosine and 5-methylcytosine. Methyltransferases can operate in partnership with restriction endonucleases, with the methylation event serving to mark the target DNA as 'self', making it exempt from cleavage by the restriction enzyme (Boyer 1964). They can also operate in isolation, having been orphaned by separation from a presumptive restriction endonuclease partner. The DNA adenine methylase, Dam, that methylates the adenines in the sequence motif 5'-GATC-3′, is one of the most widely studied orphan methyltransferases in bacteria due to its key roles in controlling the cell cycle (Boye et al. 2000) and gene expression (Casadesús and Low 2006). Dcm is the orphan cytosine methyltransferase found in *E. coli* and it has roles in controlling the expression of the RpoS sigma factor (Figure 1.20) and hence the global stress response and adaptation to stationary phase (Kahramanoglou et al. 2012), the lifecycle of bacteriophage lambda (Korba and Hays 1982), the transposition of Tn*3* (Yang et al. 1989), and the production of ribosomal proteins (Militello et al. 2012).

Unlike base substitution mutations that may become 'fixed' in the population by selection, base modifications involving methylation are reversed when the DNA is replicated and a hemimethylated state is restored temporarily. This provides a mechanism to reset the system and to test the environmental 'market' once again to assess the relative fitness of the methylated and hemimethylated states. New knowledge of bacterial epigenomes is being accumulated through the application of single-molecule, real-time (SMRT) sequencing (Beaulaurier et al. 2015, 2018, 2019). This methodology allows the different types of DNA methylation taking place in a single bacterium to be detected and analysed simultaneously. The findings include a deeper appreciation of the shear variety of methyltransferase systems, a feature that is being driven by their transmission through bacterial populations by horizontal gene transfer (HGT) (Blow et al. 2016; Kobayashi et al. 1999). Once established in a genome, a methyltransferase system can undergo evolution by mutation to alter its function and/or the timing and/or level of its expression, creating changes that drive further diversity in the bacterial populations that house these enzymes (Chen et al. 2017; Furuta et al. 2014; Krebes et al. 2014; Sater et al. 2015; Zhu et al. 2016).

Competition between a methyltransferase and a DNA-binding protein for the same site on DNA is a well-established regulatory mechanism that incorporates an element of stochasticity because the winner of the competition cannot be predicted. For example, the Dam methylase competes with SeqA to influence chromosome replication at the initiation step and, by affecting the efficiency of cohesion, the segregation of daughter chromosomes (Section 1.9). Dam also competes with the LRP protein to control Pap pilus production in a phase-variable way (Section 6.3) (Hernday et al. 2002; van der Woude et al. 1996) and it competes with the OxyR protein to influence the phase-variable expression of

antigen 43 (Agn43) (Section 6.10) (Waldron et al. 2002; Wallecha et al. 2002). Biasing of the competition outcome at the *pap* regulatory sequence has been reported: the 5′-GATC-3′ sites are presented to Dam in an unfavourable sequence context, giving an advantage to LRP in the competition to bind to the same sites (Peterson and Reich 2006). The detection by SMRT of unmethylated sites across numerous genomes is indicative of competition between methyltransferases and DNA binding proteins suggests that this regulatory mechanism is widespread (Beaulaurier et al. 2019). It is certainly very versatile because all that is required to establish a competition is for a DNA-binding protein to share part of its DNA recognition and binding site with that targeted by the methyltransferase. The broad influence of methyltransferases in the generation of variety in gene expression has led to the coining of a new collective noun: the 'phasevarion' or phase-variable regulon (Srikhanta et al. 2005, 2010). The members of the group share the properties of being expressed in a phase-variable manner and of being targets for the methyltransferase that governs the expression of the group.

Methyltransferase genes are themselves capable of being expressed in a phase-variable way, sometimes with multiple alternative outputs (Atack et al. 2018; Dybvig et al. 1998; Jen et al. 2014). This increases dramatically the degree of variety that can be achieved in gene expression between and among phasevarions.

6.27 Some Unifying Themes

It is easy to understand that an individual environmental stress is detected by a specific detector system in or on the bacterium, that this information is communicated to a regulatory protein and this in turn binds to copies of a particular nucleotide sequence that is located in the control regions of the genes that are responsible for making a response to the stress. It is also intuitive to appreciate that this system has an ability to reset itself to the unstimulated state once the response has been made. Perhaps it is a little more challenging to understand that stress response systems can overlap with one another and that large collectives of genes form networks of variable membership depending on the combination of stresses that the bacterium detects. This introduces an element of noise into the operation of the system that can make its performance difficult to predict. However, it is also entirely sensible that the bacterium should be 'wired' in this way because the organism may be faced with unpredictable combinations of stimuli by a complex environment.

In this chapter we have seen descriptions of linear regulatory systems of the type outlined in the previous paragraph. However, each has regulatory components that are shared with other collectives of genes, laying the basis for networking. This networking can become quite sophisticated. Historically, investigators anticipated that microbes were built this way for the very sound physiological reasons outlined above, but it has been only with the advent of techniques that allow gene expression to be monitored at the level of the whole genome that the picture has begun to come into focus. To appreciate this regulatory complexity, it will be necessary to review gene regulation in the context of genome structure (Chapter 8). First, the physiological regulatory circuits reviewed in the present chapter will be revisited in the context of bacterial pathogenesis (Chapter 7).

7

Gene Control: Global Regulation by H-NS

7.1 H-NS Is a Global Regulator

The nucleoid-associated protein (NAP) H-NS controls the expression of hundreds of genes in Gram-negative bacteria (Section 1.42). It has attracted attention in particular because of its role in silencing the transcription of virulence genes in enteric and other pathogens. Here the discussion of H-NS is expanded to encompass its contributions to pathogen evolution through its negative control of foreign gene expression, with a particular emphasis on the intensively investigated virulence regulons of *S*. Typhimurium, *S. flexneri* and *V. cholerae*.

7.2 H-NS and Foreign DNA

Salmonella enterica serovar Typhimurium has served as a model organism for studies of the invasive disease typhoid in mice for many years. This work has revealed the very many genes in the *S*. Typhimurium genome that contribute to the pathogenic behaviour of the bacterium, including two large gene clusters that encode distinct type 3 secretion systems and associated effector proteins called SPI1 and SPI2: 'SPI' stands for *Salmonella* Pathogenicity Island (Shea et al., 1996; Ginocchio et al., 1997; Groisman and Ochman, 1996; Zhou et al., 1991). *S*. Typhimurium uses SPI1 to invade the epithelial cells of the small intestine while SPI2 is used to allow *S*. Typhimurium to survive in the vacuole of the macrophage, an environment that would normally be lethal for an engulfed bacterium. Two landmark papers published in 2006 showed that genes in the SPI pathogenicity islands, together with other individual virulence genes and genes grouped in other clusters, islands, and islets, are silenced by the H-NS protein (Lucchini et al., 2006; Navarre et al., 2006). These H-NS targets shared the properties of having DNA with an A + T content that was higher than the average for the *Salmonella* genome and of being of foreign origin. In other words, they had been imported from an unknown, external source by horizontal transfer and integrated physically into the genome of *S*. Typhimurium.

Silencing by H-NS was proposed as a mechanism to prevent inappropriate expression of the foreign genes and to provide time for suitable regulatory mechanisms to evolve that would incorporate the new genes into the pre-existing regulatory circuits of the bacterium. Presumably, imported genes that did not present an A + T base profile that lent itself to these steps of regulatory integration created novel gene-bacterium combinations that

Structure and Function of the Bacterial Genome, First Edition. Charles J. Dorman.

were unsuccessful and were eliminated by natural selection. H-NS silencing of genes of foreign origin is found widely among bacterial species, including organisms without very close relatives of H-NS. For example, the Rok protein seems to provide an analogous function in *Bacillus subtilis* while Lsr2 acts in a related way in *Mycobacterium* spp. and in *Streptomyces* spp., with VicH providing H-NS-like activity in *Vibrio* spp. and MvaT providing corresponding functions in *Pseudomonas* spp. (Ding et al., 2015; Gordon et al., 2010; Smits and Grossman, 2010; Stonehouse et al., 2011). This is not an exhaustive list (Perez-Rueda and Ibarra, 2015).

The ubiquitous nature of NAP-mediated transcription silencing creates a requirement for mechanisms that overcome silencing in ways that are beneficial to the bacterium (Dorman, 2007; Stoebel et al., 2008b). This issue was reviewed in Section 1.42 and among its key features is the need to link the relief of transcription silencing to environmental signal detection. In the case of human pathogens, a temperature of 37 °C has been identified as an important signal and many H-NS-silenced genes become transcriptionally active at this temperature. However, the thermal signal rarely operates in isolation, with several other environmental soundings being taken before commitment to full expression is made. In the case of *S*. Typhimurium in the mammalian host, pH, osmolarity, and a requirement for magnesium ions are among the signals that influence virulence gene expression (Fass and Groisman, 2009).

7.3 H-NS and Xenogenic Silencing: Three Case Studies

The Gram-negative pathogens *V. cholerae, S. flexneri,* and *S. enterica* serovar Typhimurium cause diarrheal disease in mammals using virulence factors that are encoded by horizontally acquired genes. The H-NS protein silences transcription of these genes and their expression involves a variety of anti-silencing mechanisms (Stoebel et al., 2008b). These mechanisms respond to environmental cues that are relevant to the infection strategies employed by each of these pathogens. The versatility of H-NS for the global control of bacterial transcription is nicely illustrated by a survey of these three examples.

7.4 The H-NS Virulence Regulon in *Vibrio cholerae*

The H-NS (VicH) regulon in *V. cholerae* is extensive (Kazi et al., 2016; Nye et al., 2000; Nye and Taylor, 2003; Stonehouse et al., 2011), extending across two chromosomes and including the cholera toxin operon that is located within the genome of a filamentous bacteriophage, CTXϕ (Waldor and Mekalanos, 1996). *V. cholerae* does not encode Hha-like proteins and, unlike H-NS, VicH does not interact with Hha, showing that not all aspects of H-NS biology are recapitulated in *V. cholerae* (García et al., 2009). The *V. cholerae* example is instructive because it combines a number of horizontally acquired genetic elements whose genes influence different aspects of the bacterium's lifecycle, both in the human host and in the external environment. These genes are under very complex control and the H-NS protein is a negative influence in the expression of most of them (Figure 7.1).

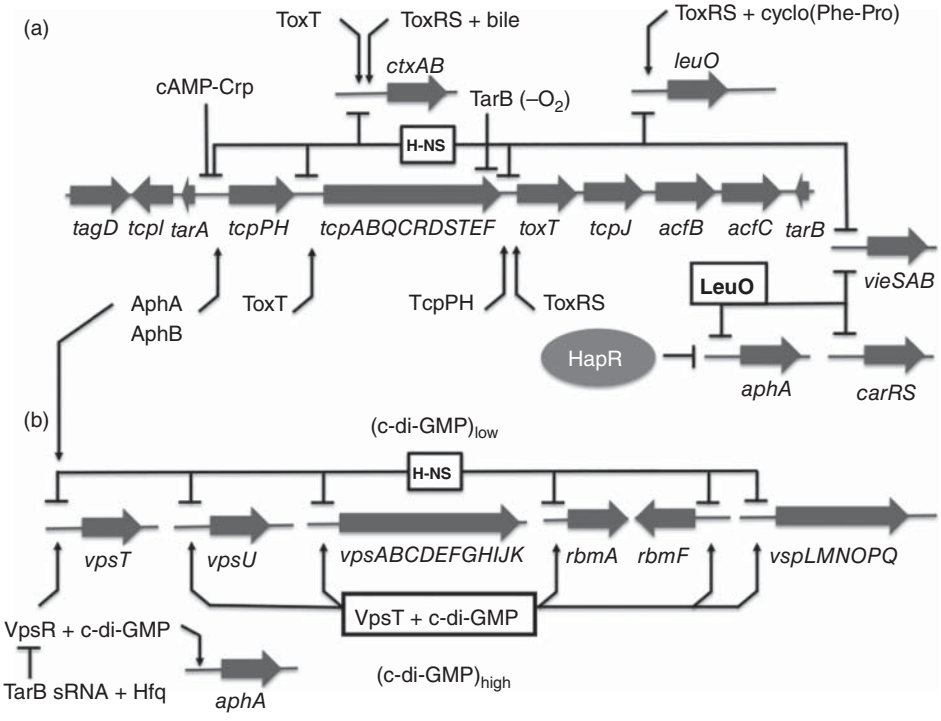

Figure 7.1 The H-NS regulon of *V. cholerae*. (a) The genes involved in the colonisation of the host brush border and the expression of cholera toxin and of the toxin co-regulated pilus (TCP) are silenced by the H-NS protein. Many of these genes are located in the VPI1 pathogenicity island where H-NS-mediated silencing is overcome by ToxRS (*toxT*), AphAB (*tcpPH*), TcpPH (*toxT*), and ToxT (*toxT, tcpABQCRDSTEF* operon). ToxT is also a positive regulator of the cholera toxin operon, *ctxAB*. The CTXϕ filamentous phage is inserted at the *dif* site of Chromosome I, although classical strains of *V. cholerae* have a second copy at the *dif* site of Chromosome II. As in *E. coli* and *Salmonella,* H-NS silences the *leuO* gene. In *V. cholerae,* this is countered by ToxRS with LeuO then acting as a repressor of *aphA* transcription, as well as *vieSAB* (biofilm and motility) and *carRS* (lipid A modification and cationic antimicrobial peptide resistance). The cAMP-CRP complex and the TarB sRNA also exert negative regulatory influences within VPI1. (b) H-NS is also a global repressor of transcription of *rmb vps* genes involved in polysaccharide biosynthesis. Silencing by H-NS occurs when c-di-GMP levels are low; at high c-di-GMP concentrations, anti-silencing by the VpsT upregulates transcription. VpsR, in association with high c-di-GMP, controls the *vpsT* gene positively. VpsR is also a positive regulator of the *aphA* gene and the AphA protein controls *vpsT* transcription positively, providing a regulatory link with events in VPI1. An additional link involves negative control of VpsR production by the TarB sRNA, acting through an Hfq-mediated RNA-RNA interaction. For further information, see Dorman and Dorman (2018).

7.5 HGT in *V. cholerae*: The CTXϕ Phage and the VPI1 Island

The CTXϕ phage uses the *dif* sequence on chromosome I (and sometimes the corresponding sequence on chromosome II) as its attachment site, exploiting the XerCD site-specific recombination system to catalyse integration (Das et al., 2013; Huber and Waldor, 2002; McLeod and Waldor, 2004). This is the same *dif* site that is used for XerCD-dependent

resolution of chromosome dimers prior to segregation during the cell cycle. The phage receptor is the toxin co-regulated pilus (TCP), a type IV pilus that is expressed on the surface of *V. cholerae* under conditions that favour the production of cholera toxin, CT (Figure 7.2) (Waldor and Mekalanos, 1996). In addition to providing a docking point on the cell surface for the phage, TCP also encourages the aggregation of the bacteria in the lumen of the

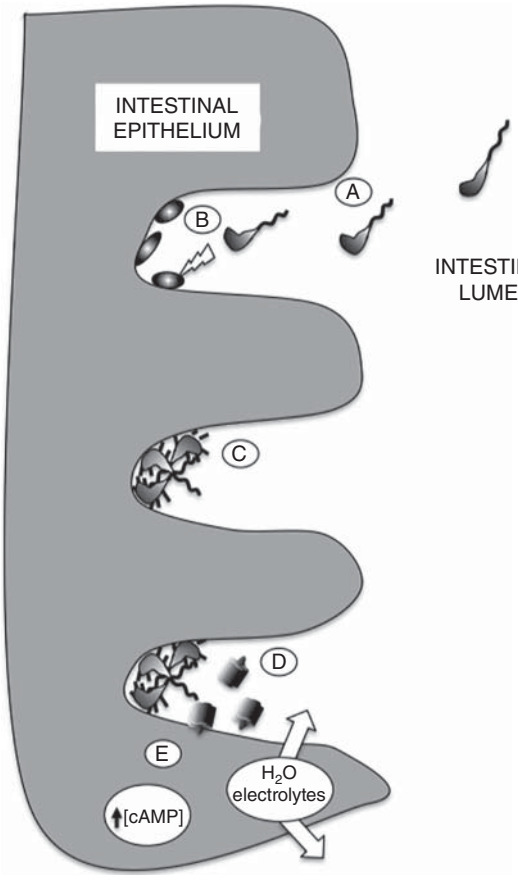

Figure 7.2 The infection of the small intestinal epithelium by *Vibrio cholerae* involves an intoxication of the epithelial cells by cholera toxin following colonisation of the brush border by *V. cholerae* bacteria. Planktonic bacteria (a) receive environmental signals (lightning flash) from the epithelial surface and other *V. cholerae* cells that initiate expression of a T6SS that eliminates other bacteria on the epithelial surface, clearing a zone (b) where *V. cholerae* can attach and adhere. (c) The bacteria now express the Toxin Co-regulated Pili (TCP) that attaches the microbes to one another and to the epithelial surface. TCP also serves as the receptor for the CTXϕ filamentous phage that carries the cholera toxin operon, *ctxAB*. (d) The attached *V. cholerae* bacteria have formed a microcolony and are expressing cholera toxin. The toxin, with its A_1B_5 structure, adheres to GM1 gangliosides on the surface of epithelial cells and the active moiety of the A subunit enters the cytoplasm where it upregulates the human adenylate cyclase by ADP-ribosylation. (e) A surge in cAMP concentrations is caused that dysregulates epithelium cell metabolism, resulting in the release of water and electrolytes. The patient will suffer the severe watery diarrhoea that characterises cholera. If untreated, this has deleterious consequences for the human host and can lead to death. For further information, see Dorman and Dorman (2018).

small intestine, allowing it to play a dual role in the development of the infection. The pilus genes are located in *Vibrio* Pathogenicity Island 1, VPI1, which is also the location of the *toxT* gene (Karaolis et al., 1998). ToxT is an AraC-like DNA-binding protein and its regulon includes *ctxAB,* the cholera toxin operon (DiRita et al., 1991; Higgins et al., 1992). The ToxT protein functions both as a conventional transcription factor that recruits RNA polymerase to its target promoters, and as an anti-repressor that overcomes the transcription silencing that is imposed by H-NS at the same promoters (Yu and DiRita, 2002).

7.6 The ToxRS, ToxT, TcpPH Regulatory Network

The *toxT* gene lies within a regulatory cascade (Figure 7.1). It is repressed by H-NS and activated by the winged helix-turn-helix (wHTH) proteins ToxR and TcpP (Higgins and DiRita, 1994; Krukonis et al., 2000). The *toxR* gene lies in the ancestral component of the *V. cholerae* genome and is constitutively expressed. ToxR forms dimers with the ToxS protein and this relationship governs the half-life of ToxR (Almagro-Moreno et al., 2015). The ToxRS complex is located in the cytoplasmic membrane with the wHTH motif in the N-terminal domain of ToxR in the cytoplasm (Fengler et al., 2012; Midgett et al., 2017; Ottemann and Mekalanos, 1996). ToxR and TcpP (encoded by the *tcpPH* operon within VPI1) interact physically through the wings of their wHTH motifs and cooperate in *toxT* activation in response to a multitude of environmental signals (Goss et al., 2010; Haas et al., 2015; Krukonis and DiRita, 2003). TcpP is also associated with the cytoplasmic membrane and has a relationship with TcpH that is reminiscent of the ToxRS partnership (Teoh et al., 2015). Further regulatory inputs are made at the *tcpPH* promoter: the AphA and AphB proteins regulate this operon positively while transcription of *tcpPH* is repressed by both H-NS and cAMP-CRP (Kovacikova and Skorupski, 2001). The latter represents a role for small molecule signalling in the operation of the virulence gene regulon of *V. cholerae*. Transcription of *aphA* is controlled negatively by the TetR-like HapR DNA-binding protein. HapR is the master regulator of quorum sensing and biofilm formation in *V. cholerae*: it promotes the former and represses the latter (Figure 7.3) (Ball et al., 2017). By acting through *aphA* repression, HapR also downregulates the virulence regulon (Zhu et al., 2002).

7.7 Control by VpsR, VpsT, and HapR

Cyclic-di-GMP concentrations play an important role in controlling the physiology of *V. cholerae* (Figure 7.1). When these are low, the H-NS protein represses the transcription of genes involved in biofilm formation; when c-di-GMP concentrations rise, a regulatory cascade consisting of the VpsR and VpsT DNA-binding proteins overcomes this transcription silencing (Ayala et al., 2015a,b; Dorman, 2015; Zamorano-Sánchez et al., 2015). The VpsT protein also inhibits expression of the RpoS sigma factor, which is consistent with adaptation to rapid growth and rising c-di-GMP levels (Wang, H., et al., 2014). The *hapA*-encoded HA protease is positively regulated by the HapR protein and this enzyme plays an important role in the escape of individual bacteria from biofilm. Biofilm escape is an important feature

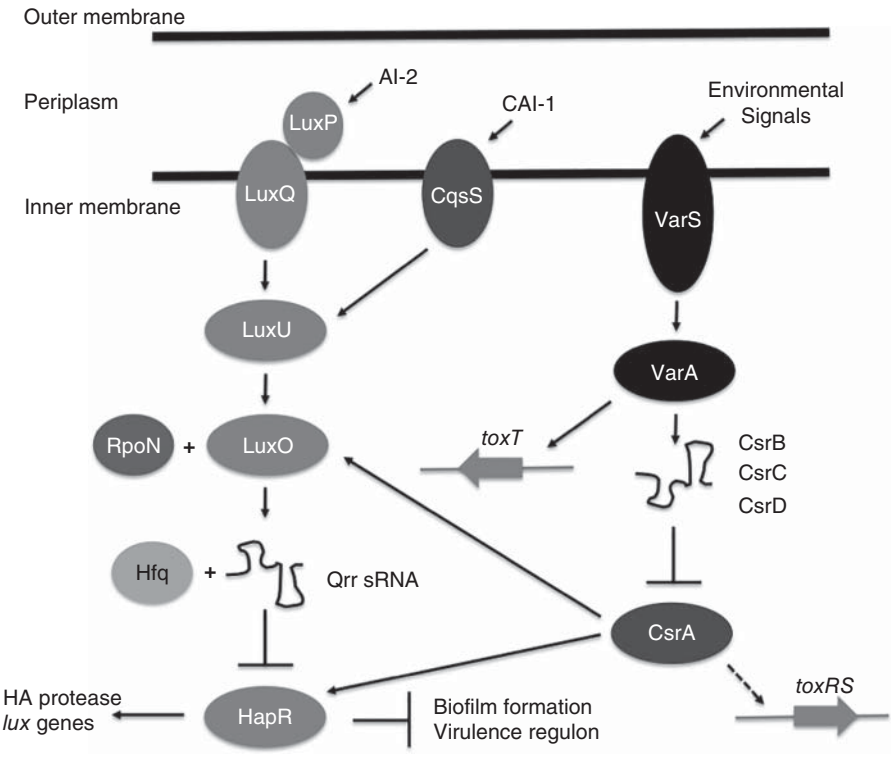

Figure 7.3 The control of virulence, motility, and biofilm expression in *V. cholerae* by quorum sensing. A summary of the principal signalling pathways is shown, extending from the external environment, through the cell envelope, to the genome. Environmental signals and autoinducer (AI) molecules can freely enter the periplasm where they are detected by sensor proteins in the cytoplasmic membrane. These in turn communicate with regulatory proteins that control the production of sRNA molecules that determine the phenotype of the bacterium. The LuxP, LuxQ, LuxU, LuxO cascade, working in association with the RpoN sigma factor, stimulates the production of Qrr sRNA in response to AI-2. Working with the Hfq RNA chaperone, Qrr blocks the production of the HapR regulatory protein that is required for the production of the HA protease. This protease is needed for escape from biofilm; if HapR and HA are absent, the bacteria will remain in biofilm rather than be shed from the host. In bioluminescent species of *Vibrio,* this pathway also controls *lux* gene expression. The CAI autoinducer also influences this process via a junction between the CqsS sensor/signalling protein and LuxU. The VarS-VarA signalling cascade has been reported to stimulate virulence gene expression via the ToxT master regulator (see Figure 7.1) and the production of the CsrB, CsrC, and CsrD sRNA molecules. These in turn are inhibitory to CsrA production. When the CsrA protein is produced, it influences the expression of the HapR transcription factor both directly and indirectly (via LuxO). In the presence of the amino acids arginine, asparagine, glutamine, and serine, CsrA may stimulate the expression of the *toxRS* operon and hence the expression of the virulence regulon. For further information, see Dorman and Dorman (2018).

of bacterial shedding by the infected host because it provides microbes to participate in further rounds of infection. HapR also promotes the expression of sigma factor RpoS, which in turn positively controls *hapA* transcription. This process is reinforced by the enhancement of HapR expression by rising cell density.

7.8 Quorum Sensing and Cholera

The infectious dose for cholera is high: 10^3–10^8 bacterial cells. This is in sharp contrast to other diarrhoeal diseases such as dysentery where the infectious dose is as low as 10 cells (Schmid-Hempel and Frank, 2007). It has been hypothesised that the lack of an XAR system for resisting severe acid stress, a system that is possessed by low-infectious-dose enteric pathogens, means that *V. cholerae* must accept very high casualties as it traverses the extremely low pH environment of the stomach, and that it therefore needs to enter the host in very high numbers to stand a chance of establishing itself in the gut (Lund et al., 2014). The pathogenic behaviour of *V. cholerae* is characterised by population-wide signalling and collective action that is coordinated by quorum sensing. The CAI-1 quorum-sensing molecule is detected by the CqsS cytoplasmic membrane protein that communicates, in turn, with the cytoplasmic LuxU protein (Figure 7.3). LuxU also receives signals originating with the AI-2 quorum-sensing molecule, acting via the periplasmic LuxP and the cytoplasmic-membrane-located LuxQ protein (Figure 7.3). LuxU communicates with the LuxO transcription factor that regulates the expression of sRNA called Qrrs from RpoN-dependent transcription units (Lenz et al., 2005). These Qrr sRNAs act in association with the Hfq RNA chaperone to downregulate HapR, thus connecting quorum sensing, biofilm formation, biofilm escape, *lux* gene expression, and the operation of the virulence regulon (Lenz and Bassler, 2007; Lenz et al., 2005; Rutherford et al., 2011) (Figure 7.3). HapR is upregulated by the CsrA global regulatory protein, which is responsive to the VarSA two-component regulatory system via the CsrB, CsrC, and CsrD sRNAs (Figure 7.3) (Tsou et al., 2011). CsrA has also been reported to have a positive influence on the expression of LuxO and ToxR, with VarSA enhancing the expression of ToxT, illustrating the high degree of interconnectedness of the *V. cholerae* virulence system (Figures 7.1 and 7.3) (Dorman and Dorman, 2018; Jang et al., 2011).

7.9 Chitin and HGT

In common with other motile bacteria, H-NS in *V. cholerae* has a positive influence on motility and chemotaxis (Wang et al., 2015). It represses expression of the type 6 secretion system that the bacterium uses to kill competitor organisms and downregulates the genes involved in chitin utilisation. Chitin plays a central role in the life cycle of *V. cholerae* and influences its ability to import foreign DNA through transformation (Figure 7.4) (Metzger and Blokesch, 2016). The bacterium colonises chitinous surfaces in its marine environment and chitin is known to promote horizontal gene transfer, and hence evolution, in this bacterium (Blokesch and Schoolnik, 2007; Le Roux and Blokesch, 2018). Once again, an important role is seen for the HapR regulatory protein. Here, HapR activates the *qstR* gene encoding the QstR regulatory protein that is required for expression of the T6SS genes and the genes for competence. The pilus structure that is used for DNA uptake is regulated positively by cAMP-CRP and a protein called TfoX (Wu et al., 2015). TfoX expression is controlled by the sRNA TfoR, in association with the Hfq chaperone. Expression of the *tfoX* and *tfoR* genes is controlled positively in response to chitin, in the case of *tfoX* by ChiS and in the case of *tfoR* by TfoS (Figure 6.4) (Dalia et al., 2014; Yamamoto et al., 2011). When chitin is absent,

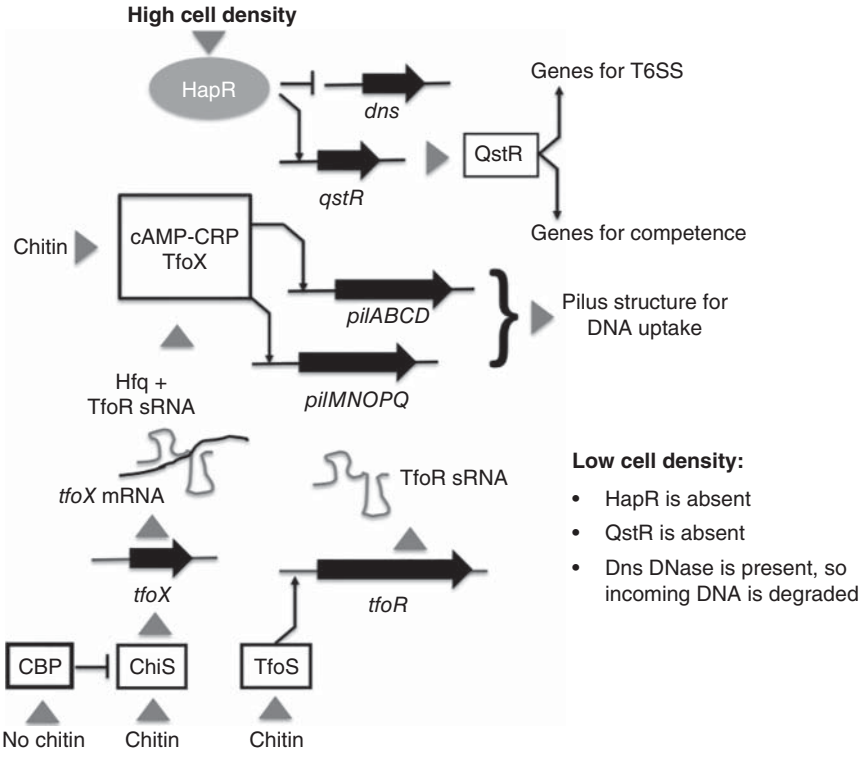

Figure 7.4 Chitin influences DNA uptake by *V. cholera*. The *dns* gene encodes the Dns DNase that degrades incoming foreign DNA. The HapR regulatory protein blocks expression of *dns* at high bacterial cell densities. Under the same conditions, HapR upregulates the expression of the *qstR* gene, encoding an sRNA (QstR) that switches on the genes for the expression of competence and the production of a T6SS for the destruction of commensal bacteria that might compete with *V. cholerae* for colonisation sites on the epithelial surface in the small intestine. This combination of circumstances allows the bacterium to engage in HGT. DNA uptake depends on the pilus structure that is encoded by the *pilABCD* and *pilMNOPQ* operons. These transcription units are under the positive control of the cAMP-CRP complex and the TfoX regulator, both of which are controlled positively in response to chitin. The TfoR sRNA positively regulates the translation of TfoX mRNA in combination with the Hfq RNA chaperone protein. The transcription of the *tfoX* gene is positively controlled by chitin via the ChiS regulator; however, in the absence of chitin the CBP protein inhibits this circuit. Chitin and TfoS activate the transcription of the *tfoR* gene, whose sRNA product (TfoR) stimulates the production of TfoX, and hence the DNA-uptake enabling pilus. When *V. cholerae* cell density declines, HapR and QstR are absent, allowing the Dns DNase to accumulate and incoming foreign DNA to be degraded. The result is downregulation of HGT. For further information, see Dorman and Dorman (2018).

the Cbp chitin-binding protein downregulates activity of ChiS (Li and Roseman, 2004). The cell division protein SlmA controls *chb* transcription (Klancher et al., 2017). DNA uptake is dependent on high cell density: when density is low, HapR and QstR are absent and the HapR-repressed *dns* gene is expressed. The product of *dns* is the Dns DNase, which degrades foreign DNA extracellularly or in the periplasm (Seitz and Blokesch, 2014).

7.10 The H-NS Virulence Regulon in *Shigella flexneri*

S. flexneri is the aetiological agent of human dysentery, a disease that is characterised by inflammation of the large intestine accompanied by bloody diarrhoea (Figure 7.5) (Ashida et al., 2015). *S. flexneri* is an aggressive pathogen with an infectious dose as low as 10 bacterial cells (Schmid-Hempel and Frank, 2007). In contrast to *V. cholerae*, *S. flexneri* maintains its principal virulence genes on a large, single-copy plasmid (Figure 7.6) (Buchrieser et al., 2000; Dorman, 2009; Lan et al., 2001). These genes are silenced at the level of transcription by the H-NS protein (Beloin and Dorman, 2003). The most important genes are clustered within a pathogenicity island on the plasmid, within a zone known as the Entry Region. They encode a type 3 secretion system and associated effector proteins that allow the bacterium to invade the epithelial layer of the lower gut (Buchrieser et al., 2000; Dorman, 2009).

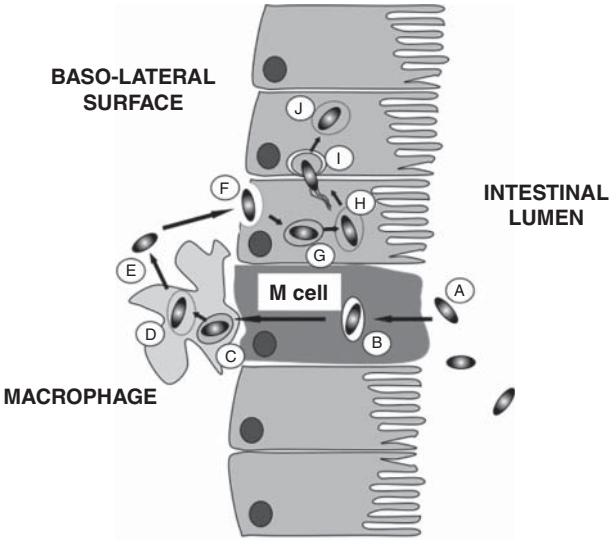

Figure 7.5 Invasion of the human large intestinal epithelium by *Shigella flexneri*. The bacterium enters the epithelial layer using its T3SS to invade the antigen-sampling M cells (a). They transit the cytoplasm and then exit the M cell via the basolateral surface (b). Macrophage engulf the emerging *S. flexneri* cells (c) but the microbes lyse the macrophage vacuole and trigger inflammatory cell death of this host defense cell (d). The bacteria are now free (e) and can invade the cells of the epithelial layer through its basolateral surface (f), residing temporarily in a vacuole in the host cytoplasm (g). Once again, the *S. flexneri* bacteria lyse the membrane of their vacuolar container (h) and cross the cytoplasm of the host cell by polymerising actin from the host cytoskeleton to form the comet tails that propel the bacterium through the cell membrane into the cytoplasm of the adjoining cell (i). They can then lyse the double membrane of this vacuole to escape to the cytoplasm where the process can be repeated. This cell-by-cell destruction of the epithelial layer, and the associated inflammatory response, contributes to the bloody diarrhoea that is characteristic of dysentery. For further information, see Dorman and Dorman (2018).

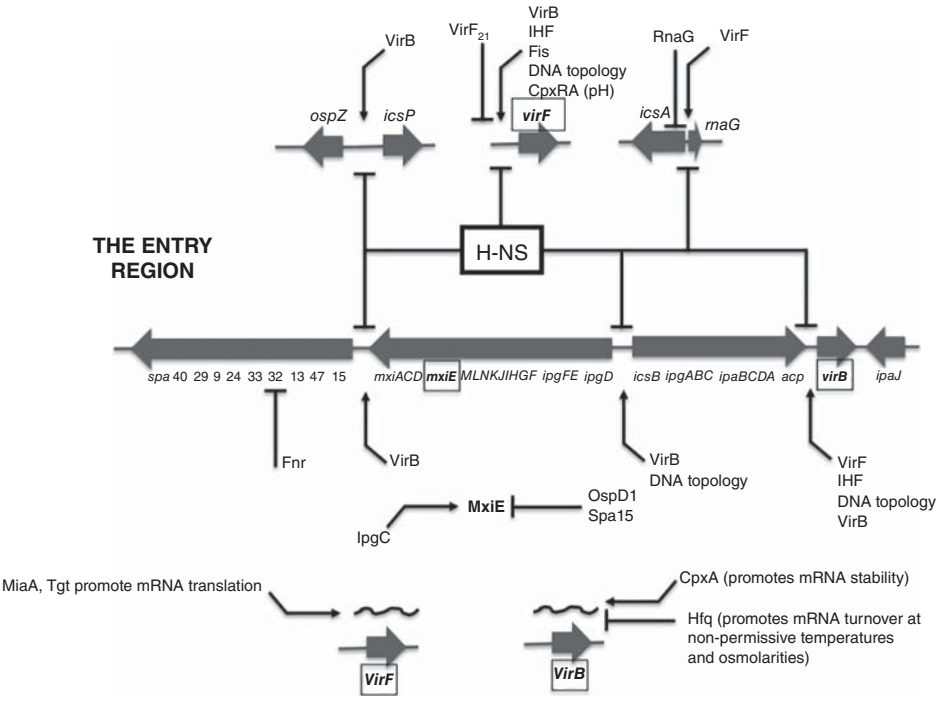

Figure 7.6 The H-NS regulon of *Shigella flexneri*. The Entry Region of the large virulence plasmid is shown at the centre of the figure. This is a pathogenicity island harbouring large operons that encode a T3SS and the associated effector proteins. Other genes that encode chaperones and other components of the T3SS machinery are found elsewhere on the plasmid and on the chromosome (not shown). The Entry Region contains two important regulatory genes: *virB* and *mxiE*. All of the transcription promoters in the Entry Region are targets for silencing by the H-NS protein, and so are other virulence gene promoters located in other places in the genome. The VirF AraC-like DNA binding protein activates *virB* transcription, initiating a regulatory cascade in which the ParB-like VirB protein acts as an anti-repressor at many of the H-NS-silenced promoters. VirB feeds back positively onto the transcription of both *virB* and *virF*. The VirF protein also regulates directly the *icsA* and *rnaG* genes, arranged divergently (top right). $VirF_{21}$, a truncated VirF that is expressed from a promoter internal to *virF,* interferes with full-length VirF production. The RnaG sRNA also controls *icsA* gene expression transcriptionally while the *virF* and the *virB* genes are subject to post-transcriptional control by a variety of agents. MxiE is a second AraC-like transcription factor and is a subject of inhibition by protein–protein interaction. In addition to H-NS, other regulatory factors encoded by genes on the chromosome contribute to the control of the virulence system: the CpxRA pH-responsive 2-component regulator, the NAPs FIS and IHF, and the anaerobic regulator FNR. FNR is thought to monitor oxygen levels, repressing the transcription of the *spa32* and *spa33* genes under anaerobic conditions. The rising oxygen levels encountered at the surface of the epithelium relieve the FNR-mediated transcriptional repression. The *virB* promoter is sensitive to changes in DNA supercoiling. For further information, see Dorman and Dorman (2018).

7.11 *Shigella* Infection

Crossing the epithelial layer via M cells brings the microbes into contact with macrophage (Figure 7.5) (Perdomo et al., 1994; Wassef et al., 1989). The bacteria kill these by inducing inflammatory cell death before moving on to invade the epithelium via its basolateral surface (High et al., 1992; Zychlinsky et al., 1992) (Figure 7.5). Once inside the host cell, *Shigella* escapes the vacuole that was created as it passed the cell membrane. It then uses the IcsA protein to recruit host actin to one pole of the bacterial cell (Mauricio et al., 2016), which is polymerised to create the characteristic comet-like tails that propel the bacterium through the cell membranes that separate the infected cell from its neighbour (Fukumatsu et al., 2012; Suzuki et al., 1996a, 1998). Once in the neighbouring cell, the bacterium escapes the double membrane vacuole that was created as it passed through the envelopes of the two adjacent cells and repeats the process of actin-propelled cell penetration (Welch and Way, 2013). In this way, *Shigella* moves along the epithelial layer, damaging host cells and inducing an inflammatory response (Ashida et al., 2015). Presumably, this intracellular lifestyle provides the pathogen with access to resources free from competition from other bacteria and protects the invader from host defences. It differs markedly from cholera where the bacterium remains in the upper gut lumen and uses a powerful protein toxin to dysregulate the physiology of host cells. However, both pathogens share a reliance of virulence genes that are part of horizontally acquired genetic elements that are silenced at the level of transcription by H-NS (Dorman and Dorman, 2018).

The master regulator of virulence gene expression in *S. flexneri* is VirF, an AraC-like protein whose gene lies outside the Entry Region of the large plasmid (Figure 7.6) (Adler et al., 1989; Porter and Dorman, 2002). The *virF* promoter is silenced by H-NS and becomes activated when the bacterium is exposed to environmental signals that are characteristic of the large intestine, with a temperature of 37 °C being of primary importance (Di Martino et al., 2016a; Porter and Dorman, 1994). Other signals have significant effects, but in the absence of the thermal signal the regulatory cascade remains inactive (Porter and Dorman, 1997b). De-repression of the *virF* promoter involves a remodelling of its DNA, with reinforcement of the activating signal coming from FIS, IHF, and DNA topology (Falconi et al., 1998, 2001; Porter and Dorman, 1997a; Prosseda et al., 2004). Once VirF is expressed, few virulence genes are regulated by it directly; the VirF-dependent intermediate regulator VirB controls most of them positively (Adler et al., 1989; Taniya et al., 2003). In *Shigella sonnei,* VirB is called InvE, but it is the same protein with the same function (Taniya et al., 2003).

VirF is required, but is not sufficient, for the activation of *virB* transcription (Tobe et al., 1993). An adjustment to the topology of the DNA in the *virB* regulatory region is needed in addition to the presence of VirF; IHF is also involved in the activation process (Porter and Dorman, 1997a; Tobe et al., 1995). Once the VirB protein is present in the cell, it will activate its target promoters in a concentration-dependent manner. This can be shown by the ectopic expression of VirB from an inducible promoter in bacteria growing at a temperature that is normally non-permissive for transcription of the virulence regulon (Beloin and Dorman, 2003). The VirB protein operates as an anti-repressor of H-NS-silenced promoters; it does not recruit RNA polymerase or assist it in forming open transcription complexes (Turner and Dorman, 2007).

7.12 The VirF AraC-Like Transcription Factor

Expression of the VirF protein provides a useful example of gene regulation in depth, with control being exerted transcriptionally and post-transcriptionally. Making transcription of *virF* sensitive to a range of environmental signals ensures that a commitment to the expression of all of the genes under direct and indirect control of VirF is only likely to occur when *S. flexneri* has reached a niche where it will benefit from doing so. The *virF* gene is capable of expressing two proteins: $VirF_{21}$ and $VirF_{30}$, with the latter being the active, full-length transcription activator (Di Martino et al., 2016b). The $VirF_{21}$ protein lacks the N-terminal domain of $VirF_{30}$ but contains the DNA-binding domain, and it is a negative autoregulator of *virF* expression (Di Martino et al., 2016b). The truncated $VirF_{21}$ protein's mRNA is transcribed as a leaderless mRNA (IImRNA) expressed from a separate promoter located internally to the *virF* gene. The purpose of $VirF_{21}$ may be to restrict the expression of full-length VirF to environments where the bacterium will benefit from the expression of the full regulon of genes that are directly and indirectly controlled by VirF (Di Martino et al., 2016b). VirF is controlled post-transcriptionally by the *miaA* and *tgt* genes whose products modify tRNA: mutants deficient in MiaA or Tgt translate *virF* mRNA poorly, with negative consequences for the expression of the virulence cascade (Durand and Björk, 2003; Durand et al., 2000). Post-transcriptional control is in operation at other points in the regulatory cascade. For example, the stability of *virB* mRNA (called *invE* in *S. sonnei*) is affected by temperature and osmolarity (Mitobe et al., 2008, 2009), by CpxA-dependent RNA processing (Mitobe et al., 2005), by the Hfq RNA chaperone (Mitobe et al., 2008, 2009), and by interaction with the cell morphology protein RodZ (Mitobe et al., 2011). An sRNA known as RnaG regulates the expression of the actin polymerisation gene *icsA,* but it does this at the level of *icsA* transcription (Giangrossi et al., 2010). The *icsA* gene is also known as *virG* and, like the adjacent *rnaG* gene, it is silenced by H-NS and activated directly by VirF (Tran et al., 2011).

7.13 VirB: A Recruit from a Plasmid-Partitioning System

The DNA sequence to which VirB binds resembles the *parS* sequences that are the binding sites of ParB-like plasmid-partitioning proteins (Taniya et al., 2003; Turner and Dorman, 2007). It seems highly likely that VirB was once a plasmid-partitioning protein (Watanabe et al., 1990) and that it became redundant in that role during the evolution of the *Shigella* large virulence plasmid (Buchrieser et al., 2000; Taniya et al., 2003; Turner and Dorman, 2007). This plasmid is a mosaic composed of at least four other plasmids and it has two active partitioning systems in addition to the hypothetically vestigial VirB one (Buchrieser et al., 2000; Dorman, 2009; Sergueev et al. 2005). VirB binds to its *parS*-like target and then polymerises along the DNA while simultaneously wrapping the DNA. These actions seem to be incompatible with DNA–protein–DNA bridging by H-NS over the same DNA segment and result in a loss of H-NS-mediated silencing of genes in the vicinity of the VirB-binding site (Turner and Dorman, 2007). VirB can act over long distances, as has been shown at the divergently transcribed *ospZ* and *icsP* genes on the virulence plasmid (Weatherspoon-Griffin et al., 2018).

7.14 The *Shigella* Virulence Plasmid

Why does *S. flexneri* maintain its key virulence genes on a plasmid? The modern virulence plasmids in *Shigella* and enteroinvasive *Escherichia coli* are not self-transmissible via conjugation (they have the vestigial remains of an F-like transmission system that is now defunct) so this is not a mechanism for horizontal dissemination of virulence genes in the way that *V. cholerae* can disseminate the cholera toxin operon by phage-mediated transduction (Buchrieser et al., 2000; Dorman, 2009; Dorman and Dorman, 2018; Makino et al. 1988; Sansonetti et al. 1982). The expression of the virulence regulon seems to correlate with the structural and genetic instability of the plasmid (Schuch and Maurelli, 1997). Subjecting *S. flexneri* to conjugation, transduction, or transformation, presumably with concomitant induction of the SOS response, also results in damage to the virulence plasmid and loss of virulence gene expression due to genetic rearrangements and other mutations (Porter and Dorman, 1997c). The efficiency of the plasmid segregation systems used by pINV may prove to be advantageous for the stable carriage of the plasmid and its vertical transmission. *S. flexneri* relies on three toxin/antitoxin post-segregational killing systems (*ccdA/B, mpvA/T,* and *gmvA/T*) to eliminate plasmid-free segregants, helping to maintain its representation in the *S. flexneri* population (Pilla and Tang, 2018). *S. flexneri* growing at 37 °C loses the plasmid at rates of up to 2–3% per generation, so the metabolic burden of expressing the toxin/antitoxin systems may be offset by ensuring plasmid maintenance in the face of such a high rate of natural loss (Sayeed et al., 2005). Another strategy for stable carriage of a plasmid that ensures high-fidelity vertical transmission is integration into the chromosome. This strategy was discussed when Hfr strains were described in Section 1.1. In *S. flexneri,* the pINV plasmid can integrate at different chromosomal sites by homologous and reversible recombination between copies of insertion sequences, by direct analogy with F plasmid integration in Hfr strains (Pilla et al., 2017). The virulence genes on the integrated plasmid are transcriptionally silent. The *S. flexneri* pINV and larger pINV plasmid of enteroinvasive *E. coli* can integrate reversibly into the chromosomes of their respective hosts at the *metB* locus, converting the bacteria to methionine auxotrophs (Zagaglia et al., 1991). The virulence genes on the integrated plasmids become silenced but are reactivated once the plasmids excise precisely from the chromosome. In the case of enteroinvasive *E. coli,* the silencing of the virulence genes on the integrated plasmid was due to H-NS-mediated repression of *virB* expression (Colonna et al., 1995). This may reflect the imposition of a transcription-silencing complex that is associated with the site of integration. Given the destabilising influence of virulence gene expression on the stability of the plasmid, its reversible integration into the chromosome and associated virulence gene silencing/reactivation seems to represent a sound strategy for stable vertical transmission of the virulence regulon.

7.15 The *Salmonella* H-NS Virulence Gene Regulon

The original studies of genome-wide H-NS binding patterns using chromatin immunoprecipitation were performed in *S. enterica* serovar Typhimurium (Lucchini et al., 2006; Navarre et al., 2006). *Salmonella* and *E. coli* separated from their common ancestor about

a million years ago, whereas *Shigella* and *E. coli* are almost the same species (Groisman and Ochman, 1997). *Salmonella* is a pathogen that maintains most of its virulence genes on the chromosome but has some important ones on a large virulence plasmid. It is a host-adapted pathogen with serovars that are specialists in their relationships with specific hosts (Branchu et al., 2018). For example, serovar Typhimurium (literally 'mouse typhoid') is a murine specialist causing a potentially fatal disease in the mouse but usually no more than mild gastroenteritis in humans (Alikhan et al., 2018). Like *E. coli, Salmonella* has played a central role in the development of bacterial genetics (see Section 1.1) and is continuing to be a useful experimental tool in the era of whole genome studies. Its H-NS regulon is very valuable in the making of comparisons with its counterparts in *E. coli/Shigella* and *V. cholerae*.

7.16 *Salmonella*'s Pathogenicity Islands (SPI)

The H-NS regulon of *S*. Typhimurium includes the (at least) 17 horizontally acquired genetic elements that confer pathogenic traits and these have attracted considerable attention from those interested in understanding virulence gene regulatory mechanisms (Figure 7.7) (Ilyas et al., 2017). *S*. Typhimurium uses a T6SS encoded by SPI6 to kill competing bacteria in the lumen of the host gut (Sana et al., 2016). The SPI6 genes are repressed by H-NS so overcoming this transcription silencing is an important early step in the process of establishing an infection (Brunet et al., 2015). As it establishes itself in the gut lumen, *S*. Typhimurium seems to deliberately induce inflammation. It does this by using proteins that are secreted through the SPI1-encoded T3SS and the resulting inflammation releases host resources into the lumen that allow the bacteria to thrive metabolically (Santos et al., 2009). Attempts by epithelial cells lining the gut to kill *Salmonella* using defensin peptides while the bacteria are still in the lumen are thwarted by modifications to the bacterial cell surface carried out by genes under the control of the PhoPQ and PmrAB sensor-regulator systems (Bevins and Salzman, 2011). At the epithelial surface, binding to host fibronectin is promoted by genes in SPI3 while SPI4 helps to enable entry of the bacteria into host cells (Dorsey et al., 2005; Gerlach et al., 2007). Entry itself involves a bacterium-directed restructuring of the host cytoskeleton by effector proteins that are translocated through the SPI1-encoded T3SS (Que et al., 2013). Appropriate activation of the virulence genes centres around some key AraC-like transcription factors (HilA, HilC, and HilD) that are encoded by SPI1 and that operate in associated with NAPs such as IHF, HU, and FIS (Kelly et al., 2004; Mangan et al., 2006, 2011; Schechter et al., 2003) in processes that include overcoming H-NS-mediated transcription silencing (Figure 7.8) (Ali et al., 2012; Dorman, 2007; Stoebel et al., 2008b). There is also a need to coordinate gene expression within SPI1 with the expression of genes at other locations (SPI2, SPI5, SPI14, the SpoEϕ prophage etc.) (Bustamante et al., 2008; Cameron and Dorman, 2012; Jiang et al., 2017; Martínez et al., 2014). The result is a sophisticated and complicated regulatory network that operates both transcriptionally and post-transcriptionally (Colgan et al., 2016; Kröger et al., 2012, 2013). Once *Salmonella* is internalised in a vacuole in the epithelial cell, it can cover its tracks by restoring the epithelial surface to a pre-ruffled configuration and also diminish the level of inflammation in its vicinity (McGhie et al., 2009). *Salmonella* is not necessarily

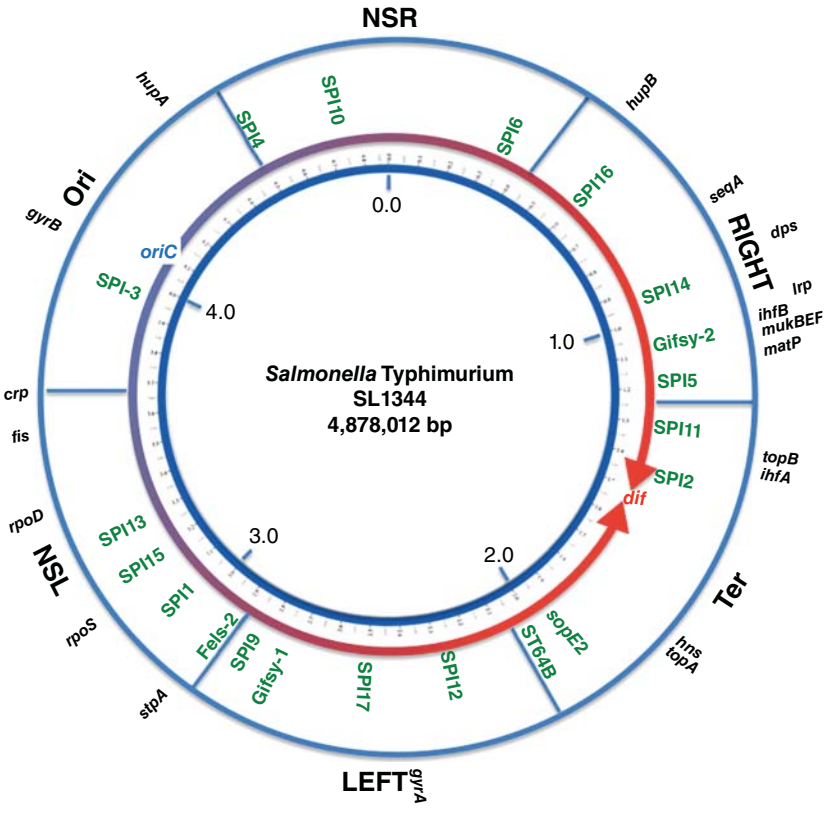

Figure 7.7 The macrodomain structure of the *Salmonella* chromosome (*S. enterica* serovar Typhimurium SL1344). The macrodomains are indicated by the segments labelled Ori, Right, Ter, and Left, as are the Left and Right Non-structured regions. The large curved arrows represent the Left (anticlockwise) and Right (clockwise) replichores, respectively. Horizontally acquired genetic elements are labelled with green lettering: SPI (*Salmonella* pathogenicity islands) and prophage (Fels and Gifsy). The circular chromosome is calibrated in 1-Mbp units and the positions of genes mentioned in the text are shown around its periphery. (*See colour plate section for colour representation of this figure*)

fated to remain in its vacuole: in around 20% of cases, the bacteria can escape and begin to proliferate vigorously in the epithelial cytosol, leading to the death of the host cell and the release of the microbes (Knodler, 2015; Malik-Kale et al., 2012). The *Salmonella* that stay in intact vacuoles are trafficked to the basolateral surface for release. Regardless of the route taken, the bacteria can enter phagocytic cells either by direct action on the part of the *Salmonella* or by engulfment by the phagocytic cells (Haraga et al., 2008). The SPI2-encoded T3SS and associated effector proteins protect the bacteria as they reside in the *Salmonella*-containing vacuole (SCV) (Figueira and Holden, 2012). Effector proteins to be secreted by the SPI2 T3SS are encoded by genes at other locations, such as SPI5, SPI12, and prophage, indicating a need for coordination of expression across the genome (Ilyas et al., 2017).

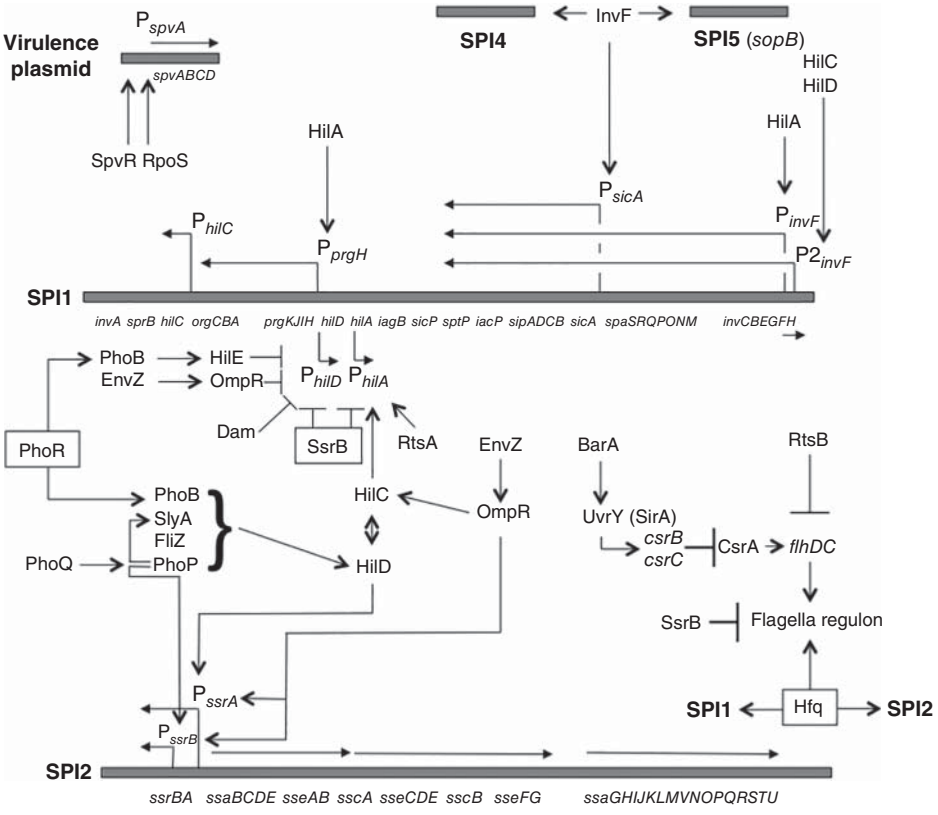

Figure 7.8 The H-NS virulence regulon of *Salmonella enterica* serovar Typhimurium. Simplified representations of the *Salmonella* pathogenicity islands SPI1, SPI2, SPI4, and SPI5 on the bacterial chromosome and the *spv* virulence locus on the pathogenicity plasmid are shown. Transcription units are indicated with gene names and the extent and orientations of major transcription units are shown using horizontal arrows with filled arrowheads. Angled arrows labelled 'P' represent important transcription promoters. Arrows with open arrowheads denote positive regulatory inputs and negative ones are represented by 'T' shapes. The H-NS NAP (not shown) silences the genes shown and additional regulators are involved in positive or negative control at the transcriptional level (e.g. transcription factors OmpR, PhoB, PhoP, and anti-silencing protein SlyA) or the post-transcriptional level (e.g. Hfq RNA chaperone and the CsrB and CsrC sRNAs). An important feature of the network is the extent to which genes and operons are crossregulated. SPI1 and SPI2 each encode dedicated regulators (e.g. HilA, HilC, HilD for SPI1, and SsrB for SPI2) but HilD also controls SPI2 gene expression while SsrB plays a role in governing gene expression in SPI1. Regulatory proteins encoded by genes in the ancestral genome, such as PhoB, PhoP, and OmpR, also contribute to the control of virulence gene regulation, showing the extent to which the horizontally acquired virulence genes have become integrated into the regulatory circuits of *Salmonella*. SPI1 and SPI2 each encode a T3SS and it is interesting to note that SPI1 and SPI2 gene expression is networked with that of the flagellar regulon, the third T3SS in the bacterium. The role of the InvF AraC-like protein in regulating transcription in SPI4 and SPI5 is a further example of inter-island cross regulation. For the genomic locations of the pathogenicity islands, see Figure 7.7.

7.17 SlyA, PhoP/Q, and SPI Gene Expression

Studies aimed at understanding the mechanisms by which H-NS-mediated transcriptional silencing is overcome in *Salmonella* have revealed the existence of specialist 'remodelling' proteins that clear a path for transcription factors at H-NS target promoters. The SlyA protein is an important member of this group and it belongs to the MarR-like wHTH family of DNA-binding proteins (Dolan et al., 2011; Ellison and Miller, 2006a). It relies chiefly on indirect readout in selecting its binding sites and these sites have many features in common with H-NS targets in DNA, facilitating the natural, mutual antagonism that is displayed by SlyA and H-NS (Dolan et al., 2011; Dorman and Dorman, 2017; Haider et al., 2008). The PhoP/PhoQ regulon overlaps partially with that of H-NS (Navarre et al., 2005; Norte et al., 2003; Shi et al., 2004). This two-component system consists of a cytoplasmic-membrane-located sensor kinase, PhoQ, and a cytoplasmic DNA-binding response regulator protein, PhoP (Kato and Groisman, 2008). It responds to magnesium starvation, transmitting information about a lack of magnesium cation availability to PhoP by phosphorylation (García Véscovi et al., 1996). It also responds to antimicrobial peptides at low pH (Bader et al., 2005; Bearson et al., 1997). Its responsiveness to this combination of signals is useful in helping *Salmonella* to adapt to the hostile environment of the macrophage SCV. The activated form of PhoP binds to promoters that have DNA sequences that match the PhoP box consensus and the promoters can be activated or repressed, depending on the position and orientation of the boxes, with respect to the binding sites used by RNA polymerase (Perez and Groisman, 2009; Zwir et al., 2012). PhoP-regulated genes are found in the ancestral and the horizontally acquired parts of the *Salmonella* genome. In the case of the latter, the promoters are typically silenced by H-NS and before phosphorylated PhoP can interact with them, the SlyA protein must first remodel the H-NS-DNA complex appropriately (Perez et al., 2008). Work in *E. coli* has shown that SlyA and H-NS display mutual antagonism: not only can SlyA overcome transcription silencing by H-NS, but the NAP can also displace SlyA in a protein-concentration dependent manner (Lithgow et al., 2007). This work reveals a continuously mutually antagonistic relationship between the two proteins and is consistent with the possibility that conventional transcription factors like PhoP are required to await the outcome of the competition before being able to influence the expression of the target gene. This is not the case at genes in the ancestral chromosome where the SlyA-H-NS interaction does not occur; here activated PhoP can control transcription independently of remodelling (Perez et al., 2008; Will et al., 2014).

7.18 Gene Control in SPI1 and SPI2

Among the targets of PhoP in the H-NS regulon are the virulence genes in the SPI1 and SPI2 pathogenicity islands (Figure 7.8) (Bajaj et al., 1996). These horizontally acquired, A + T-rich DNA insertions are located at either the NSL region (SPI1) or in the Ter macrodomain (SPI2), the home of the *hns* gene. Each encodes a T3SS with a distinct role in the infection process: SPI1 is used to invade the epithelium of the small intestine (Galán and Curtiss, 1989), while SPI2 is used to modify the *Salmonella*-containing vacuole of the

macrophage to protect the bacterium (Fass and Groisman, 2009). The effector proteins that are secreted through each T3SS are encoded both within the respective islands and elsewhere in the *Salmonella* genome. Collective control of the expression of all of these genes involves a complex regulatory network, of which PhoP and H-NS are just two players. Within SPI1, a series of genes encodes transcription factors that control the SPI1 promoters in response to environmental signals (Figure 7.8) (Galán, 2001). There is also crosstalk with SPI2 (Bustamante et al., 2008), with the SPI1-encoded HilD regulator acting as an antagonist of H-NS at the *ssrB-spiR* regulatory locus (Martinez et al., 2014) that encodes the SsrB/SpiR two-component regulatory system that governs the transcription of both the regulatory and the structural genes within the SPI2 island (Ochman et al., 1996; Shea et al., 1996). SpiR is a cytoplasmic-membrane-associated sensor kinase that detects environmental signals associated with *Salmonella* adaptation to the macrophage vacuole. SpiR detects the lowering of periplasmic pH that accompanies acidification of the macrophage vacuole through a mechanism that depends on histidine amino acids in that part of the protein (Mulder et al., 2015). Signal transmission involves auto-phosphorylation of SpiR followed by phosphorylation of its response regulator partner, SsrB (Fass and Groisman, 2009). SpiR is also known as SsrA (Valdivia and Falkow, 1997), a designation that may cause confusion with the ssrA tag that targets incompletely synthesised proteins for turnover. The 11-amino acid tag (amino acid sequence: AANDENYALAA) is added co-translationally by the tmRNA to the C-terminus of the defective protein (Moore and Sauer, 2005; Karzai et al., 2000). Its addition provides a mechanism for nonsense protein turnover by ATP-dependent proteases and the release and recycling, or 'rescuing', of ribosomes (Farrell et al., 2005; Lies and Maurizi, 2008) (Section 5.7).

SsrB is a response regulator that acts both as an anti-repressor to overcome H-NS-mediated transcription silencing, and as a conventional transcription factor (Walthers et al., 2007). SsrB works in association with SlyA and autoregulates its own gene and that encoding SpiR (Feng et al., 2003; Linehan et al., 2005). PhoP binds to the promoter of the *ssrB* gene but controls *spiR* expression post-transcriptionally: the physiological conditions under which PhoP regulates SPI2 gene expression are not completely clear (Bijlsma and Groisman, 2005; Fass and Groisman, 2009; Lee et al., 2000). Unphosphorylated SsrB is not inactive but regulates an alternative group of genes outside SPI2 involved in biofilm formation, activating their expression by an anti-H-NS-silencing mechanism of action (Desai et al., 2016). This may represent a lifestyle switch for the bacterium, allowing it to cycle between a planktonic and a biofilm-related attached state. SsrB contributes to SPI2-SPI1 crosstalk by regulating the expression of the *hilA* and *hilD* regulatory genes (Figure 7.8) (Pérez-Morales et al., 2017). As we have seen, HilD acts reciprocally to downregulate transcription within SPI2 (Bustamante et al., 2008) providing a molecular mechanism for a two-way conversation between these pathogenicity islands that helps ensure that when one is being expressed, the other is repressed. Within SPI2, SsrB works in cooperation with OmpR, another response regulator that is encoded by the core genome (Feng et al., 2003; Garmendia et al., 2003). Like SsrB, OmpR uses a wHTH motif to bind to DNA. OmpR is phosphorylated by the EnvZ sensor kinase, located in the cytoplasmic membrane. It responds to osmotic stress and acidic pH and plays an important part in the adaptation of *Salmonella* to the acidified vacuole of the macrophage (Bang et al., 2002; Chakraborty et al., 2017; Quinn et al., 2014). OmpR is also involved in SPI1

gene regulation: it activates transcription of the *hilC* regulatory gene while repressing *hilD* transcription (Cameron and Dorman, 2012). The protein PmrA binds to the *ssrB* promoter: PmrA is a regulator of lipopolysaccharide modification and a determinant of *Salmonella* resistance to Fe^{3+}, aluminium, serum, and defensin peptides (Nishino et al., 2006). Loss of control by PmrA makes *Salmonella* hypervirulent, suggesting that its role may be to enhance the persistence of the pathogen in host tissues (Choi and Groisman, 2013).

These examples serve to illustrate the ubiquitous nature of the H-NS protein as a local organiser of DNA and as a global regulator of transcription in three different Gram-negative pathogens of mammals. The three cases demonstrate the intimate association between H-NS and horizontally acquired genes and the myriad ways in which the bacteria have evolved mechanisms to overcome H-NS silencing is response to infection-relevant environmental cues.

8

An Integrated View of Genome Structure and Function

8.1 Networks versus Hierarchies

The connectedness of the biochemical pathways in living organisms is reflected in a corresponding connectedness in the processes that govern the expression of the genes that encode the enzymes in those pathways, the transport systems that import the raw materials for their operation or translocate the pathways' products. Connectedness extends beyond the molecular components of the cell to the information that directs its operations. Information is sampled from the interior and exterior of the cell and used to fine-tune biochemical operations. It is also written into the base sequence and structure of DNA in the genome.

From an adaptive perspective, genomically encoded information may seem to be neutral when it is not being read. In contrast, reading and expressing it, on cue and in the correct circumstances, should benefit the cell, while misexpressing it can be deleterious. However, the physical nature of the information in the genome prevents it from being neutral in cost/benefit terms. DNA is a macromolecule that must be replicated accurately if the cell is to copy itself faithfully, and it must be folded appropriately if it is to be stored, read, copied, and segregated with high fidelity (Chapter 1). We have seen that the processes by which DNA is replicated and read affect one another, and the structural and functional aspects of the genome are completely integrated with each other. The mutual influences of DNA supercoiling and gene transcription provide just one example (Chapter 3). The tiny universe of the bacterial cell's interior consists of very intimate interdependencies where everything that happens appears to have an impact on everything else. Given all of the interconnectedness, how meaningful is it to think in terms of regulatory hierarchies or networks?

The concept of essentiality has been used to establish a pecking order among cellular processes. If something is essential, its removal always results in the death of the cell, so that process outranks one that is sometimes dispensable. A complication of the principle of essentiality is that it is conditional: an essential process under one set of growth conditions may be non-essential under different circumstances. Following this logic, the top processes in the ranking should be those that are essential under all conditions. However, one may discover within a process that appears to be absolutely essential that there are steps or subroutines that contain redundant elements. That said, no amount of sophistry will allow one to avoid the conclusion that there are indeed hierarchies of essentiality among the processes taking place in bacterial cells (or of any other type of cell). A cell that loses

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the ability to replicate its chromosome or to transcribe its genes will not survive for long. Losing the ability to transport and/or metabolise a seldom-encountered carbon source is likely to be much less serious. It seems safe to conclude that the networked operations of the cell include players and processes that can be ranked in terms of their essentiality.

Gene regulation can be considered using the analogies of governance and management. In many human organisations these activities involve hierarchies, but this is not always the case: some organisations resemble networks rather than hierarchies. Military and some ecclesiastical organisations operate in a hierarchical way, as do most governments and their civil service bureaucracies. There are clear reporting lines and chains of command. Less formal organisations, such as clubs or monastic communities, operate as networks with the leader emerging as primus inter pares. The two models can be contrasted in several ways, but one important distinction concerns their vulnerability to disruption. Decapitation is an effective way to incapacitate a hierarchy, whereas a network is likely to be more robust as a functioning entity following the sudden loss of its leader. Tellingly, 'decapitate' and 'incapacitate' both have the Latin word *'caput'*, meaning head, as the root.

The genes in bacterial cells have been grouped in different ways according to the interests of the investigator. The concept of 'homology' aggregates genes based on their relatedness to a common ancestor; functional grouping gathers together genes based on contributions to a common process or because they encode products with related ways of working (e.g. transcription factors with wHTH [winged helix-turn-helix] motifs); geographical grouping uses physical co-location in the genome and regulatory grouping makes collectives of genes that are subject to control by the same signal or regulatory molecule. It is possible for two genes to be grouped together under all four of these headings (as well as under additional ones that the reader might propose). The rise of computer-assisted genomic studies has seen a proliferation of ever more complex Venn diagrams displaying the different ways in which genes can be grouped together. One of the interesting features of these groupings is that the membership can change according to the circumstances under which the collective was assembled. It is also striking to note that a given gene can turn up in many different groups according to the criteria that have been set for group membership. Here, the collectivization of genes will be considered from the perspective of gene expression control.

8.2 Regulons, Stimulons, and Heterarchies/Netarchies

Genes that are under the control of a common regulator are said to be members of the same regulon. In the regulon's original formulation, the regulator was a protein; it is clear that sRNAs (small RNA) can also command regulons, as we have seen (e.g. the sRNA RhyB, Section 6.11). The DNA-binding protein is usually responsive to an environmental signal that alters its interaction with its target genes, and these genes possess a signature DNA sequence that is used by the regulator as its recognition and binding site. By accumulating binding sites used by different regulatory proteins, a gene can belong simultaneously to several regulons and be controlled in response to multiple signals. This is a sound strategy for bacteria that inhabit complex and dynamic environments where signals are seldom received individually. By taking multiple soundings of the environment and then making a finely nuanced response, the bacterium improves its chances of giving the right answers

to the challenges that it faces. Building in additional regulation at the post-transcriptional and post-translational levels allows maximum refinement of the response while reducing the likelihood of making an inappropriate response. Thus, regulons, be they transcriptional or post-transcriptional, are networked.

A group of genes that responds to the same signal represents a stimulon. This grouping is distinct from a regulon because the signal can be transmitted by more than one regulatory molecule. Thus, one can speak of an osmotic stress stimulon, an acid stress stimulon, a heat stress stimulon, etc.; the common feature of the stimulon members is their responsiveness to the named stress and not to a common regulator. This concept has been extended to other types of groupings, e.g. the 'virulon', where a collective of genes involved in a common enterprise are co-regulated by signals associated with that activity (e.g. infecting a host).

Who are the dominant players in these regulatory groupings and between the groupings? The common regulator is an obvious candidate for dominant player in a regulon, because, by definition, it is its governing molecule. If the regulator is eliminated by mutation, the subservient genes will still exist and one may find that they can still contribute to the original response through other regulatory inputs, revealing an element of redundancy in the system. Alternatively, compensatory mutation may result in a rewiring of the regulon so that it can continue to make a useful contribution (Stoebel and Dorman 2010; Stoebel et al. 2009). The more the system displays evidence of regulatory redundancy, the less it resembles a strict hierarchy and the more it seems to be a network (Ferguson 2018). Regulatory cascades not only link regulons together in networks but also blur the distinctions between networks and hierarchies.

The heterarchy is a concept that acknowledges the conditionality of the regulatory relationships in bacterial cells. In a heterarchy, the dominant player is not fixed and regulators can move up and down in the pecking order according to the physiological state of the microbe. The mutual influences of transcription and DNA supercoiling that have been described earlier offer an example. The underwinding of DNA by DNA gyrase facilitates open complex formation as the RNA polymerase holoenzyme begins to scrunch the duplex at the gene promoter, while the elongating core enzyme creates a local domain of DNA negative supercoiling that can modulate transcription initiation at the next promoter along the chromosome. This phenomenon has been described experimentally for the promoter relay in the neighbourhood of the *leuABCD* operon in both *Escherichia coli* and *Salmonella* Typhimurium (Chen et al. 2005). In a case like this, which is the dominant influence: transcription-mediated or gyrase-mediated decreases in DNA linking number? The 'netarchy' concept builds on the idea of the heterarchy, positing that whole networks can shift up or down in terms of their relative ranking of importance as the circumstances of the bacterium change. The chicken-egg nature of the problem is obvious and leads to the conclusion that the answer depends on the nature and circumstances of the genes in question and the prevailing growth conditions.

8.3 Transcription Burstiness and Regulatory Noise

Due to technical limitations, studies of gene regulation and gene expression in bacteria were conducted exclusively using bulk populations for many decades. Consequently, gene

expression outputs have represented population-wide averages. The advent of techniques allowing investigations to be conducted at the levels of single cells and single genes has revealed a picture of great copy-to-copy variation in the expression of given genes from cell to cell. A gene may be transcribed in bursts in a subset of cells and not at all in others, with different cells contributing to the transcription of this gene from moment to moment (Chong et al. 2014; Chubb and Liverpool 2010; Raj and van Oudenaarden 2008; Sanchez and Golding 2013). All of this diversity remains undetectable when the investigation is carried out with ensemble techniques.

Transcription burst size refers to the number of transcripts that are generated within a single episode and burst frequency reports the number of transcriptional bursts per unit time. These reflect the activities of transcriptional regulators and RNA polymerase, with the regulators modulating RNA polymerase. Typically, bacterial transcription bursts produce 10 or fewer transcripts (Taniguchi et al. 2010; Wang et al. 2018). DNA supercoiling is one of the principal contributors to bursting in bacterial transcription; a failure to relax topological constraints arising from transcription elongation causes a cessation in that process with negative feedback onto transcription initiation, temporarily silencing the gene (Chong et al. 2014; Levens and Larson 2014; Ma and Wang 2016). Other contributors include the availability of transcription factors and sigma factors in sufficient local concentrations to initiate transcription, with the transcription factors being in a mode that makes them proficient to act (e.g. appropriately phosphorylated, where this is a requirement for activity). RNA polymerase appears to find promoters by direct binding rather than by facilitated diffusion along DNA (Friedman et al. 2013), so the visibility to RNA polymerase of the promoter in the folded and dynamic chromosome is another contributor to variation. Each contributor creates additional layers of stochasticity in the transcription process, leading to the observed physiological diversity across genetically uniform bacterial populations.

8.4 The Significance of Gene Position

The preceding chapters have summarised some key information about the structure of the chromosome in model bacteria, the processes of chromosome replication and segregation, the architectural elements that help to impose order on the folded chromosome, and the principal mechanisms that influence the expression of the genes along the chromosome. There has also been a discussion of the environmental and physiological challenges that bacteria face and how these are met at the levels of gene expression and genome structure. How do all of these factors combine to influence the emergence of a genomic structure that is, presumably, optimised for the survival of the bacterium?

Bioinformatic analysis suggests a correspondence between gene position on the chromosome and the role of the gene product in physiology (Sobetzko et al. 2012). If one considers the locations of genes contributing to such major cellular functions as transcription, translation, DNA replication, and chromosome organisation there is a compelling correspondence between the genetic map and the order in which those genes are expressed during the growth cycle. Moreover, this order is conserved across the gamma-proteobacteria in terms of gene distance from the origin of replication (*oriC*) and in terms of membership of a specific replichore (Left or Right) (Sobetzko et al. 2012).

Among the genes located closest to *oriC* are the members of the *atpIBEFHAGDC* operon (Figure 1.1b). These genes encode the F_1F_0 ATP synthase that is powered by proton motive force and generates ATP. The synthesis of ATP is maximal during growth on glucose in the presence of oxygen, conditions that support rapid cell growth and genome replication. Repeated initiations of chromosome replication in the fast-growing cells will elevate the number of copies of *oriC*-proximal genes (such as those in the *atp* operon) relative to that of genes in the Ter macrodomain. This may be regarded as a useful way of increasing the output of ATP synthase copies in the cytoplasmic membrane and hence the capacity of the bacterium to generate ATP. The production of the subunits of ATP synthase in the stochiometrically correct amounts is, however, determined at the level of individual gene translation (Larson et al. 2014).

Consistent with this model is the presence of the *dnaA* gene in the vicinity of *oriC,* given its key role in priming new rounds of chromosome replication (Figures 1.2 and 1.5). The genes for DNA gyrase represent an anomaly. The functional form of this type II topoisomerase has an A_2B_2 subunit composition, yet the *gyrB* gene (B subunit) is *oriC*-proximal while *gyrA* (A subunit) is in the Left macrodomain, far away from *oriC* (Figure 1.1a). This situation obtains in the model bacteria *E. coli, Salmonella*, and their relatives, yet in other bacteria the two genes are in an operon. For example, *Mycobacterium* spp., *Streptomyces* spp., *Listeria* spp., *Borrelia* spp., *Staphylococcus aureus, Clostridium* spp., *Leptospira* spp. and others possess a *gyrBA* operon. This arrangement is more intuitively appealing given that the gene products have to find one another in order to assemble into a functioning enzyme and that they are required in a precise stoichiometry. It is presumed that the genomically dispersed locations of the *gyrA* and *gyrB* genes in *E. coli* et alia represent a successful strategy for gyrase expression in the context of those organisms' lifestyles, but the underlying molecular rationale is currently unclear.

The *dusB-fis* operon is located in the Left Non-structured domain, in a part of the chromosome where several parts of RNA polymerase and its modulators are encoded (Figure 1.1a). FIS (Factor for Inversion Stimulation) acts to boost the transcription of genes and operons that encode the major components of the translation machinery and it has a positive role in the initiation of chromosome replication (Ryan et al. 2004). This NAP (Nucleoid-associated protein) is expressed at peak levels as bacterial growth accelerates at the end of the lag phase and the onset of logarithmic growth. The promoter of the operon is stimulated by negative DNA supercoiling and is under stringent control. Thus, a position for *dusB-fis* in the Ori-proximal quadrant of the chromosome is consistent with the scheme of Sobetzko et al. (2012). The expression of FIS is linked to the level of oxygen available to the bacterium: in microaerobic environments, FIS expression is sustained into stationary phase, albeit at lower levels than those seen in highly aerated cultures (Cameron et al. 2013; O Cróinín and Dorman 2007). In this context, it is interesting to note that the *arcB* gene is positioned in the same macrodomain as *dusB-fis* and that the *arcA* gene is found at a corresponding point on the opposite replichore in the Ori macrodomain (Sobetzko et al. 2012). The ArcA/ArcB two-component system is a master regulator of the bacterial response to oxygen limitation: the ArcA protein regulates positively the genes that encode the *cydBA* operon encoding the high-affinity, oxygen-scavenging cytochrome bd oxygenase used by *E. coli* under microaerobic growth conditions (Cotter et al. 1997). FNR, the other 'half' of the aerobic/anaerobic respiration master control system, is encoded by a gene (*fnr*) that is

located in the Ter macrodomain (Figure 1.1b). The Ter macrodomain also houses the *topA* and *topB* genes encoding, respectively, Topo I and Topo III, the enzymes that oppose the negative supercoiling activity of DNA gyrase by relaxing negatively supercoiled DNA. This part of the chromosome is also the location of the *hns* gene: its product, H-NS, is an abundant NAP that silences transcription throughout the genome, targeting in particular genes that have been acquired by HGT (horizontal gene transfer) (Dorman 2007; Lucchini et al. 2006; Navarre et al. 2006).

Is the Ter macrodomain an especially appropriate depot for the supply of H-NS to the genome? It has been pointed out that the changes to the physical location of chromosome macrodomains as a function of the growth cycle might alter the spheres of influence of globally acting regulatory proteins encoded by particular chromosomal regions (Sobetzko et al. 2012). Thus, FIS and H-NS, originating at opposite sides of the chromosome, might exert different effects during the different major phases of the growth cycle in addition to those that can be linked to protein concentration (FIS is expressed at about 60 000 copies in early log phase and then declines rapidly in numbers whereas H-NS is present at about 20 000 copies per chromosome throughout the cycle). If the FIS and H-NS proteins (or any other proteins) are free to diffuse and can do this with rapidity, then the positions of their genes in the folded chromosome might not matter beyond the influence of gene copy number as a function of distance from *oriC* (Figure 1.1a).

The issue of gene position was considered in Section 1.33 from the perspective of DNA supercoiling levels around the chromosome. Experimental and bioinformatic data indicate that different portions of the chromosome are likely to be supercoiled to different extents through the growth cycle (Lal et al. 2016), with possible implications for the expression of genes in the affected regions. This model is consistent with the finding that the binding sites for DNA gyrase are not distributed evenly in the *E. coli* genome (Jeong et al. 2004; Sobetzko et al. 2012; Sutormin et al. 2019). Bryant et al. (2014) found that DNA-supercoiling-sensitive transcription varied from place to place on the chromosome. Gerganova et al. (2015) repositioned the *dusB-fis* operon on the *E. coli* chromosome and detected moderate changes in its expression together with phenotypic changes, including altered antibiotic resistance, new environmental-stress-resistance profiles, and shifts in the topoisomer distributions of reporter plasmids. Other investigations were unable to establish clear links between gene position, DNA topology, and transcription levels beyond copy number effects arising from gene distance from the origin of chromosome replication (Block et al. 2012; Brambilla and Sclavi 2015; Chandler and Pritchard 1975; Miller and Simons 1993; Pavitt and Higgins 1993; Schmid and Roth 1987; Sousa et al. 1997; Thompson and Gasson 2001; Ying et al. 2014).

8.5 Messenger RNA May Not Be Free to Diffuse Far in Bacteria

Experiments with *Caulobacter crescentus* and *E. coli* suggest that mRNA does not diffuse far from the gene that encodes it (Montero-Llopis et al. 2010). This work applied improved methods of labelling mRNA and protein and visualising them in bacteria. The results show that for a number of genes the transcript is constrained physically to remain in the neighbourhood of the gene from which it was expressed. This in turn constrains the

movement of ribosomes because these become associated with the constrained mRNA during translation. These constraints break down to some extent when the mRNA is encoded by a highly expressed gene, such as a heat shock gene in a bacterium undergoing thermal stress. Untranslated RNA, including sRNAs, may be exempt from the constraints that apply to transcripts that are associated with polyribosomes. This may assist sRNAs in being highly effective at regulating gene expression throughout the folded genome.

Presumably, the exemption of mRNA expressed from highly transcribed genes from constraints on movement also applies to the messages specifying the abundant NAPs FIS and H-NS. Data from experiments in which the genes coding for FIS or H-NS were moved to different locations around the chromosome in *E. coli* indicate that the effect of *hns* gene displacement is negligible and the effect of *fis* gene relocation is marginal at best (Brambilla and Sclavi 2015; Gerganova et al. 2015). In the case of the effect of moving the *fis* gene in *E. coli* to the Ter macrodomain, one of the novel phenotypes was a shift in average DNA supercoiling levels, which was accompanied by a negative alteration in competitive fitness (Gerganova et al. 2015).

Rewiring the *hns* gene in a new chromosomal location does produce strong phenotypes (Fitzgerald et al. 2015). Exchanging the *hns* open reading frame for that of the paralogous *stpA* gene at the *stpA* gene position at approximately the midpoint of the Left Replichore of the *Salmonella* chromosome (Figure 1.1a) produces an organism that is inherently fitter in competition. This is due to a rescheduling of the expression of the RpoS stress and stationary phase sigma factor with a concomitant impact on the expression of the RpoS regulon. This can be explained in part by dysregulation of the *stpA* gene, whose open reading frame has been reciprocally exchanged with that of *hns* because loss of StpA correlates with early appearance of RpoS (Lucchini et al. 2009).

8.6 RNA Polymerase Activity and Genome Organisation

An *E. coli* cell has between 1500 and 5000 copies of RNA polymerase (Grigorova et al. 2006), increasing to 8400 copies in rapidly growing cells (Patrick et al. 2015). Super resolution microscopy has indicated that RNA polymerase can be partitioned into two populations: one that is free to move around in the cell and one that is attached to DNA either at promoters or within genes (Stracy et al. 2015). Treatment with the antibiotic rifampicin, which inhibits transcription by RNA polymerase, shifts most of the RNA polymerase into the mobile population. Cessation of transcription also correlates with de-compaction of the chromosome and this facilitates increased diffusion in the nucleoid, not only of RNA polymerase, but also of the Pol1 component of DNA polymerase (Stracy et al. 2015). In the drug-treated cells, between 400 and 550 promoters retain bound RNA polymerase, presumably unable to initiate transcription due to the inhibitory effect of rifampicin (Grainger et al. 2005; Stracy et al. 2015).

Experiments with promoters on plasmids have shown how powerfully an active transcription complex can displace a gene (and the plasmid within which it resides) across a cell (Sánchez-Romero et al. 2012). In the *E. coli* nucleoid, transcription occurs at the nucleoid surface (Spahn et al. 2015) and it has been proposed that entropic forces drive active transcription complexes from the bulk nucleoid to its surface (Stracy et al. 2015).

Other processes that involve bulky assemblies of proteins and other factors also occur outside the main body of the nucleoid, such as DNA repair (Lesterlin et al. 2014) and much of the translation activity of the cell.

Transcription is not excluded from the bulk nucleoid, but it seems that the most heavily transcribed genes are displaced to the nucleoid surface, leaving weakly transcribed genes that are associated with single copies of RNA polymerase within. In contrast, clusters of RNA polymerase are associated with the heavily transcribed genes and operons at the nucleoid surface. If transcription is interrupted by rifampicin treatment, these units are no longer transcribed and return to the bulk nucleoid (Stracy et al. 2015).

It might seem that one of the practical benefits of gene displacement to the nucleoid surface would be contact between the transcript and the translational machinery (Jin et al. 2013). While it is true that active polyribosome complexes are excluded from the nucleoid, 30s and 50s ribosome subunits can be detected in the bulk nucleoid and translation can at least begin there, followed by migration to the nucleoid surface (Bakshi et al. 2014; Sanamrad et al. 2014).

In the case of protein-coding genes, the assembly of polyribosomes along the message and the act of translation enhance the entropic effect. Certainly, the assembled ribosomes and their associated translation factors will accumulate to produce bulky assemblies along the transcript (Shajani et al. 2011). Genes that do not specify proteins, such as stable RNA genes, are usually heavily transcribed, allowing a queue made up of scores of transcribing RNA polymerases to enhance the displacement of the DNA template towards the surface of the nucleoid (Endesfelder et al. 2013). The products of such genes include ribosomal and transfer RNAs, molecules that are needed to support the process of translation that is taking place principally in the space between the nucleoid and the cytoplasmic membrane.

Proteins that are destined for cytoplasmic membrane insertion, or to traverse the membrane on their way to the periplasm or beyond, create physical links between the nucleoid and the cell envelope through a phenomenon called 'transertion' (Woldringh 2002; Woldringh et al. 1995). Chloramphenicol treatment, which abolishes translation, results in chromosome compaction (Begg and Donachie 1991; van Helvoort et al. 1996), suggesting that the loss of the DNA-mRNA-protein-membrane bridge might allow the nucleoid to collapse on itself. Studies of specific genes that encode secreted proteins have provided data that support this proposal: the *lacY* (Lac permease) and *tetA* (tetracycline efflux pump) genes encode unrelated cytoplasmic membrane proteins and these genes move closer to the membrane when they are expressed (Libby et al. 2012). Gene repositioning occurs rapidly following induction of gene expression and inhibition of transcription with rifampicin, or of translation with kasugamycin, abolished the gene repositioning effect (Libby et al. 2012). Treatment with chloramphenicol does not interfere with the movement of active transcription units to the nucleoid surface, indicating that the transertion process is not required for this displacement (Stracy et al. 2015).

Clusters of RNA polymerase appear in rapidly growing cells and it has been proposed that these are equivalent to the transcription factories seen in eukaryotes (Cabrera and Jin 2003; Papantonis and Cook 2013). Transcription, especially of heavily transcribed genes, has the power physically to move genes within the cell and may bring together genes or groups of genes that are far apart on the circular chromosome. The spatially reorganised genes may contribute to related processes and/or express products that need to interact.

This level of organisation, which is process-dependent, is distinct from, and additional to, organisation that depends on operon structure and/or gene–gene neighbourhoods along the chromosome. The geographical neighbourhoods are fixed but the process-dependent ones are contingent on growth conditions that drive transcription. Thus, spatial organisation that is transcription driven will vary as a function of the environmental and metabolic circumstances of the cell, making it a valuable contributor to the creation of physiological variety across the bacterial population.

See Section 1.33 for a discussion of the influence of transcription on nucleoid architecture at the level of chromosome interaction domains.

8.7 Gene–Gene Interactions in the Folded Chromosome

Gene expression and chromosome organisation exert influence on one another and changes to one have consequences for the other (Cagliero et al. 2013; Dorman 2013; Le et al. 2013; Le and Laub 2016; Lioy et al. 2018; Meyer et al. 2018). Despite this relationship of mutual integration, the genome is remarkably robust, absorbing changes to gene expression programmes, including the physical positioning of transcription units, without fatal consequences for the cell. However, there is a significant difference between being viable and being competitively fit, and the latter is critical for the long-term survival of the genome. Fitness can be affected by changes that are rather subtle and the effects may not be detected until an appropriate challenge is imposed. Are there hints in genome architecture about the optimal arrangements of genes that contribute to the same or related processes?

The operon is an obvious example of gene colocation for collective regulation of expression. There is an anti-correlation between the frequency with which operons occur in genomes and the regulatory sophistication of those genomes: on average, large genomes with many transcription regulators have fewer operons (Nuñez et al. 2013). This may facilitate greater dispersal of genes contributing to common pathways but they will still need to be controlled collectively in time if not in space. The presence of operons contributing to motility and chemotaxis in opposite replichores of the chromosome shows that physical distance between functionally related genetic elements is not a barrier to their effective co-regulation. Similarly, *Salmonella* operates a sophisticated virulence programme using genes and operons located in both replichores and on a large plasmid, where both cross-regulation and co-regulation are essential (Figure 7.7). These genes respond to a multitude of chemical and physical signals that are transmitted directly (e.g. via riboswitches), through DNA-binding proteins and sRNA molecules, and through the topological state of the DNA. This regulatory complexity and the associated geographical complexity may make for a variety of responses to environmental signals across the *Salmonella* population, something that may aid the pathogen as it tries to evade host responses. Ribosomal operons provide a further example: they are found in both replichores, oriented away from, and at similar distances from, *oriC* (Figure 1.1b).

Bioinformatic studies have detected a periodicity in the positioning of genes in the bacterial chromosome that might be expected to communicate with one another and/or to participate in a common biological process (Figure 8.1). The genes are described as being members of evolutionarily conserved gene pairs. The data suggest that there may be three

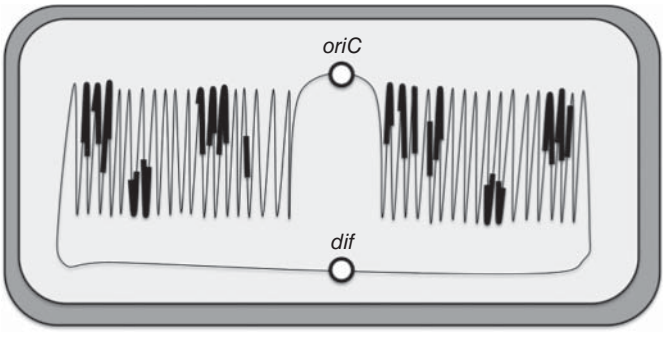

Figure 8.1 Evolutionarily co-located genes in the *E. coli* chromosome. The chromosome is represented as two toroidal solenoids aligned with the long axis of the bacterial cell, with the origin of replication (*oriC*) positioned between the Left and Right replichores. Each replichore is composed of 19–20 helices, each with a periodicity of 117 kb; the replichores are connected by the Ter macrodomain with the *dif* site for XerCD-mediated dimer resolution at its midpoint. Thickened sections of the periodically organised chromosome show the locations of evolutionarily co-located genes and operons. Not drawn to scale. Source: For further reading, see Wright et al. (2007).

levels of spatial organisation for transcription and that these operate over ranges of around 16 kb, from 100 to 125 kb, and from 600 to 800 kb (Jeong et al. 2004; Junier et al. 2012; Képès 2004; Wright et al. 2007; Xiao et al. 2011). These short-, medium-, and long-range distances are approximately equivalent to the scale of a chromosomal looped microdomain (10–12 kb), a helix of periodicity 117 kb, and to a chromosomal macrodomain, respectively (Deng et al. 2005; Postow et al. 2004). Other investigations have detected a 33 kb periodicity in addition to the 117 kb one (Mathelier and Carbonne 2010). These relationships seem to imply that strict limits may constrain the extent to which chromosome rearrangements can be tolerated without affecting vital gene expression and regulation processes. However, the frequency of rearrangements of the existing genetic complement due to amplification (Andersson and Hughes 2009) and the commonplace nature of HGT (Syvanen 2012) suggest that the genome can endure reorganisation and that it is the impact on competitive fitness that will determine if a new structure is a success.

8.8 DNA Supercoiling as a Global Regulator

All of the genes in the bacterial genome are made of DNA, so adjustments to the structure of the chromosome have the potential to influence the expression of every gene in the cell. The structural variable that will be considered in this section is DNA supercoiling. The parameters of DNA supercoiling were reviewed in Section 1.27 and the roles of DNA topoisomerases in the management of DNA topology were described in Section 1.28.

Overwinding and underwinding the DNA duplex around its helical axis has the potential to influence transactions taking place within DNA, such as gene transcription. However, on its own, variable DNA topology is probably too blunt an instrument for the effective and specific regulation of gene expression. However, it can play a general role in setting the background against which the more refined controls operate. A useful analogy is provided

by the effect of variable interest rates on the multitude of financial transactions taking place in a national economy. Cheap money will encourage commerce while high interest rates will act as a brake on economic activity. It is for the central bank (or equivalent agency) to make prudent choices about the cost of doing business by manipulating the interest rate. A fiscal policy, as set by the government, exerts wide-ranging influence on the economy through taxation and this too can be used to encourage or to discourage commercial activity. A range of factors informs these monetary and fiscal choices, including the condition of the local and global economic environments.

Much like a sovereign nation state in the process of managing its economy, the bacterium monitors its internal and external environments and then uses the accumulated information to modulate its programme of gene expression. The goal is to maximise competitive fitness, a goal that would be applauded by chancellors and central bankers. It is commonplace to describe ATP as a biological currency and the bacterium exploits the ATP dependence of DNA gyrase to link the supercoiling of its DNA to the 'real economy' of the cell. A thriving bacterium, supplied with a preferred carbon source such as glucose and experiencing a high metabolic flux, will have a higher [ATP]/[ADP] ratio than one languishing on a less-preferred carbon-and-energy source such as glycerol (Balke and Gralla 1987; Jensen et al. 1995; Snoep et al. 2002; Westerhoff et al. 1988). In general, rapid growth is associated with more negative supercoils in DNA and DNA relaxation correlates with slow growth or the cessation of growth (Bordes et al. 2003; Conter et al. 1997). This correlation is observed regardless of whether the negative supercoils are introduced by DNA transactions such as transcription and replication or due to the enhanced activity of DNA gyrase. The genes that encode DNA gyrase and DNA topoisomerase I have promoters that respond to DNA supercoiling and this is thought to provide the basis for a homeostatic control of DNA supercoiling (DiNardo et al. 1982; Pruss et al. 1982). The gyrase gene promoters are stimulated by DNA relaxation (Menzel and Gellert 1983, 1987; Straney et al. 1994; Unniraman and Nagaraja 1999) while the transcription of *topA,* the gene for DNA topoisomerase I, is stimulated by negative supercoiling (Ahmed et al. 2016; Tse-Dinh and Beran 1988).

Exposing bacteria to environmental stress produces changes to the supercoiling of their DNA. In several cases, these changes have been linked to alterations to the [ATP]/[ADP] ratio (Hsieh et al. 1991a,b; Jensen et al. 1995; Snoep et al. 2002; Westerhoff et al. 1988). However, the analysis is complicated by the fact that in addition to ATP synthesis by the cytoplasmic-membrane-associated F_1F_0 ATPase, ATP is also produced in stressed bacteria by the *adk*-encoded adenylate kinase (Balke and Gralla 1987; Gutierrez and Csonka 1995). Among the stresses that produce a shift in DNA topology are osmotic upshock (Alice and Sanchez-Rivas 1997; Bordes et al. 2003; Cheung et al. 2003; Higgins et al. 1988; Hsieh et al. 1991b; Meury and Kohiyama 1992; O'Byrne et al. 1992; Sheehan et al. 1992), acid stress (Bang et al. 2002; Colgan et al. 2018; Karem and Foster 1993; Quinn et al. 2014), thermal stress (Goldstein and Drlica 1984), anaerobic growth (Bebbington and Williams 2001; Cameron et al. 2013; Cortassa and Aon 1993; Dixon et al. 1988; Dorman et al. 1988; Hsieh et al. 1991a; Malkhosyan et al. 1991; Yamamoto and Droffner 1985), oxidative stress (Weinstein-Fischer et al. 2000), and environmental influences experienced by bacteria during intracellular growth within the host (Colgan et al. 2018; Ó Cróinín et al. 2006).

The pathogenic bacterium *Mycoplasma genitalium* has a minimal genome and encodes few transcription factors, yet it possesses a full set of DNA topoisomerases and regulates the

expression of its genes (Fraser et al. 1995). Some of this regulation depends on variations in the negative supercoiling of its DNA, suggesting that, in the absence of more sophisticated regulatory machinery, the organism can effect some level of gene control by making DNA topological changes (Dorman 2011; Zhang and Baseman 2011a,b). This points to variable DNA supercoiling as a crude and primitive form of gene control, perhaps one that has predated the emergence of more refined regulatory mechanisms (Dorman et al. 2018).

In modern model organisms such as *E. coli,* variable DNA topology that is environmentally responsive works with conventional transcription regulators to tune the gene expression profile of the organism (Dorman and Dorman 2016). The same is true of pathogenic bacteria, with their specialist virulence genes whose expression is activated on receipt of the required set of environmental signals from the host during infection (Dorman 1991, 1994, 1995; Dorman et al. 2016). DNA supercoiling has been linked to virulence gene expression in an impressive array of pathogens, including *B. pertussis* (Graeff-Wohlleben et al. 1995), *Campylobacter jejuni* (Scanlan et al. 2017), *Dickeya dadantii* (Hérault et al. 2014; Ouafa et al. 2012), *E. coli* (Carmona et al. 1993; Dove and Dorman 1994, 1996; Jia et al. 2017; Nieto et al. 1997), *Haemophilus influenzae* (Gmuender et al. 2001), *Helicobacter pylori* (Ye et al. 2007), *Mycobacterium tuberculosis* (Datta et al. 2019b), *Pseudomonas aeruginosa* (DeVault et al. 1991), *Pseudomonas syringae* (Neale et al. 2018), *Shigella flexneri* (Dorman et al. 1990; McNairn et al. 1995; Ní Bhriain and Dorman 1993; Tobe et al. 1995), *S. aureus* (Sheehan et al. 1992), *Salmonella enterica* (Cameron and Dorman 2012; Cameron et al. 2011; Colgan et al. 2018; Galán and Curtiss 1990; LeClerc et al. 1998; Tam et al. 2005), *Streptococcus pneumoniae* (Balsalobre et al. 2011; Ferrándiz et al. 2016), *Vibrio cholerae* (Parsot and Mekalanos 1992), and *Yersinia enterocolitica* (Rohde et al. 1994). These disease-causing organisms must express genes during infection that encode products that are often physiologically expensive, such as type three secretion systems and their effector proteins. If the bacteria fail to do this on cue, they run the risk of missing out on the advantages of life on or in the host (e.g. access to resources and/or to a protected environment that is free from competitors or antimicrobial actors such as the host's antibodies). If they express their virulence traits inappropriately, in spatiotemporal terms, they may be replaced by better-prepared organisms or be eliminated by the host defences. Variable DNA supercoiling can contribute to the decision-making process by licensing the activation of virulence trait production without making the final decision: that is left to conventional gene regulatory proteins responding to specific host-associated signals. The licensing process involves the adjustment of the DNA structure at the site of transcription factor interaction to tip the balance in favour of gene activation. This DNA structural adjustment works in reverse to diminish the likelihood of virulence gene activation in environments where the expression of pathogenic traits would bring no advantages to the bacterium.

The activation/silencing decision at expensive genes often involves genes that have been acquired by the pathogen by HGT (Srinivasan et al. 2013). In Gram-negative enteric pathogens, the H-NS NAP and/or its homologues, such as StpA, usually silence such genes transcriptionally. They possess an A + T-rich character that makes them candidates for silencing and many of them have promoters that respond to changes in DNA supercoiling (Higgins et al. 1988). Thus, H-NS and DNA supercoiling work together to optimise the programme of virulence gene expression by minimising risk and optimising the life chances of the pathogen.

8.9 Modelling the Nucleoid

Nucleoid modelling, guided by experimental data, provides testable hypotheses about nucleoid structure (Hacker et al. 2017). Modelling a molecule as large and complex as the bacterial chromosome is a considerable challenge. Coarse-grained models of the *E. coli* chromosome have explored its macrodomain structure, looped domain structure, and gene position along the folded chromosome (Buenemann and Lenz 2010; Fritsche et al. 2012; Junier et al. 2014). The models have also been applied to an investigation of the locations of the ribosome-rich portion of the cytoplasm in relation to the nucleoid (Bakshi et al. 2014; Mondal et al. 2011). Generic models that ignore much structural detail have explored problems such as the tendency of polymer rings to repel one another when they are stored together under confinement – a proxy for chromosome segregation driven by mutual repulsion, especially in bacteria such as *E. coli* that lack active protein-dependent segregation systems (Jun and Mulder 2006). Generic models have also been used to investigate the influence of chromosomal folding on bridging interactions (Junier et al. 2010; Scolari and Lagomarsino 2015). The coarse nature of these models rules out the inclusion of fine detail such as DNA supercoils. Details of this type can be included in models at a smaller, sub-genomic scale (Benedetti et al. 2014, 2015; Hong et al. 2013; Krajina and Spakowitz 2016). Hacker et al. (2017) applied the rich detail of sub-genomic modelling to the full chromosome by creating a model in which one bead was equivalent to one nucleotide. Their model recapitulates all of the features of B-DNA in the bacterial cell: it includes the major and minor grooves of the DNA, together with DNA twist and writhe, and is in good agreement with experimental data from analyses of the chromosome at different scales. In addition to aiding studies of natural chromosomes, finely detailed models of this type will prove to be invaluable in guiding work aimed at creating artificial chromosomes in synthetic biology.

8.10 Synthetic Biology

Microbial cells, including bacterial ones, have played a foundational role in synthetic biology. It is important to discover the rules that govern the architecture of natural genomes and the regulatory events taking place in them so that any artificial ones being constructed have the best possible chance of operating successfully. Genome structural planning must take into account the size of the desired genome, the number of chromosomes over which the genetic information should be distributed, how the expression of this information will be controlled, the rate of genome replication, and the measures in place to ensure that this proceeds smoothly and with a minimum of error. Nature has already solved all of these problems, so it is probably prudent to pay close attention to how things are done in existing microbes.

One approach to synthetic biology has been to focus on very small and simple genomes. The *Mycoplasma* genome has been very useful in this regard and has provided a basis for proof-of-principle experiments. Fraser et al. (1995) determined the complete genome sequence of *M. genitalium*, and this information was used to describe the composition of

a minimal microbial genome, with 382 of its 482 genes being found to be essential (Glass et al. 2006). The mycoplasmas were also exploited for whole genome transplantation, with the genome of *Mycoplasma mycoides* being used to replace the native genome of *Mycoplasma capricolum*. The genomic transfer process was achieved by polyethylene glycol (PEG)-mediated transformation (Lartigue et al. 2007). Later, the transfer protocol was improved by the introduction of direct transfer via PEG-assisted cell-to-cell fusion to reduce the risk of genomic DNA shearing (Karas et al. 2014). The *Mycoplasma* genome was also used as a blueprint for the synthesis of a fully synthetic copy of the genome. This synthetic genome was then used to 'reboot' a bacterial cell from which the native genome had been eliminated (Gibson et al. 2008a, 2010). This project also provided valuable experience in assembling a whole genome from a series of synthetic sub-assemblies (Gibson et al. 2008b). Further progress was made when bacterial genomes were cloned in their entirety in the yeast *Saccharomyces cerevisiae*. This breakthrough offers the possibility of working with the genomes of unculturable bacteria in a eukaryotic cell that is easy to manipulate genetically. The first series of experiments saw the complete genomes of *M. genitalium* (0.6 Mb), *M. mycoides* (1.1 Mb), and *Mycoplasma pneumoniae* (0.8 Mb) being cloned as yeast centromeric circular plasmids (Benders et al. 2010). Once established in yeast, the cell cycle of the eukaryotic host could be used to drive progressive genome minimisation in the bacterium through cycles of gene deletion (Suzuki et al. 2015).

Bacterial genomes can be modified in their native cellular containers through genome editing protocols such as CAGE (Conjugative Automated Genome Engineering) and MAGE (Multiplex Automated Genome Engineering), respectively (Carr and Church 2009; Gibson 2014; Isaacs et al. 2011; Wang et al. 2009). MAGE targets ssDNA oligonucleotides to the genes to be modified, exploiting the bacteriophage lambda Red ssDNA-binding protein β to perform the recombineering (Ellis et al. 2001; Wang et al. 2009). CAGE takes the MAGE-modified DNA segments and assembles them sequentially (Carr and Church 2009; Isaacs et al. 2011). Applying these methodologies to bacterial genomes can produce profound changes in the biology of the organism. For example, reprogramming of the genetic code in living *E. coli* MG1655 to convert stop codons to sense codons expands the range of biological functions that they can elaborate (Lajoie et al. 2013). Regulatory proteins can be repurposed or reprogrammed. For example, the VirB DNA binding protein from the *S. flexneri* virulence gene regulon (Section 7.13) has been redirected to regulate the transcription of the *proU* operon in response to temperature; *proU* is a genetic element that is normally controlled by osmotic stress (Kane and Dorman 2011); the Lac repressor has been re-engineered to respond to four new regulatory signals, turning it into a tool for the control of synthetic regulatory circuits (Taylor et al. 2016). Completely novel bacterial transcription factors with new ligand-binding activities have been generated by fusing soluble periplasmic binding proteins to DNA-binding domains, an approach that is likely to find wide application (Juárez et al. 2018).

Molecular microbiology has arrived at a stage in its development where most barriers to progress are intellectual rather than technical. The modular nature of the molecular machines in bacterial cells opens the door to almost limitless possibilities for reprogramming and repurposing: the investigator only has to imagine the application and the required molecular tool can either be found or can be fashioned from pre-existing

components. The chief bottlenecks in the field in the immediate future will be providing the tools and the operators to handle and interpret all of the information being generated in studies of bacteria and their genomes being conducted worldwide. In the final analysis, our capacity to make meaningful progress will be determined by our skill in guiding the intellectual formation of the investigators that will undertake the research.

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