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CHAPTER 1

Capsular Polysaccharides in *Escherichia coli*

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Contents

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I. INTRODUCTION

The expression of extracellular polysaccharide (EPS) material is a common feature of many bacteria. This EPS coats the outside of the bacterial cell and as a consequence plays an intimate role in mediating interactions between the bacterium and its immediate environment. In certain cases the polysaccharide may be tightly associated with the cell surface forming a discrete structure termed a capsule, or it may be shed in the form of EPS or slime. A number of roles have been assigned to polysaccharide capsules and it is clear that in a variety of environments the expression of a capsule confers a selective advantage to the host. The major components of bacterial capsules are highly hydrated, high molecular weight acidic polysaccharides that confer upon bacteria an overall negative charge and hydrophilic properties. There is great structural diversity in capsular polysaccharides both between different bacterial species but also within the same bacterial species. This diversity is a consequence of not only differences in the repeat monosaccharide components but also differences in linkage between the different repeating monosaccharide units. The selective pressure that has driven this diversity is unclear as are the mechanisms by which it has been achieved. However, a consequence of this structural diversity is that there exists a library of diverse polysaccharide structures within the microbiome that may be exploited to engineer novel polysaccharide molecules with particular biochemical, pharmacological, or immunological properties.

II. FUNCTIONS OF BACTERIAL CAPSULES

A number of functions have been assigned to bacterial capsules in different bacteria including adhesion, transmission, resistance to innate host defenses, resistance to the host's adaptive immune response, and intracellular survival (Roberts, 1996). In certain cases it is possible to directly correlate the function of the bacterial capsule with the chemical structure of the capsular polysaccharide. For instance, the adhesion of Group A Streptococci to pharyngeal cells mediated via the interaction between the hyaluoronic acid capsule and CD44, the hyaluronic acid binding protein (Cywes and Wessels, 2001). In the case of invasive pathogens an ability to survive innate host defenses is essential. It has been known for a long time that the expression of a polysaccharide capsule confers some measure of resistance to complement-mediated killing (Roberts, 1996) even though mechanistically the basis for this is not always clear. In the case of capsules that contain sialic acid, binding of factor H and the inhibition of the complement activation cascade can explain this resistance, but with

other capsules it may be due to steric effects and masking the cell surface form the membrane-attack complex. What is clear is that complement mediated resistance is likely to involve a number of cell surface structures which contribute to the overall effect (Burns and Hull, 1998, 1999). The ability of capsules to confer resistance to phagocytosis by polymorphonuclear (PMNL) cells has long been assigned to the negatively charged polysaccharide capsule and the repulsive effect on the negatively charged cell surface of the PMNL (Roberts, 1996). However, it is likely that poor opsonization with complement of encapsulated bacteria will also play a role in this protection (Roberts, 1996). The *Escherichia coli* K1 capsule is vital for intracellular survival and crossing the blood brain barrier (Kim *et al.*, 2003). Specifically, the K1 capsule moderates the maturation process of *E. coli* containing vacuoles inside endothelial cells preventing fusion with lysosomes (Kim *et al.*, 2003). As such, expression of the K1 capsule is critical to the pathology of the disease.

III. CAPSULAR POLYSACCHARIDES IN *E. coli*

E. coli is a facultatively anaerobic, Gram-negative bacillus that forms part of the commensal human bowel flora, but in the environment can be found in soil and water, usually as the result of fecal contamination. Although considered a commensal organism and widely used as a workhorse in molecular biology research, *E. coli* is capable of causing a range of diseases in humans and animals, including gastro-intestinal and urinary tract infections, meningitis, and septicemia. A common feature of *E. coli* isolates responsible for extraintestinal infections is the expression of a polysaccharide capsule or K antigen. The expression of certain K antigens is strongly associated with particular infections. For example, the K1 capsule is the most common capsule type found in isolates of *E. coli* causing neonatal meningitis and urinary tract infection. The K5 polysaccharide is associated both with urinary tract infection and sepsis, but not with meningitis. In both cases, these capsules are more often found associated with infection than in the normal intestinal flora of healthy individuals (Kaijser and Jodal, 1984).

There are more than 80 different K antigens in *E. coli*, and originally, they were divided into Groups I and II on the basis of serological, biosynthetic and genetic data (Jann and Jann, 1997). The system has since been restructured to take account solely of biochemical and genetic data, comprising four groups: Group 1 (Ia), Group 2 (II), Group 3 (III), and Group 4 (Ib) (Whitfield and Roberts, 1999). The following sections briefly consider the genetics, biosynthesis and evolution of the capsular polysaccharides of the related Groups 1 and 4, then Group 3, followed by a detailed analysis of the Group 2 capsular polysaccharides.

IV. *E. coli* GROUP I CAPSULES

Group 1 capsules, encoded by the *cps* locus located near *his*, are typified by *E. coli* K30, which is a polymer of galactose, mannose, and glucuronic acid. They are similar to those expressed by *Klebsiella* strains, although in *E. coli* the capsular polysaccharide is expressed in two forms. The first comprises one to several repeat units of the K polysaccharide linked to lipid A-core, and is termed K_{LPS} (Dodgson *et al.*, 1996). This is not synthesized by *Klebsiella* spp. (Whitfield and Roberts, 1999). Lipid A and core are two conserved constituent domains of LPS, the third being the highly variable O antigen. All three components are synthesized separately and ligated together later. Lipid A is formed from UDP-GlcNAc and fatty acids that are transferred to a Kdo disaccharide. The core is an oligosaccharide linker that is formed on lipid A by the sequential transfer of glucose, galactose, and GlcNAc from their nucleotide precursors. The second higher molecular weight polysaccharide forms the capsule proper. In each case the repeating unit of the polysaccharide is identical.

The Group 1 capsule biosynthetic locus is a 16 kb region of DNA encoding 12 ORFs located in the same region of DNA as the typical O antigen biosynthetic locus in *E. coli* K-12 and strains bearing capsules from Group 2, 3, or 4 (Drummelsmith and Whitfield, 1999; Rahn *et al.*, 1999). The Group 1 gene cluster is distinguished by the presence of an essential polymerization and translocation region dedicated to capsule expression (*wzi–wzc)* that is conserved between different strains of *E. coli* expressing Group 1 capsules and *K. pneumoniae* (Whitfield, 2006). Strains bearing Group 1 capsules are unable to co-express colanic acid, the first evidence for which emerged when it was found that multicopy RcsB in *E. coli* K30, K1, K5, and K-12 resulted in a mucoid phenotype at 37 °C, but only in serotype K30 was mucoidy the result of serotype-specific capsular polysaccharide expression: in all of the other strains this was due to colanic acid expression (Drummelsmith and Whitfield, 1999; Keenleyside *et al.*, 1992). Unlike bacteria belonging to Groups 2, 3, and 4, the genes responsible for synthesis of this EPS have been lost from Group 1 strains, probably through extensive DNA re-arrangements involving replacement of the O-antigen synthesis region by a large segment of DNA laterally transferred from *K. pneumoniae* (Rahn *et al.*, 1999; Whitfield, 2006).

It is not known how Group 1 capsules are linked to the bacterial cell surface, but unlike K_{LPS}, it does not involve LPS (Whitfield, 2006). The repeat units of these capsules are formed on the cytoplasmic face of the bacterial inner membrane followed by export across the inner membrane and polymerization to form the capsular polysaccharide. The precursor monosaccharides are first synthesized by the appropriate enzymes (e.g., ManB and ManC are responsible for generating UDP-mannose).

The glycosyltransferase enzyme WbaP then transfers galactose from free UDP-galactose in the cytoplasm to undecaprenyl phosphate, a lipid carrier molecule (Drummelsmith and Whitfield, 1999; Roberts, 1996). A further glycosyl transferase, namely WbaZ, then completes the formation of the repeating unit backbone, -2)-α-Man-(1–3)-β-Gal-(1-. A side-branch also exists, formed from repeating glucuronic acid and galactose residues by the glycosyltransferase WcaN, which is linked to the main polysaccharide chain by WcaO (Drummelsmith and Whitfield, 1999). The repeat units are flipped across the bacterial inner membrane by an unknown process involving Wzx before being attached to the reducing terminus of the nascent undecaprenyl phosphate-linked polysaccharide at its reducing terminus by Wzy on the periplasmic face of the inner membrane. The Wzy protein is believed to function as a polymerase, although this role has not been directly demonstrated (Whitfield, 2006). Mutations in Wzy abolish capsule expression and reduce the length of K_{LPS} to one repeat unit (Drummelsmith and Whitfield, 1999). At some point the length of the nascent polymer must trigger export, and either Wzy or WaaL may play a role in determining the chain length (Whitfield, 2006). Strains carrying mutations in *wzy* are acapsular and add only one repeat unit onto K_{LPS} (Drummelsmith and Whitfield, 1999). Polymerization is terminated for K_{LPS} by WaaL-mediated transfer of the polymer to lipid A-core.

Translocation of the finished polymer involves the products of the genes *wza*, *wzb*, and *wzc*, encoded within a polymerization and translocation locus located upstream of the serotype-specific biosynthetic loci. Wzi (formerly *orf3* or *orfX*) is not essential for capsule expression (Drummelsmith and Whitfield, 1999), but *wzi* mutants show a significant reduction in cell associated and cell-free polymer (Rahn *et al.*, 2003). Wza is a surface-exposed outer membrane lipoprotein that forms octameric structures in the outer membrane, the bulk of which are exposed in the periplasm, and is essential for surface presentation of capsule (Collins *et al.*, 2007; Dong *et al.*, 2006; Drummelsmith and Whitfield, 2000; Nesper *et al.*, 2003). It represents the outer membrane accessory (OMA) protein of Group 1 capsules. OMAs carry a conserved signal peptidase motif that, after cleavage, is modified at a conserved cysteine residue to yield a lipoprotein (Paulsen *et al.*, 1997). Failure to acetylate Wza results in a failure of capsule export and intracellular accumulation of capsule polymer (Nesper *et al.*, 2003). Wza is found associated with Wzc, interacting via their periplasmic domains (Collins *et al.*, 2007). Wza-Wzc interaction is believed to hold Wza in an open conformation conducive to capsule export, as the Wza octomer encloses a large central cavity with a 22Å pore (Collins *et al.*, 2007). However, in the absence of Wzc, the Wza ring is closed at both the periplasmic and external faces (Beis *et al.*, 2004; Dong *et al.*, 2006). Wzc is a tyrosine autokinase protein similar to the chain-length regulating protein Wzz found in strains from other capsule groups.

Wzc differs however, by an extension at its C-terminus containing walker A and B box motifs and a tyrosine-rich region (Collins *et al.*, 2007; Nesper *et al.*, 2003; Wugeditsch *et al.*, 2001), and as such is a representative of the MPA-1 family of cytoplasmic membrane-periplasm auxiliary proteins (Paulsen *et al.*, 1997). At least four of these tyrosine residues must be phosphorylated for function, although no individual residue is essential (Paiment *et al.*, 2002; Wugeditsch *et al.*, 2001). Wzb is a protein tyrosine phosphatase that dephosphorylates Wzc. Both *wzb* and *wzc* mutants result in an acapsular phenotype, so it is likely that a cycle of phosphorylation and dephosphorylation is important in properly regulating capsule expression. Additionally, multicopy *wzb* causes a near four-fold reduction in capsule production (Paiment *et al.*, 2002; Whitfield, 2006).

V. *E. coli* GROUP 4 CAPSULES

Group 4 capsules are found in enteropathogenic (EPEC) and some enterotoxigenic (ETEC) *E. coli*, but similar capsule types are not known to exist in other bacteria (Peleg *et al.*, 2005; Whitfield, 2006). Members of this group are alternatively known as O-antigen capsules as they are very similar to LPS, with up to 50% of the capsule polymer expressed by these strains linked to lipid A-core (Dodgson *et al.*, 1996; Roberts, 1996). LPS normally consists of a lipid A-core moiety linked to an O polysaccharide. These bacteria are said to possess smooth LPS. Mutants that fail to produce the O-specific polysaccharide and thus display only lipid A and the core oligosaccharide are termed rough mutants and are virtually avirulent. The capsular O antigens, e.g., K40, are able to mask co-expressed O8/O9 antigen in serological analyses in a similar manner to other K antigen capsules, hence them originally being given a K antigen designation (Whitfield and Roberts, 1999). Unlike strains bearing Group 1 capsules, the colanic acid locus of Group 4 strains is intact, and colanic acid can be co-expressed with the capsular polysaccharide (Whitfield, 2006).

Biosynthesis of the Group 4 capsular antigen occurs in the same manner as described for Group 1 capsules except that the initiating glycosyltransferase enzyme is Wca, transferring GlcNAc or *N*-acetyl galactose (GalNAc), not WbaP (Whitfield and Roberts, 1999). The mechanism by which Group 4 capsules are transported involves homologues of the Group 1 transport genes Wza, Wzb (Etp), and Wzc (Etk) located at 22 minutes on the *E. coli* chromosome (Peleg *et al.*, 2005). Wza at this locus is also able to function, albeit modestly, in the export of Group 1 capsules (Drummelsmith and Whitfield, 2000; Wugeditsch *et al.*, 2001). Four additional genes that form part of the same operon (*ymcABCD*) are all essential for capsule expression, although their function is unknown (Peleg *et al.*, 2005).

Unlike the capsule, the polysaccharide chain length of K_{LPS} is under the control of the enzyme Wzz in strains with Group 4 capsules. Wzz is absent from Group 1 strains, which explains why Group 1 K_{LPS} bears only low molecular weight polysaccharides. In the absence of Wzz, K_{LPS} chain length is uncontrolled, usually resulting in shorter oligosaccharide chains. Polymer extension is terminated by transfer of the nascent polymer to the lipid A-core acceptor molecule by the enzyme WaaL (Whitfield and Roberts, 1999).

VI. *E. coli* GROUP 3 CAPSULES

Prime examples of this group of *E. coli* capsular polysaccharides include the K10 and K54 antigens, formerly assigned to Group III (Clarke *et al.*, 1999; Russo *et al.*, 1998). Gene clusters and capsules of Group 3 show similarities to those of Group 2 in terms of their lack of thermostability, possession of homologues to the Group 2 biosynthetic genes KpsM, T, C, and S—the latter two of which are functionally interchangeable between the two groups—and similarity to the capsules of *N. meningitidis* and *H. influenzae* (Pearce and Roberts, 1995; Whitfield and Roberts, 1999). Unlike Group 2 capsule gene clusters, those of Group 3 are not thermo-regulated, and the cluster shares little sequence identity with the Group 2 genes (Whitfield, 2006). The Group 3 cluster is significantly re-arranged compared to Group 2 clusters, being located downstream of a truncated *kpsM* gene homologous to that found in Group 2 (Russo *et al.*, 1998). This is followed by region 1 containing *kpsD*, *kpsM*, *kpsT*, and *kpsE*, the region 2 biosynthetic genes, and a third region composed of *kpsC* and *kpsS*. A remnant of IS110 from *Streptomyces coelicolor* is located immediately downstream of the cryptic *kpsM* in both K54 and K10, and in K10 there is an IS3 element upstream of the cryptic *kpsM* (Clarke *et al.*, 1999; Russo *et al.*, 1998) and a prophage-like element downstream of the capsule gene cluster. It has been suggested that the similarity between the truncated *kpsM* gene and *kps*M of Group 2 capsules indicates that Group 3 strains are descended from a Group 2 progenitor that has acquired a new capsule gene cluster through horizontal transfer. The presence of several IS sequences in these clusters may provide clues to the evolution of the Group 3 capsules (Clarke *et al.*, 1999; Russo *et al.*, 1998).

VII. *E. coli* GROUP 2 CAPSULES

Group 2 capsules are expressed by many extraintestinal isolates of *E. coli*, and closely resemble those found in *N. meningitidis* and *H. influenzae* (Frosch *et al.*, 1991). Capsular polysaccharides of this group are linked

via their reducing termini to phosphatidyl-Kdo, which may play a role in anchoring the capsular polysaccharide to the cell surface (Finke *et al.*, 1991). Group 2 capsules are distinguished by their temperature-regulated nature, being expressed at 37 °C, but not below 20 °C, and this control of expression has been shown to be transcriptional in nature (Rowe *et al.*, 2000; Simpson *et al.*, 1996). The differential expression allows for selective expression of the capsule when bacteria encounter a host, and newly synthesized capsule appears within 25–30 min of a temperature upshift (Jann and Jann, 1997).

A. Genetics and evolution of *E. coli* Group 2 capsules

Group 2 capsule gene clusters are organized into three principal regions. Regions 1 and 3 contain the genes responsible for transport of newly synthesized capsular polysaccharide from the cytoplasm to the bacterial cell surface, and are conserved in all Group 2 capsule gene clusters. Region 2 encodes the genes responsible for the synthesis of the polysaccharide and its precursors (Fig. 1.1) (Whitfield, 2006). Region 1 comprises a single transcriptional unit containing six genes, *kpsFEDUCS*. There is a σ^{70} promoter (PR1) located 225 bp upstream of *kpsF* (Simpson *et al.*, 1996). No alternative sigma factor recognition sites are present in this promoter, although it does harbour a binding site for the DNA architectural protein, integration host factor (IHF) (Rowe *et al.*, 2000). Transcription from PR1 generates an 8 kb polycistronic message that is later processed to yield a separate 1.3 kb *kpsS*-specific transcript, and this processing has been proposed to play a role in the differential expression of this gene

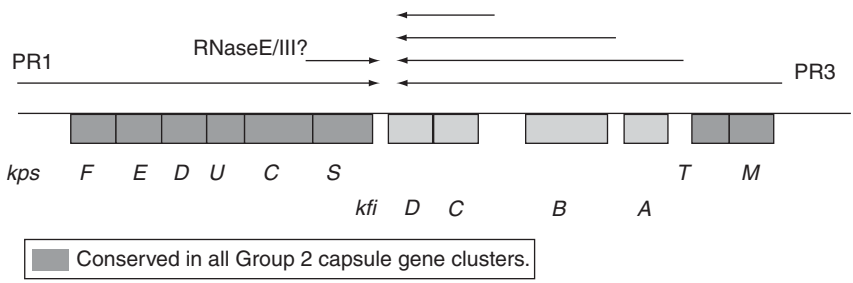

FIGURE 1.1 Genetic organization and transcription pattern of the *E. coli* K5 capsule gene cluster. The *kps* genes are conserved in all Group 2 capsule gene clusters and encode the proteins for polysaccharide export. The *kfi* genes are K5-specific and would be replaced by other capsule specific genes in different K antigen expressing *E. coli*. The two major promoters are shown as PR1 and PR3 and the arrows denote the detected transcripts at 37 °C. The mechanism by which the *kpsS*-specific transcript is generated is unclear.

(Fig. 1.1) (Roberts, 2000). The *kpsF* gene contains a Rho-dependent transcriptional terminator. Rho is an RNA-binding protein that exists as a hexamer, requiring a particular DNA sequence –$CACA(Y)_3$ and $C(N)_3CC$, also known as *rut*—approximately 200 bp upstream of the termination site (Ciampi, 2006). The Rho protein is proposed to track behind RNA polymerase as it proceeds through transcription. If RNA polymerase pauses, transcription is terminated when the Rho protein catches up (Ciampi, 2006). These intragenic terminators are believed to play a role in reducing unnecessary transcription during times of physiological stress.

Region 3 contains two genes, *kpsMT*, which are involved in transport of the capsular polysaccharide across the cytoplasmic membrane (Fig. 1.1) (Bliss and Silver, 1996). The region 3 promoter (PR3) is located 741 bp upstream of the first of the two genes in region 3, *kpsM*. Transcription from this promoter is driven through region 2, past a Rho-dependent transcriptional terminator that exists between *kpsT* and *kfiA*, the first gene of region 2.

Unlike regions 1 and 3, region 2 is serotype-specific (Roberts, 1996). In the K5 gene cluster, this region consists of *kfiA*, *kfiB*, *kfiC*, and *kfiD*. A fifth ORF was identified, although expression of the predicted 16 kDa gene product could not be demonstrated (Fig. 1.1) (Petit *et al.*, 1995). In serotype K1, region 2 consists of six genes, *neuDBACES* (Whitfield, 2006). Region 2 of the K5 capsule gene cluster has a higher A+T content (66%) than either region 1 or 3 which possess 50% and 57% AT respectively, values more typical for the *E. coli* chromosome (Blattner *et al.*, 1997; Petit *et al.*, 1995). This suggests that region 2 has evolved through the acquisition of capsule gene DNA sequences from other bacteria. It is not known precisely how this has happened, but it is believed that homologous recombination events between regions 1 and 3 of incoming and resident capsule DNA clusters may account for this (Roberts, 1996). Such *en bloc* acquisition of biosynthetic loci is supported by the fact that the C-terminal regions of *kpsS* (region 1) and *kpsT* (region 3) from K1 and K5 which flank, and therefore form junctions with, region 2 harbor the greatest divergence in sequence of the conserved regions (Boulnois and Jann, 1989; Pavelka *et al.*, 1994; Petit *et al.*, 1995; Roberts, 1996). Additionally, it has been suggested that *kfiD* might have been acquired from a Streptococcal species (Munoz *et al.*, 1998). Relatively long intergenic regions exist in region 2: *kfiA* and *kfiB* are separated by 340 bp, and *kfiB* is separated from *kfiC* by 1293 bp (Petit *et al.*, 1995). The purpose of these regions has not been determined, although three promoters have been identified in region 2, each upstream of *kfiA*, *kfiB*, and *kfiC*, generating 8, 6, and 3.5 kb transcripts, respectively. These promoters are relatively weak (Petit *et al.*, 1995), and are probably insufficient for levels of the biosynthetic proteins necessary to express a K5 capsule (Stevens *et al.*, 1997). Instead, the vast majority of region

2 message is produced by read-through transcription from PR3, generating a polycistronic transcript containing both the regions 3 and 2 genes (Stevens *et al.*, 1997).

B. Biosynthesis of *E. coli* Group 2 capsular polysaccharides

Synthesis of Group 2 capsular polysaccharides occurs on the inner face of the cytoplasmic membrane (Roberts, 1996). The current model for capsule synthesis is one that is linked to polysaccharide export via a hetero-oligomeric synthesis-export complex of the region 2 proteins KfiABC and D bound to the bacterial inner membrane together with the Kps export proteins which span the periplasm and outer membrane (Fig. 1.2) (McNulty *et al.*, 2006; Rigg *et al.*, 1998; Whitfield, 2006). In serotype K1, the polysialic acid capsule is synthesized from activated UDP-NeuNAc acid produced from *N*-acetylmannosamine (ManNAc)

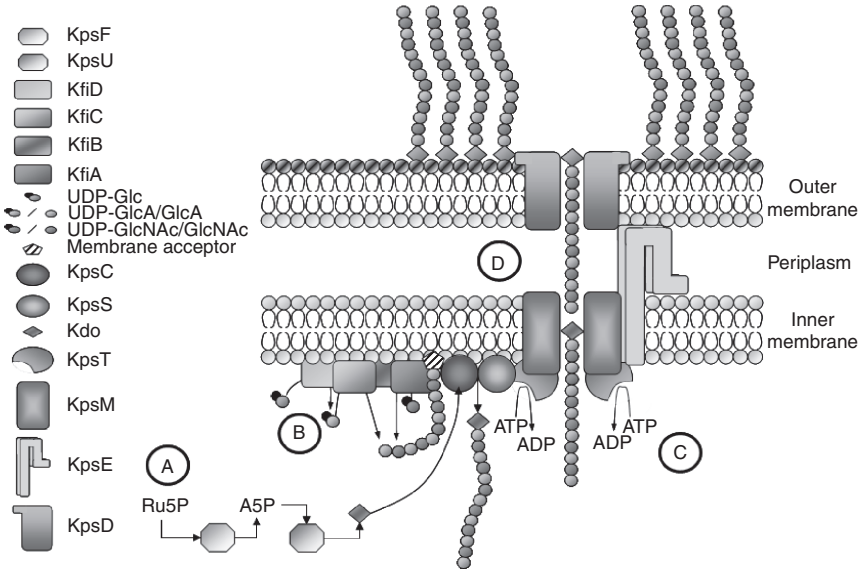

FIGURE 1.2 Synthesis and export of the K5 capsular polysaccharide. Synthesis of the K5 polysaccharide occurs on the inner face of the cytoplasmic membrane (A) by successive transfer of GlcA and GlcNAc residues at the nonreducing end of the nascent polysaccharide by KfiC and KfiA. KpsF converts ribulose 5-phosphate (Ru5P) to arabinose 5-phosphate (A5P), which is an intermediate in the production of CMP-Kdo by KpsU (B) KpsS and KpsC are believed to play a role in the transfer of Kdo to the reducing terminus of the finished polysaccharide, and initiation of polymer export. Transport across the cytoplasmic membrane is achieved by KpsM and KpsT (C), whilst transport to the cell surface requires KpsE and KpsD (D). (See Color Plate Section in the back of the book.)

and phosphoenolpyruvate (Bliss and Silver, 1996). Neither lipid cofactors nor lipid-linked intermediates (e.g., undecaprenyl phosphate) are required for K5 polysaccharide synthesis, but a polyisoprenoid lipid intermediate is used in K1 synthesis (Bliss and Silver, 1996). The initiating sugar, and its acceptor molecule upon which the polymerization reaction occurs, have not been confirmed (Jann and Jann, 1997), although recent observations have suggested that initiation involves the attachment of GlcNAc to an unidentified membrane acceptor in K5 (Roman *et al.*, 2003).

Synthesis of the K5 polysaccharide occurs via the step-wise addition of alternating GlcA and GlcNAc residues from their nucleoside-sugar precursors to the nonreducing end of the polysaccharide (Fig. 1.2). KfiA is an α-UDP-GlcNAc glycosyltransferase enzyme whose function is to catalyze the addition of GlcNAc to the nonreducing terminus of a nascent K5 polysaccharide chain via an α-1,4-glycosidic bond (Hodson *et al.*, 2000; Roman *et al.*, 2003). Glycosyltransferase enzymes function by two main mechanisms, namely retention or inversion of the α- or β-configuration of the anomeric carbon atom on the sugar substrate, depending on whether an α- or β-linked product is to be formed. In this case, it is presumed that KfiA retains the α-configuration of the donor sugar (Saxena *et al.*, 1995). The role of KfiB has not as yet been elucidated, but it may be a structural one, supporting the biosynthetic complex: mutation of *kfiB*, as with the other genes of region 2, abolishes K5 capsule expression (Hodson *et al.*, 2000). KfiD is the enzyme responsible for dehydrogenation of UDP-glucose, itself formed from glucose-6-phosphate, to form UDP-GlcA, a key substrate for the K5 polysaccharide, but one which is not otherwise required by *E. coli* K5 (Roman *et al.*, 2003; Sieberth *et al.*, 1995). Overexpression of KfiD reduces the amount of K5 polysaccharide produced, possibly because the increased production of UDP-glucose reduces intracellular stocks of glucose-6-phosphate. This molecule exists in equilibrium with fructose-6-phosphate, the precursor of UDP-GlcNAc, and so the reduction of the precursor could lead to stalling of initiation and/or polymerization of the capsule polysaccharide at the point of GlcNAc addition (Roman *et al.*, 2003). Catalysis of the addition of UDP-GlcA to the nascent polysaccharide is the responsibility of the β-UDP-GlcA β-glycosyltransferase enzyme, KfiC (Griffiths *et al.*, 1998). The *kfi* gene products are also structurally important for the synthesis-export complex, as targeting of KfiA to the cytoplasmic membrane requires KfiC, targeting of KfiC requires both KfiA and KfiB, and targeting of KfiB requires KfiA and KfiC (Hodson *et al.*, 2000). KpsF, the product of the first gene in region 1, catalyzes the conversion of ribulose 5-phosphate to arabinose 5-phosphate, an intermediate in Kdo synthesis (Meredith and Woodard, 2006; Tzeng *et al.*, 2002). The capsule gene clusters of *N. meningitidis* lack *kpsF* homologues, thus explaining the defects that these bacteria suffer in capsule and LOS expression when the *kpsF* homologue located elsewhere

on the chromosome is mutated (Tzeng *et al.*, 2002). Nonpolar mutations in *kpsF* reduce surface presentation of capsule 10-fold in *E. coli* K1, with a concomitant intracellular accumulation of capsular polysaccharide (Cieslewicz and Vimr, 1997), although it is unclear why this should be given that Kdo has no known role in K1 expression. KpsF is not required for expression of the K5 capsule (Simpson *et al.*, 1996), probably due to the presence of the *kpsF* homologue *ybrH* (but not *gutQ*) located elsewhere on the chromosome (Simpson *et al.*, 1996; Tzeng *et al.*, 2002). Similar accumulation of polysaccharide is not observed in *N. meningitidis*, whose *kpsF* mutants can be complemented by K1 *kpsF* (Tzeng *et al.*, 2002).

At 37 °C, strains bearing Group 2 capsules display increased CMP-Kdo synthetase activity when compared to growth at 20 °C, and to other *E. coli* serotypes. This is due to up-regulation of *kpsU*, the enzyme responsible for formation of CMP-Kdo, a precursor in the attachment of Kdo to phospholipid (Rosenow *et al.*, 1995b). As with KpsF, KpsU is not essential for capsule expression as a homologue, *kdsB*, is encoded elsewhere on the chromosome (Roberts, 1996). The KpsC and KpsS proteins are able to associate with the inner membrane in the absence of other capsule proteins (Rigg *et al.*, 1998), and believed, on the basis of similarity with the LipA and LipB proteins of Meningococci, to be responsible for the attachment of phosphatidyl-Kdo to the reducing terminus of the finished capsular polysaccharide immediately before export (Arrecubieta *et al.*, 2001; Jann and Jann, 1997; Rigg *et al.*, 1998). In addition to this, they play a vital role in stabilising the proposed capsule synthesis complex on the bacterial inner membrane, as KpsC and KpsS (in addition to KpsM and KpsT) are required for the targeting of KfiA and KfiC to the inner membrane (Rigg *et al.*, 1998). A recent study with *E. coli* K1 has suggested that KpsC forms part of a minimal set of proteins, including NeuE and NeuS, required for *de novo* synthesis of polysialic acid *in vitro* and *in vivo* (Andreishcheva and Vann, 2006). Polymerization is dependent on KpsC, suggesting a role for it in initiation of capsule biosynthesis. In the same study, KpsS was shown to increase the yield of capsule polymer by an unknown mechanism (Andreishcheva and Vann, 2006).

VIII. EXPORT OF *E. coli* GROUP 2 POLYSACCHARIDES

Translocation of capsular polysaccharides is a significant problem for bacteria due to their very high molecular weight and negative charge, and the fact that it must be moved across both the inner and outer membranes via the periplasm. Transfer across the cytoplasmic membrane is achieved by KpsM and KpsT, whereas KpsD and KpsE are responsible for transport across the periplasm (Fig. 1.2). The capsule export pathway does not recognize a specific motif in the structure of the capsular

polysaccharide that would enable it to be transported out of the cell. The K5 region 3 genes can complement loss of function in K1 capsular genes, and capsule expression in hybrid systems combining components of the *Actinobacillus pleuropneumonia* and *E. coli* export pathways has been demonstrated (Roberts, 1996; Silver *et al.*, 2001). KpsM and KpsT form an ATP-binding cassette type 2 (ABC-2) transporter. KpsM has at least six transmembrane domains and a total of five loops. Three of the loops are located in the periplasm and two in the cytoplasm. One model proposes that two molecules of KpsM form a pore in the inner membrane. A molecule of KpsT, which contains an ATP-binding domain, is bound to each KpsM molecule, whilst simultaneously being associated with the inner membrane. This complex functions to hydrolyze ATP to provide energy for transport of the polysaccharide. Due to the presence of homologues of KpsM and KpsT, a similar mechanism is proposed to exist in *H. influenzae* and *N. meningitidis* (Rigg *et al.*, 1998; Roberts, 1996). Bliss and Silver (1996) proposed a co-insertional model for the function of KpsM and T. Both proteins are required for the initiation of polysaccharide export, and this relies on the interaction of KpsT with the polysaccharide (possibly mediated through KpsC or KpsS), ATP and KpsM. The interaction of the conserved KpsT with various polysaccharide structures is achieved through the recognition of the reducing-terminal phospholipid moiety. Binding of ATP to KpsT results in a conformational change in the protein leading to the insertion of KpsT into the inner membrane, whilst KpsM forms a pore around it. Following this, the bound ATP is hydrolyzed, removing the KpsM/T complex from the membrane and releasing the polysaccharide into the periplasm (Nsahlai and Silver, 2003; Pigeon and Silver, 1997; Silver *et al.*, 2001).

KpsE and KpsD mutants accumulate K5 polymer in the periplasm, confirming their role in transportation of the capsular polysaccharide across the periplasm (Bronner *et al.*, 1993). Typically, ABC-2 transporters act in conjunction with MPA-2 proteins, forming a physical linkage between an ABC-2 transporter and an outer membrane porin, of which KpsE is an example (Paulsen *et al.*, 1997). KpsE possesses an N-terminal transmembrane domain followed by a large periplasmic domain and a C-terminus that is exposed in the periplasm and also associated with the inner membrane (Arrecubieta *et al.*, 2001; Rosenow *et al.*, 1995a). KpsE has been shown to exist as a dimer by chemical cross-linking experiments (Arrecubieta *et al.*, 2001). Translocation of the finished capsular polysaccharide has been suggested to be dependent on an unlinked porin in a similar manner to haemolysin A in *E. coli* which utilizes the unlinked *tolC* gene for export (Schlor *et al.*, 1997; Thanabalu *et al.*, 1998). This is because the *kps* gene cluster lacks an apparent OMA protein, which is typically required to complete the transport process started by KpsM and KpsT (Silver *et al.*, 2001; Whitfield and Roberts, 1999). KpsD shows only limited

similarity to OMAs such as Wza, and is not a lipoprotein (Arrecubieta *et al.*, 2001; Whitfield, 2006), whereas the capsule gene clusters of *H. influenzae* and *N. meningitidis* both possess OMAs (*bexD* and *ctrA* respectively) (Whitfield and Roberts, 1999). Support for the porin theory stems from the observation that over-expression of KpsD in *E. coli* K-12 is lethal—if the hypothesized role was correct, this over-expression would recruit too many porins to the outer membrane, resulting in disruption of the cell's osmotic balance (Bliss and Silver, 1996). If porins are involved, it is not known how the polysaccharides would be transported through the relatively small pores that they generate (Whitfield and Roberts, 1999). OmpT, LamB, OmpF, OmpA, and OmpC, have all been ruled out as candidates in this role (Arrecubieta *et al.*, 2001). Detection of KpsD in both the cytoplasmic and outer membranes lead to the hypothesis that KpsD might assist capsule export in a manner that involves cycling of the protein between both membranes whilst chaperoning the polysaccharide to an outer membrane transport protein (Arrecubieta *et al.*, 2001). In the presence of KpsE, Lpp, and the K5 polysaccharide, KpsD is exposed on the cell surface, anchored in the outer membrane by its N-terminus and has a large C-terminal domain exposed in the periplasm (Arrecubieta *et al.*, 2001; McNulty *et al.*, 2006). However, whether or not KpsD is directly involved in K5 export remains unclear, because it does not form heat stable oligomers in the way that OMAs such as Wza do, and has not yet been shown to form pores in lipid bilayers *in vitro* (McNulty *et al.*, 2006).

The RhsA protein was identified as being necessary for maximal levels of K5 biosynthesis, but does not affect transcription of the biosynthetic genes itself (McNulty *et al.*, 2006). In the absence of RhsA, KpsD, and KpsE are no longer targeted to the poles of the cell, suggesting that RhsA may be required to stabilize the proposed biosynthetic-export machinery (McNulty *et al.*, 2006). It is less likely to be involved with transport *per se*, as *rhsA* mutants are still able to present a K5 capsule, whereas *kpsD* or *kpsE* mutants result in periplasmic accumulation of polymer (Bronner *et al.*, 1993). The finding that RhsA is involved in K5 (Group 2) capsule expression is curious. RhsA has been suggested to be a sugar binding protein and possibly be located in the periplasm (Wang *et al.*, 1998). However the low levels of RhsA expression have made detection of the cellular location of RhsA difficult. As such the role of RhsA in Group 2 capsule expression awaits further clarification.

A. Group 2 capsular polysaccharide synthesis and export are linked

Biosynthesis and export are likely to be linked, and this linkage is believed to be conserved between capsule groups. Coupling of synthesis and export would avoid the problem of assembling a >100 kDa polymer

and exporting it in separate processes at separate sites, and would increase the efficiency of the process by increasing the effective concentration of the required proteins at any one site in the cell (Rigg *et al.*, 1998; Whitfield and Roberts, 1999). The theory is supported by the fact that a trans-envelope complex appears to be formed from the synthetic and export proteins in both Group 1 and Group 2. Mutations in any gene in region 1 or 3 of Group 2 capsule gene clusters adversely affects capsule biosynthesis, indicating that the processes of synthesis and export are linked (Bronner *et al.*, 1993; Roberts, 1996). Mutation of *kpsC*, *M*, *S*, or *T* prevents KfiA and KfiC from associating with the inner membrane. Indeed, KfiC is known to be unstable if it fails to localize to the inner membrane (Griffiths *et al.*, 1998; Rigg *et al.*, 1998). More recently, western blot analysis revealed that interactions between the three proteins KfiA, C and D were essential for their stable localization to the bacterial inner membrane (Hodson *et al.*, 2000). KpsD and KpsE localize to the poles of the cell, as shown by immunofluorescence microscopy, suggesting that this is where the biosynthetic complex is formed. It has been shown that polysaccharide export occurs at these sites at the pole of the cell after which the polysaccharide spreads around the surface of the bacterium to form a capsule (McNulty *et al.*, 2006). The mechanism by which this diffusion occurs is unknown, it may be an active process or reflect the lateral diffusion of lipid linked polysaccharide in the outer-leaflet (McNulty *et al.*, 2006). Although KpsD is intrinsically polar, this is not true for KpsE that is held at the pole probably through interactions with KpsD and the capsule biosynthetic complex on the inner-membrane. The biosynthetic complex may itself be targeted to the pole of the cell via the action of KpsS which intrinsically localizes to the poles of the cell, although the mechanism by which this occurs is unknown. *In vivo* chemical cross-linking of KpsS has provided direct evidence of a trans-envelope complex, stretching from the cytoplasmic face of the inner membrane to the exterior of the cell. The complex includes at least KfiA, KpsM, KpsT, KpsS, KpsE and KpsD (McNulty *et al.*, 2006).

Further evidence comes from Group 1 strains. In *E. coli* K30, Wza and Wzc interact, forming a continuum between the cytoplasm and exterior surface of the cell (Collins *et al.*, 2007), and *wza* mutants, in addition to failing to express capsule, do not accumulate polymer internally (Nesper *et al.*, 2003). In *wzc* mutants, the activity of WbaP is markedly reduced (Collins *et al.*, 2007; Nesper *et al.*, 2003), and Wzc has been shown to extend into the cytoplasm, although not in a manner which would form a pore. The exposed ''legs'' of Wzc may form attachment sites for the rest of the synthetic-export complex (Collins *et al.*, 2007). Transport of capsular polysaccharide to the cell surface is believed to occur at Bayer patches—areas where the inner and outer membranes come into close apposition (Bayer, 1991; Bayer and Thurow, 1977) although the nature of such sites is

controversial because they can only be visualized with certain methods of preparation (Bayer, 1991). It is possible, but not proven, that the sites at which the capsule export machinery is located coincides with a Bayer junction (McNulty *et al.*, 2006), and recent studies have suggested that the periplasm is compressed at the site of Wza-Wzc interaction (Collins *et al.*, 2007).

IX. REGULATION OF CAPSULE EXPRESSION IN *E. coli*

Given the substantial energy investment that the biosynthesis and export of a capsular polysaccharide represents to bacteria, and that expression of a capsule is not appropriate for bacteria in all circumstances, it is to be expected that capsule expression is tightly regulated. For example, although polysaccharide capsules protect bacteria from the effects of serum-mediated killing, the capsule often prevents intimate adhesion of bacteria to host tissues, and further dissemination into the host. The following sections review the current understanding of capsule regulation in *E. coli*.

A. Regulation of the *E. coli* K30 capsule and expression of colanic acid (Slime) in *E. coli* K-12 strains

The principal role of colanic acid appears to be in protection from the external environment, being necessary both for prevention of desiccation and biofilm formation, and its expression is induced in response to osmotic shock (Ophir and Gutnick, 1994; Sledjeski and Gottesman, 1996). Colanic acid can be co-expressed with *E. coli* polysaccharide capsules of Groups 2, 3, and 4, but not with Group 1. However, the regulation of both colanic acid and Group 1 K antigens is similar, involving the Rcs phosphorelay system, a highly conserved regulatory system amongst the γ-proteobacteria.

The Rcs phosphorelay system was originally considered a two-component regulatory system involving RcsC, RcsB and an accessory protein, RcsA (Gottesman and Stout, 1991). Two-component regulatory systems allow bacteria to couple a stimulus, detected by a sensor protein, to a specific response in the form of altered gene expression. This occurs via a response regulator protein, typically a transcriptional regulator, whose activity the sensor protein controls through phosphorylation of conserved aspartate residues (Stock *et al.*, 2000). However, the model has recently been expanded (Majdalani and Gottesman, 2005), and the system now includes: RcsF, an outer membrane lipoprotein and sensor which is able, by an unknown mechanism, to transduce a signal to RcsC; RcsC is an inner membrane-bound sensor histidine kinase; RcsD,

a membrane-bound phosphotransfer protein; and RcsB, a transcriptional response regulator. The accessory protein RcsA is also involved in colanic acid gene expression, and provides a layer of regulation independent of the phosphorelay system (Majdalani and Gottesman, 2005). Briefly, a phosphorelay system usually involves the transfer of phosphate from a histidine residue within the sensor, which is typically autophosphorylating, to a conserved aspartate residue within its target. Atypically, RcsC contains a response regulator domain in addition to a histidine kinase domain, but it lacks a histidine phosphotransfer domain. This function is provided by RcsD (Takeda *et al.*, 2001). Upon activation, phosphate is transferred from the kinase domain to the response regulator domain within RcsC. The phosphate moiety is then transferred to a histidine residue within RcsD, and from there to the response regulator RcsB, resulting in its activation. Activated RcsB is able to bind to promoter regions as a homodimer, binding near the –35 region, probably through interaction with RNA polymerase (Majdalani and Gottesman, 2005). However, RcsB is also capable of forming heterodimers with RcsA, which is a target for the ATP-dependent protease Lon (Majdalani and Gottesman, 2005). As a result, RcsA is typically present at very low levels in the cell. RcsAB heterodimer formation shields RcsA from the action of Lon, and permits subsequent direct activation of RcsA-dependent target promoters, including the colanic acid biosynthetic operon. Strains inactivated for *lon* become independent on RcsC for colanic acid overexpression and present a mucoid phenotype, as the half-life of RcsA is dramatically increased (Gottesman and Stout, 1991). This also suggests that an alternative mechanism for activating RcsB must exist. The binding site is altered to a distantly upstream region containing what has been termed the RcsAB box with a core consensus sequence of TaAGaatatTCctA (Wehland and Bernhard, 2000). Group 1 capsular polysaccharide expression is also upregulated by the Rcs phosphorelay system, resulting in a mucoid phenotype. However, it is not involved in wild type levels of capsule expression as grown under laboratory conditions (Jayaratne *et al.*, 1993). Unlike the colanic acid biosynthetic operon of *E. coli* K-12, Group 1 capsular polysaccharides are not temperature-regulated and possess no RcsAB box immediately upstream of the capsule gene cluster. Basal transcription of these genes is unaffected by the Rcs phosphorelay system (Rahn and Whitfield, 2003). In these strains, there is an RcsAB box upstream of *galF*, whose gene product is involved in the synthesis of UDP-glucose, a sugar precursor for capsule synthesis. Multicopy RcsB was found to induce the transcription of *galF*, concomitant with an increase in K30 expression and a mucoid phenotype (Rahn and Whitfield, 2003). Thus, Group 1 capsule expression is in part regulated by the availability of activated sugar precursors, which in turn may be increased through activation of the Rcs phosphorelay system

(Rahn and Whitfield, 2003). In addition, both *E. coli* Group 1 capsule gene clusters and the colanic acid gene cluster are dependent upon a sequence downstream of their promoters known as JUMPStart (Just Upstream of Many Polysaccharide gene Starts) (Hobbs and Reeves, 1994). This is a conserved 39 bp sequence present in the 5′ untranslated region (UTR) of many polysaccharide genes, and contains a further conserved GGCGGTAG motif known as *ops* (operon polarity suppressor) which must be present on the transcribed mRNA of a gene in order to function (Nieto *et al.*, 1996). During transcription, RNA polymerase pauses at the *ops* sequence whilst the anti-termination factor RfaH interacts with the exposed, noncoding DNA strand in the melted transcription bubble. Transcription elongation is then accelerated, and the RNA polymerase-RfaH complex is subsequently less sensitive to transcription termination signals (Artsimovitch and Landick, 2002; Bailey *et al.*, 2000). Consequently, RfaH is often essential for the transcription of large polysaccharide gene clusters (Bailey *et al.*, 1997). This is true for the K30 gene cluster, which contains a stem-loop transcription-terminating structure between the *wzc* and *wbaP* genes. Transcription of the genes downstream of *wzc*, and eventual expression of the K30 polysaccharide requires RfaH (Rahn and Whitfield, 2003).

B. Regulation of expression of Group 2 (K5) capsule gene clusters

Transcription of the K5 polysaccharide gene cluster is driven by two convergent temperature-regulated promoters located upstream of regions 1 and 3 (Fig. 1.1) (Simpson *et al.*, 1996; Stevens *et al.*, 1997). This temperature regulation appears to be controlled at the level of transcription, as no detectable transcription originates from either promoter at 20 °C (Cieslewicz and Vimr, 1996; Rowe *et al.*, 2000). Transcriptional regulation of this gene cluster involves the regulatory proteins H-NS, BipA, and IHF, which are discussed in detail below.

Transcription of the region 2 genes is driven by PR3 (Fig. 1.1) and because the region 2 promoters are too weak to enable the bacterium to express a capsule (Petit *et al.*, 1995), expression of the K5 capsular polysaccharide is absolutely dependent upon RfaH, which acts on an *ops* sequence present in the 5′ UTR of *kpsM* (Stevens *et al.*, 1994, 1997). Unlike PR3, there is no dependency upon RfaH for transcription elongation at PR1, and no JUMPStart sequence is present upstream of *kpsF* (Simpson *et al.*, 1996; Stevens *et al.*, 1997). The IHF protein is required for maximum transcription from PR1 at 37 °C and binds to a single site located 130 bp downstream of the transcription start point at PR1 (Fig. 1.1) (Rowe *et al.*, 2000; Tippner *et al.*, 1994; Tupper *et al.*, 1994). Two additional regulators,

H-NS and BipA play an unusual dual role in the temperature regulation of transcription at this promoter: both are required for maximum transcription at 37 °C, yet both also contribute to repression of transcription at 20 °C, and the transcriptional effects are mirrored in the levels of K5 capsule produced by the respective mutants (Rowe *et al.*, 2000). The effect of the *bipA* mutant is unlikely to be the result of modulation of H-NS expression as quantitative western blots showed the level of H-NS to be unchanged by mutation of *bipA* (Dame *et al.*, 2001; Grant *et al.*, 2003). H-NS and BipA play similar roles at PR3, but there is no evidence of a role for IHF at this promoter (Rowe *et al.*, 2000; Stevens *et al.*, 1997). Unlike many of the other capsule types discussed here, the Rcs phosphorelay system is not involved in expression of the K5 capsule gene cluster (Keenleyside *et al.*, 1993; Stevens *et al.*, 1994).

It has been suggested that downregulation of region 1 transcription and capsule expression occurs when UPEC type 1 fimbriae bind to mannose receptors such as those that exist on bladder epithelial cells (Schwan *et al.*, 2005). Preliminary data from strains expressing a fusion of PR1 to a promoterless *lacZ* gene also indicate that capsule gene transcription is down-regulated during biofilm formation on cellulose-containing surfaces, and that in planktonic phase cultures, sub populations of cells expressing very low or no capsule may exist, and that these are the cells which interact with surfaces and initiate biofilm formation (Ebah and Roberts, 2001).

Recently a more detailed understanding of the control of transcription from PR1 has been elucidated which demonstrates that transcription involves a novel interaction between H-NS and the transcriptional activator SlyA (Corbett *et al.*, 2007). These studies have shown that at 37 °C maximal expression from PR1 requires the binding of both H-NS and SlyA at the promoter (Corbett *et al.*, 2007). This requirement for H-NS for SlyA mediated activation explains the observation that at 37 °C, *hns* mutants have reduced capsule expression. Whereas reduced SlyA expression at 20 °C results in an H-NS dominated nucleoprotein complex repressing transcription from PR1. This model for regulation of transcription in which expression depends on the relative cellular levels of H-NS and SlyA has recently been suggested for regulation of the *hlyE* gene in *E. coli* (Lithgow *et al.*, 2007). Recent unpublished data would also suggest that a similar situation exists for the regulation of PR3 (Que and Roberts, unpublished results). The role played by BipA in the regulation of Group 2 capsule gene clusters still awaits elucidation but is likely to be via an as yet unidentified transcriptional activator. The current understanding of the regulation of Group 2 capsule gene clusters is shown in Fig. 1.3.

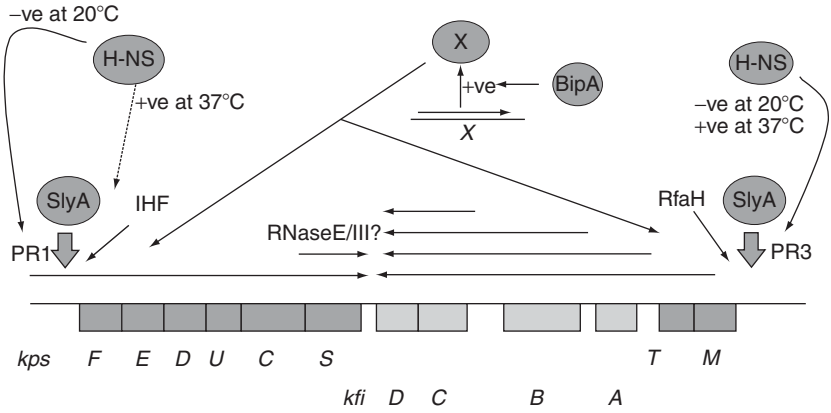

FIGURE 1.3 Regulation of Group 2 capsule gene expression. At 37 °C SlyA and H-NS activate transcription from both PR1 and PR3, with SlyA activity being dependent on the binding of H-NS. At 20 °C H-NS acts to repress transcription. BipA is a translational regulator, loss of which results in a drop in transcription from PR1 and PR3. As such BipA is postulated to regulate the translation of an as yet unknown transcriptional activator termed X.

X. CONCLUSIONS

In the last five years there has been dramatic progress made in our understanding of the expression of capsular polysaccharides by *E. coli*. The application of structural biology to studying Group 1 polysaccharide export has proved fundamental information on the mechanism by which such polysaccharides are exported out of the cell. It is predicted that similar approaches currently being used to study polysaccharide export of Group 2 polysaccharides will prove equally rewarding. It is also apparent that we are beginning to dissect the complex regulatory pathways that control capsule expression in *E. coli*, this being particularly true for the regulation of Group 2 capsules. The challenge is to translate these *in vitro* data into understanding the regulation of polysaccharide expression in the host under conditions of both health and disease. By understanding how capsule expression is regulated *in vivo* we will be able to better understand the role trhat capsules play during survival and growth in the host.

The engineering of polysaccharides in *E. coli* has meet with some success (Priem *et al.*, 2002; Samain *et al.*, 1999; Yavuz *et al.*, 2008). It is our view that polysaccharide engineering represents an area of great unfulfilled potential, especially when one considers that many polysaccharides of biomedical importance, such as heparin, are still extracted from animal sources. As such there is a clear need for an alternative safe

biotechnological route to synthesize these molecules. *E. coli* in many ways represents the ideal host, with it long history as a laboratory organism and clearly *E. coli* capsular polysaccharides are one source of enzymes for generating diverse polysaccharides in *E. coli*. However it will be the efficient expression in *E. coli* of heterologous polysaccharide biosynthesis genes that will offer the route to greatest diversity. The long-term aspiration has to be to exploit *E. coli* capsular transport functions to effectively export heterologous novel polysaccharide in *E. coli* thereby facilitating their rapid purification.

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CHAPTER 2

Microbial PAH Degradation

Evelyn Doyle, Lorraine Muckian, Anne Marie Hickey, and **Nicholas Clipson**

Contents

I. INTRODUCTION

Since the Industrial Revolution, human activity has created a legacy of environmental contamination widespread through industrialized economies (Baveye *et al.*, 1999). As modern economies move to postindustrial economic activity, and heavy industry retreats, more sites affected with by-products from industrial processes are revealed, especially as environmental protection, legislation, and monitoring become more effective, and also as ''brown'' land is released for other uses (Philp *et al.*, 2005).

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One of the challenges facing postindustrial societies is to develop effective strategies to rehabilitate polluted environments.

Many strategies have been advanced to counter these problems, including physical and chemical approaches such as incineration or soil washing. A potentially effective strategy for some polluted sites is the development of biologically based treatments that exploit the degradative ability of microorganisms. The US EPA has estimated that an average of 294,000 contaminated sites require remediation in the next 25 years, at a cost of approximately $209 billion (US EPA, 2004). Between 1982 and 2002, bioremediation was only used at 6% of contaminated sites on the US national priority list. Clearly, industry faces very large costs to remediate sites under the ''polluter pays'' principle, and there is a need to develop the most cost-efficient approaches for ''clean-up.'' Bioremediation technologies are often more cost efficient than physical or chemical approaches to site remediation (Levin and Gealt, 1993; Wood, 1997).

Although biologically based processes have clear cost and environmental advantages, there is incomplete understanding of how these work at the microbial level. For large sites, *in situ* remediation is often desirable where natural microbial communities are exploited and stimulated. In the last 10 years, developments in molecular ecology mean that more sophisticated analysis of degradative communities is possible.

II. POLYCYCLIC AROMATIC HYDROCARBONS (PAHs)

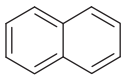
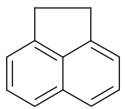
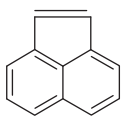
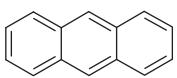
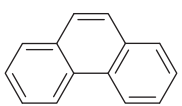
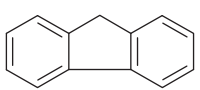
PAHs are a diverse group of over 100 organic compounds containing two or more fused benzene and/or pentacyclic ring structures, in linear, angular, or cluster arrangements (Table 2.1). They are thermodynamically stable due to their negative resonance energy and possess high melting and boiling points together with low water solubilities and vapor pressures (WHO, 1998).

The chemical properties and environmental fate of PAHs depend on the number of aromatic rings present, and the nature of the linkage between these rings. PAHs are generally divided into low molecular weight PAHs (compounds containing up to three aromatic rings) and high molecular weight PAHs (containing four or more aromatic rings). The hydrophobicity, recalcitrance, and toxicity of these compounds increase as the number of aromatic rings present increases (Cerniglia, 1992). Low molecular weight PAHs tend to be more soluble and volatile, and have less affinity for surfaces than high molecular weight PAHs and thus have lesser tendency to accumulate (WHO, 1998).

A. Toxicity

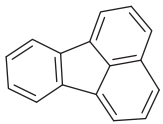
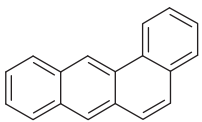
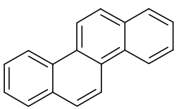
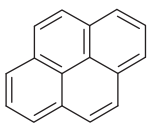
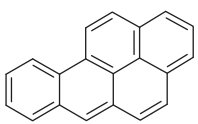
Exposure to PAHs has long been recognized as a significant health risk (Miller and Miller, 1981) and many of these compounds are listed as priority pollutants by both the US EPA and the European Union due to

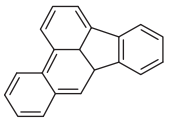
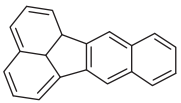
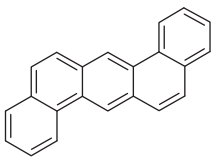
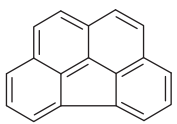
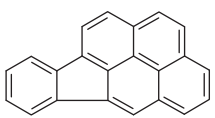
TABLE 2.1 Chemical and physical properties of the 16 PAHs considered priority pollutants (ATSDR, 2005)

PAH	Chemical formula	Molecular weight	Boiling point (°C)	Melting point (°C)	Solubility (mg l^{-1})	Physical appearance
 Naphthalene	$C_{10}H_8$	128	218	77	31	White crystals
 Acenaphthene	$C_{12}H_{10}$	154	279	95	3.8	White or pale yellow crystalline powder
 Acenaphthylene	$C_{12}H_8$	152	280	94	16.1	Yellow crystalline powder
 Anthracene	$C_{14}H_{10}$	178	340	218	0.045	Off-white to pale green crystals
 Phenanthrene	$C_{14}H_{10}$	178	340	99.5	1.1	White crystals
 Fluorene	$C_{13}H_{10}$	166	295	116	1.9	White crystals

(*continued*)

TABLE 2.1 (*continued*)

PAH	Chemical formula	Molecular weight	Boiling point (°C)	Melting point (°C)	Solubility (mg l^{-1})	Physical appearance
 Fluoranthene	$C_{16}H_{10}$	203	375	111	0.26	Light yellow needles
 Benzo[*a*]anthracene	$C_{18}H_{12}$	228	438	158	0.011	Colorless to yellow-brown flakes
 Chrysene	$C_{18}H_{12}$	228	448	253	0.0015	Crystalline powder
 Pyrene	$C_{16}H_{10}$	202	404	145	0.132	Colorless to light yellow solid
 Benzo[*a*]pyrene	$C_{20}H_{12}$	252	495	179	0.0038	Yellow crystals

 Benzo[*b*]fluoranthene	$C_{20}H_{12}$	252	481	168	0.0015	Colorless crystals
 Benzo[*k*]fluoranthene	$C_{20}H_{12}$	252	480	216	0.0008	Yellow crystals
 Dibenzo[*a,h*] anthracene	$C_{22}H_{14}$	278	524	266	0.0005	White to yellow crystalline solid
 Benzo[*g,h,i*]perylene	$C_{22}H_{12}$	276	550	277	0.00026	Large pale yellow–green crystals
 Indeno[1,2,3-cd]pyrene	$C_{22}H_{12}$	276	536	162	0.062	Crystalline solid

their toxic, mutagenic, or carcinogenic properties (ATSDR, 2005). PAH exposure can occur via inhalation, ingestion, or dermal contact, and PAHs have also been reported to cross the placenta causing adverse effects to the embryo and fetus (Collins *et al.*, 1991; Srivastava *et al.*, 1986).

Minimal risk levels for human exposure to PAHs have been determined where sufficient toxicological data is available (Table 2.2). Once ingested, PAHs are rapidly absorbed by the gastro-intestinal tract owing to their high lipid solubility. Metabolism of PAHs in mammals results in the production of reactive epoxide and quinone intermediates via a cytochrome P450 monooxygenase mediated reaction (Sutherland *et al.*, 1995). These intermediates may subsequently be oxidized/hydrolyzed prior to forming covalent adducts with DNA, which can result in mutations and ultimately cancer (Bigger *et al.*, 1983). Cases of lung, liver, intestine, pancreas, and skin cancer have all been associated with PAHs (Samanta *et al.*, 2002). The degree of toxicity is generally related to the molecular weight of the PAH, with the higher molecular weight compounds often exhibiting greater toxicity, although the route of exposure and presence of other toxic substances will influence the toxicological effects. Carcinogenicity is particularly associated with high molecular weight PAHs such as benzo[*a*]pyrene. Mutagenicity studies have indicated that the only PAHs that are clearly not mutagenic are naphthalene, fluorene, and anthracene (Bamford and Singleton, 2005; Cerniglia, 1992; Juhasz and Naidu, 2000).

TABLE 2.2 Minimal risk levels (MRLs) for human exposure to PAHs (ATSDR, 2008)

PAH	Exposure route	Duration of exposure	MRL
Acenaphtene	Oral	Intermediate	0.6 mg/kg/day
Anthracene	Oral	Intermediate	10 mg/kg/day
Fluoranthene	Oral	Intermediate	0.4 mg/kg/day
Fluorene	Oral	Intermediate	0.4 mg/kg/day
Naphthalene	Inhalation	Chronic	0.0007 ppm
	Oral	Acute	0.6 mg/kg/day
	Oral	Intermediate	0.6 mg/kg/day
1-methyl naphthalene	Oral	Chronic	0.07 mg/kg/day
2-methyl naphthalene	Oral	Chronic	0.04 mg/kg/day

Acute = 1–14 days. Intermediate =>14–364 days; Chronic 365 days or longer. MRLs represent the minimal level of human exposure to a compound that is likely to be without appreciable risk of adverse noncancer health effects over a specified duration of exposure. Values are derived only for compounds where reliable and sufficient data exist for a specific duration for a given route of exposure to the substance.

B. Sources of PAHs

PAHs originate from both natural and anthropogenic sources (Blumer, 1976; Mueller *et al.*, 1996). They are natural components of fossil fuels such as petroleum and coal, and may enter the environment as a result of accidental spills and natural leakage of these products. PAHs are also formed during the incomplete combustion of organic matter during volcanic activity, forest and prairie fires, fossil fuel combustion, waste incineration, and to a lesser extent, the cooking of food (Maliszewska-Kordybach, 1999). Other significant sources of PAHs include coal tar and creosote, both by-products of coke production. Coal tar residue is a by-product of coal gasification and up to 300 g kg^{-1} total PAHs have been reported in soils at abandoned coal gasification sites (Sutherland *et al.*, 1995). Creosote, a high temperature distillate of coal tar, has been used for over a century in the wood preservation industry, and consists of a complex mixture of organic chemicals including phenols (5%), N-, S-, and O-heterocyclics (10%), and polycyclic aromatic hydrocarbons (85%) (Rasmussen and Olsen, 2004). Due to the known hazards associated with many of the components of creosote, many countries have introduced restrictions regarding its use. In the US and most European countries only people licensed to use pesticides can apply creosote or handle creosote-treated wood.

C. Environmental contamination

Due to their ubiquitous nature, PAHs are found in a wide range of environments including soils, sediments, groundwaters, and the atmosphere. They have been detected in motor engine emissions, tobacco smoke, and in foods such as charcoal-grilled or smoked meats, smoked fish, leafy vegetables, and fats and oils (Maliszewska-Kordybach, 1999; Trapido, 1999). Over 95% of the PAHs (1000 tonne/year) emitted from UK sources into the atmosphere, emanate from unregulated fires, vehicle emissions, and domestic coal consumption (Wright and Welbourn, 2002).

In aquatic environments, PAHs tend to adsorb to particulate organic matter and most PAH contamination in these environments is concentrated in sediments, or associated with the presence of suspended solids in surface waters (Basu *et al.*, 1987). For example, Mediterranean sediments have been reported to contain between 1 and 20,500 ng g^{-1} PAHs (Baumard *et al.*, 1998). The concentration of PAHs in surface and groundwaters is typically lower, with Menzie *et al.* (1992) reporting total PAH concentrations ranging from 0.1 to 829 ng l^{-1} in surface waters in the USA.

It is estimated that most of the total environmental PAH load (~90%) is found in terrestrial ecosystems, and more specifically, the top 20 cm of the soil horizon (Jones *et al.*, 1989; Maliszewska-Kordybach, 1996, 1999). Soils surrounding crude-oil refineries, fuel storage depots, manufactured gas

plants, and wood preservation facilities are some of the more common sites where industrial scale PAH pollution has been detected (Mahro *et al.*, 1994; Wilson and Jones, 1993). Criteria for acceptable levels of PAHs in soil vary and many countries have yet to publish soil guideline values (SGVs). Where they exist, guidelines are based on an assessment of the risk posed to human health from exposure to contaminated soil and values depend on planned end use (i.e., agricultural, residential, industrial, etc). In some jurisdictions, guidelines refer to the cumulative concentration of several PAHs, whereas others use the concentration of the carcinogenic PAH, benzo[*a*]pyrene alone (Mueller *et al.*, 1996). Guidelines generally refer to soil intervention and target values. Soil concentrations above the intervention value are considered to pose an unacceptable risk to human health, and target values represent background levels of PAHs in soils. The Dutch Ministry of Housing, Spatial Planning, and Environment (VROM) apply an intervention value of 40 mg total PAHs kg^{-1} dry weight of soil and a target value of 1 mg kg^{-1} (Swartjes, 1999; VROM, 2000); these criteria are often used as a reference point by countries which have yet to develop guidelines.

III. DEGRADATION OF PAHs

Numerous abiotic and biotic factors affect PAH degradation in environments such as soil (Fig. 2.1). The number of aromatic rings present and the chemical properties of the PAH have a major effect on its environmental fate, with higher molecular weight compounds persisting for longer

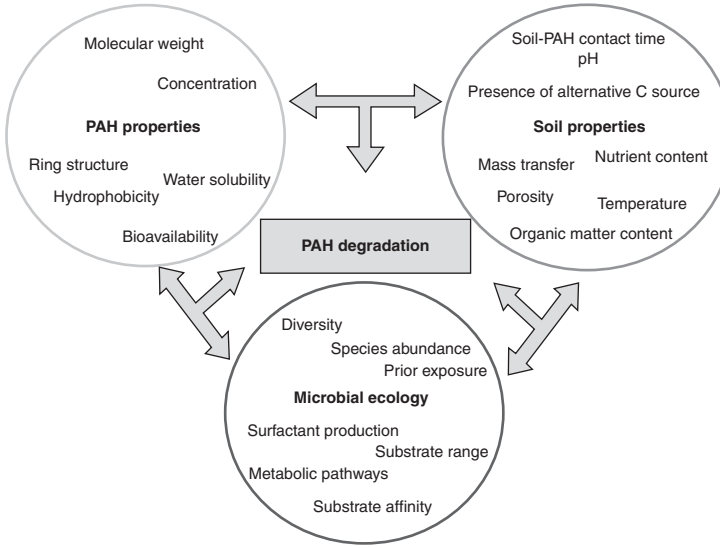


FIGURE 2.1 Abiotic and biotic factors affecting PAH degradation in soil.

periods than their lower molecular weight counterparts. For example, the half-life of the 3-ring PAH phenanthrene in soil varied between 16 and 126 days, whilst the 5-ring PAH benzo[*a*]pyrene had a half life of up to 4 years (Dabestani and Ivanov, 1999; Wickle, 2000). Although abiotic factors such as pH, moisture content, oxygen levels, and nutrient availability influence PAH degradation, it is clear that microbial processes are largely responsible for most degradation (Alexander, 1999; Bossert and Compeau, 1995). If *in situ* microbial processes are to be effectively exploited to accelerate PAH removal from contaminated environments, greater understanding is required of organisms involved and the processes they mediate.

A. Pure culture studies

Culture-based approaches have been used to profile PAH degrading microbial activity in polluted environments. These have included isolation and enrichment of pollutant degrading microorganisms (Heitkamp and Cerniglia, 1988; Mueller *et al.*, 1997), most probable number (MPN), estimates of degraders (Sarkar *et al.*, 2005; Vinas *et al.*, 2005), and estimates of microbial community activity using enzyme assays (Eggen, 1999; Margesin *et al.*, 2000). These approaches have resulted in the isolation of a range of PAH degrading microorganisms, which have been used to elucidate the metabolism of PAHs by specific microbial isolates. Examples of the more commonly isolated organisms are outlined in Table 2.3.

Metabolism of PAHs is best understood for low molecular weight compounds, with a considerable amount of information now existing for the pathways, enzymes, and genetics of PAH degradation particularly amongst the pseudomonads (Cerniglia, 1993; Davies and Evans, 1964; Ensley *et al.*, 1982; Gibson and Parales, 2000). Microorganisms have been found to degrade PAHs as carbon and energy sources, as a means of reducing PAH toxicity and as co-metabolic substrates (Johnsen *et al.*, 2005; Wilson and Jones, 1993). The rate and extent of degradation depend on the number of rings in the specific PAH, with high molecular weight PAHs more resistant to degradation. To date, few bacteria have been isolated that are capable of utilizing PAHs with four or more aromatic rings as sole sources of carbon and energy (Kanaly and Harayama, 2000).

Bacterial degradation of PAHs usually proceeds via an initial oxidation of the PAH to a catechol, which is subsequently hydrolyzed to TCA intermediates (Fig. 2.2). Bacteria typically incorporate two molecules of oxygen into one of the benzene rings through the action of intracellular dioxygenases, forming a *cis*-dihydrodiol that is subsequently converted to a catechol. *Ortho*- or *meta*-cleavage of the catechol yields aliphatic products, which may then be channeled into central metabolism. Once the

TABLE 2.3 Examples of PAH-degrading bacteria, fungi, and algae

Organism	Selected reference
Bacteria	
Pseudomonas spp., *Rhodococcus* spp., *Mycobacterium* spp., *Sphingomonas* spp., *Acinetobacter* spp., *Alcaligenes* spp., *Flavobacterium* spp., *Nocardia* spp., *Xanthomonas* spp., *Comamonas* spp., *Agrobacterium* spp., *Aeromonas* spp., *Arthrobacter* spp., *Acidovorax* spp., *Brevibacterium* spp., *Corynebacterium* spp., *Micrococcus* spp., *Streptomyces* spp., *Vibrio* spp., *Bacillus* spp., *Moraxella* spp., *Stentrophomonas* spp., *Aquamicrobium* spp., *Rhizobium* spp.	Mueller *et al.* (1989, 1990), Davies and Evans (1964), Hickey *et al.* (2007), Walter *et al.* (1991), Juhasz *et al.* (1997), Kelley *et al.* (1993), Boldrin *et al.* (1993), Ye *et al.* (1996), Ryu *et al.* (1989), Kiyohara *et al.* (1976a,b), Wikada *et al.* (2002), Trzesicks-Mlynaz and Ward (1995), Zeinali, 2007; Hamann *et al.* (1999), Aitken *et al.* (1998), Kueth and Rehm (1991), Samanta *et al.* (1999), Dua and Meera (1981), Ghosh and Mishra (1983), Sutherland *et al.* (1990), West *et al.* (1984), Stucki and Alexander (1987), Andreoni *et al.* (2004), Weissenfels *et al.* (1990)
Fungi	
Phanerochaete chrysosporium, Pleurotus ostreatus, Penicillium spp., *Phanerochaete sordida, Cunninghamella* spp., *Cunninghamella elegans, Aspergillus* spp., *Rhizopus* spp., *Bjerkandera* sp.	Cerniglia (1992), Allard and Neilson (1997), Harayama (1997), Eggen (1999), Johnsen *et al.* (2005)
Cyanobacteria and Algae	
Anabaena sp., *Aphanocapsa* sp., *Petalonia* sp., *Chlamydomonas* sp., *Chorella* sp., *Coccochloris* sp., *Dunaliella* sp., *Nostoc* sp., *Oscillatoria* sp., *Porphyridium* sp.	Cerniglia *et al.* (1985), Narro *et al.* (1992a, b)

initial hydroxylated aromatic ring is degraded, the second ring is attacked in the same manner (Kelley *et al.*, 1991, 1993).

Fungi appear to oxidize PAHs as a means of detoxifying these compounds rather than as an initial step in their assimilation (Cerniglia *et al.*, 1985). Nonligninolytic fungi generally oxidize PAHs via cytochrome P450 monoxygenases, resulting in the production of an arene oxide that is

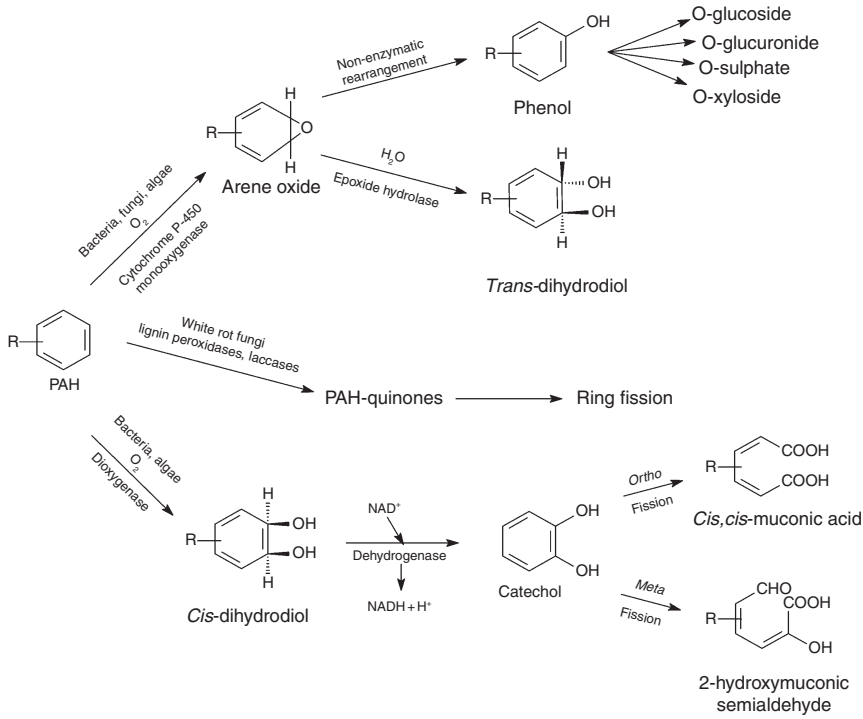


FIGURE 2.2 Possible pathways used by microorganisms to degrade PAHs (Cerniglia, 1992).

subsequently hydrolyzed by an epoxide hydrolase to a *trans*-dihydrodiol (Fig. 2.2). While many of these fungi can transform PAHs to *trans*-dihydrodiols and other oxidized products such as phenols, tetralones, quinones, dihydrodiol epoxides, and various conjugates of the hydroxylated intermediates, few have the ability to mineralize PAHs (Mueller *et al.*, 1996). Fungal metabolites produced are often more water-soluble and chemically reactive than the parent PAH, thus increasing their potential for mineralization by indigenous soil bacteria (Cerniglia, 1997). Ligninolytic fungi, such as *Phanerochaete chrysosporium* and *Trametes versicolor*, use extracellular enzymes involved in lignin degradation, such as lignin peroxidase, manganese peroxidase, and other H_2O_2-producing peroxidases or laccases, to degrade PAHs (Bezalel *et al.*, 1997; Bumpus, 1989; Hammel *et al.*, 1992). These enzymes oxidize a wide range of organic compounds in a nonspecific radical-based reaction. This results in the production of quinones and acids, rather than dihydrodiols.

Prokaryotic and eukaryotic algae have also been reported to oxidize PAHs from hydroxylated intermediates (Fig. 2.2). Cyanobacteria appear to employ monooxygenases for the oxidation of low molecular weight

PAHs, producing metabolites similar to those of mammals and fungi. In contrast, the production of *cis*-dihydrodiols by the eukaryotic alga *Selenastrum capricornutum* growing on benzo[*a*]pyrene indicated a dioxygenase mode of attack, similar to heterotrophic prokaryotes (Narro *et al.*, 1992a,b; Schoeny *et al.*, 1988; Warshawsky *et al.*, 1988, 1995).

PAH degradation is most extensive under aerobic conditions, but is known to occur to some extent in anaerobic environments (Bianchin *et al.*, 2006; Coates *et al.*, 2002; Davidova *et al.*, 2007; Hayes and Lovley, 2002; Spormann and Widdel, 2000; Zhang *et al.*, 2000). Nitrate and sulphate have been reported as alternative electron acceptors (Heider *et al.*, 1998; Holliger and Zehnder, 1996). Although pathways for the anaerobic degradation of naphthalene and phenanthrene have been proposed, details of the mechanisms involved remain to be elucidated (Annweiler *et al.*, 2002).

Metabolism of PAHs by pure cultures has provided information on the types and diversity of metabolic pathways. Nevertheless, it is important to appreciate how this knowledge transfers to microbial processes degrading PAHs in real environments such as polluted soils, sediments, and waters. Biodegradatory studies are now focused on elucidating how microbially mediated degradation works at an environmental level.

B. Culture-independent analysis

In contaminated environments, microorganisms exist amongst complex communities. Nutrient availability can be diverse, with a complexity of available carbon and other nutrient sources that may be used in preference to PAHs. Additionally, most environments would be considered oligotrophic (Hamer, 1992). Although culture-based studies have indicated the types of PAH metabolism that take place, the environmental fate of PAHs is not well understood. A complicating factor in understanding microbial mediation of PAH metabolism in the environment is that less than 1% of microorganisms are culturable (Torsvik and Ovreas, 2002), so that studies carried out on isolates do not necessarily reflect environmental microbial activity.

Recent advances in molecular techniques have extended our ability to profile microbial communities in natural environments using culture-independent methods (Liu *et al.*, 1997; Mills *et al.*, 2003; Torsvik and Ovreas, 2002). Such approaches have focused either on describing microbial diversity and community structure, or on elucidating gene activity involved in PAH degradation. Most attention, to date, has been paid to elucidating microbial community structures utilizing molecular analysis of the rRNA gene complex or other phylogenetically separable genes. After extraction of environmental nucleic acids, specific primers are selected to amplify rRNA gene diversity by PCR, prior to either cloning and sequencing or the use of fingerprint techniques such as denaturing

gradient gel electrophoresis (DGGE), automated ribosomal intergenic spacer analysis (ARISA), and terminal restriction fragment length polymorphism (TRFLP). These approaches generate both phylogenetic information and profiles of overall community structure. For a fuller view of the techniques typically used, the reader is referred to Hugenholtz *et al.*, 1998; Konstantinidis and Tiedje, 2004; Torsvik *et al.*, 1998; Xu, 2006. The challenge for microbial ecologists is to link microbial diversity to individual functional components of PAH metabolism in environments.

Microbial communities have been examined in a range of PAH contaminated environments including soil, rhizosphere, water, sediments, anoxic sediments, and bioreactors (Table 2.4). Most studies, to date, have been concerned with bacterial communities from aerobic environments, although the effects of PAHs on fungal (Salvo *et al.*, 2005), archaeal (Chang *et al.*, 2005), and protozoan community structures (Lara *et al.*, 2007) have also been reported. There are many reports of shifts in microbial community structure occurring during PAH degradation (Grant *et al.*, 2007; Vinas *et al.*, 2005). Bacterial community composition in a creosote-contaminated site was found to be correlated to the type of PAH present rather than with PAH concentration (Muckian *et al.*, 2007). For example, community structure in the presence of naphthalene (2-ring) was quite distinct from that in soil containing multicomponent mixtures of PAHs (2-, 3-, and 4-ring). Plant roots have also been found to influence PAH degradation, with the highest levels of degradation being observed closest to roots; community structure near to root surfaces was quite different from zones further away (Corgie *et al.*, 2004, 2006a). Using a PLFA (phospholipid fatty acid analysis) approach rather than a molecular approach, Yang *et al.* (2007) observed that bacterial PFLA patterns varied in PAH contaminated soils in the presence of different vegetation and over the growing period, whilst Parrish *et al.* (2005) observed differences over the growing period.

Comparison of culture-based and molecular diversity estimates is informative. The culturable component of bacterial communities from PAH contaminated environments tends to belong to a limited number of taxonomic groups and is generally dominated by *Sphingomonas* and *Mycobacterium* spp. (Bastiaens *et al.*, 2000; Johnsen *et al.*, 2005, 2007; Leys *et al.*, 2005; Mueller *et al.*, 1990). It has been suggested that *Sphingomonas* spp. are associated with the initial degradation of high concentrations of low molecular weight PAHs, whereas *Mycobacterium* spp. are involved in degradation of less available higher molecular weight PAHs (Friedrich *et al.*, 2000). PLFA analysis of a creosote contaminated site indicated that those associated with Gram negative bacteria were the only ones not negatively correlated with PAH concentration (Törneman *et al.*, 2008). *Sphingomonas* spp. have been identified in a variety of PAH contaminated environments using genus specific primers (Leys *et al.*, 2004), but interestingly, inoculation of contaminated

TABLE 2.4 Molecular based studies used to examine microbial communities in PAH contaminated environments

Environment	Technique(s) used	Targeted groups	Reference
PAH contaminated soils	DGGE	Bacteria, Eubacteria, and Mycobacteria	Vinas *et al.* (2005), Uyttebroek *et al.* (2007b)
	Functional gene (*phnAc*) diversity	Naphthalene degrading bacteria	Wilson *et al.* (2003)
	Quantitative real time PCR of *nahAc* gene	Naphthalene degraders and Bacteria	Park and Crowley (2006)
	Culture-based analysis and 18S rDNA clone library	Protozoa	Lara *et al.* (2007)
	Competitive PCR of *nahAc* and *phn* genes	Bacteria degrading low molecular weight PAHs	Laurie and Lloyd-Jones (2000)
Aged PAH contaminated soils	DGGE and 16S rDNA clone libraries	Bacteria, *Sphingomonas* and *Mycobacteria* spp.	Uyttebroek *et al.* (2007a)
	TGGE	Mycobacteria	Cheung and Kinkle (2001)
Fertilized and slurried aged PAH contaminated soil	DNA-SIP combined with DGGE and 16S rDNA clone libraries	Pyrene degrading bacteria	Jones *et al.* (2007)
Batch and biofilm enrichment from a PAH contaminated soil	Culture-based analysis, DNA and cDNA clone libraries, functional gene diversity	Bacteria and fungi	Stach and Burns (2002)

Bioreactor treating soil and groundwater from a gasworks site	DGGE (16S rRNA and NDO genes)	Bacteria	Ferguson *et al.* (2007)
Bioreactor treating PAH contaminated soil	DNA-SIP combined with DGGE, 16S rDNA clone libraries, and quantitative PCR with group specific primers	Bacteria	Singleton *et al.* (2005, 2006, 2007)
Naphthalene, phenanthrene, and pyrene enrichments from PAH contaminated soil	DGGE, ARDRA, and functional gene analysis	PAH dioxygenase population	Ni Chadhain *et al.* (2006)
PAH contaminated soil inoculated with PAH degrading bacteria	DGGE Functional gene (*nahAc*) analysis	Bacteria	Coppotelli *et al.* (2007), Cunliffe and Kertesz (2006), Piskonen *et al.* (2005)
Pristine soil amended with bioremediated soil	Real time PCR of *pdo1* gene	Mycobacteria	Johnsen *et al.* (2007)
Rhizosphere of PAH contaminated soil	Phospholipid fatty acid analyis (PLFA)	Mycorrhizae	Joner *et al.* (2001)
	TGGE, DNA and RNA	Bacteria	Corgie *et al.* (2004, 2006a, 2006b)

(*continued*)

TABLE 2.4 (*continued*)

Environment	Technique(s) used	Targeted groups	Reference
	Functional gene (*alkB*, *ndoB* and *xylE*) analysis and DGGE	Bacteria	Nakai *et al.* (1983), Siciliano *et al.* (2002)
PAH contaminated urban mangrove sites	DGGE using *ndo* primers	Bacteria	Gomes *et al.* (2007)
PAH contaminated sediments	16S rDNA clone libraries	Bacteria and Archaea	Chang *et al.* (2005)
	BrdU Immunocapture and T-RFLP DGGE	Bacteria	Edlund and Jansson (2006)
	Functional gene (*nagAc*, *nahAc*, *nahR*, *alkB*, *nahA*, *nahH*, *tolC1/C2*) diversity	Naphthalene degrading bacteria	Hilyard *et al.* (2008), Dionisi *et al.* (2004), Ghiorse *et al.* (1995), Langworthy *et al.* (1998).
	FISH using group specific probes	Archaea, α, β, and γ Proteobacteria, sulphate reducing bacteria and specific bacterial groups	Rogers *et al.* (2007)
Groundwater from a coal tar waste contaminated site	RT-PCR of *nahAc* sequences	Naphthalene degrading bacteria	Wilson *et al.* (1999)

Treatment plant treating PAH contaminated water	Functional gene (*nahAc*, *phnAc*, *C12O*, and *C23O)* analysis	Bacteria degrading low molecular weight PAHs	Wang *et al.* (2007)
Open lagoons containing ship bilge wastes	Functional gene (*nahAc*) analysis	Naphthalene degrading bacteria	Olivera *et al.* (2003)
Anaerobic methanogenic enrichments of a wastewater sludge	FISH	Bacteria, Archaea, and *Methanobacteriales*	Christensen *et al.* (2004)

soil with *Sphingomonas* spp. did not stimulate PAH degradation (Coppotelli *et al.*, 2007; Cunliffe and Kertesz, 2006).

Most *Mycobacterium* spp. isolated from PAH contaminated environments have been placed in the phylogenetic branch of the fast growing mycobacteria. These bacteria have been reported to metabolize high molecular weight PAHs such as pyrene and benzo[*a*]pyrene in culture (Heitkamp *et al.*, 1988; Kim *et al.*, 2005; Uyttebroek *et al.*, 2007a) and are adapted to oligotrophy (Wick *et al.*, 2002, 2003). The fast growing mycobacteria appear to be involved in the degradation of sorbed PAHs, which has been attributed to their mycolic-rich cell walls that allow them to attach to PAH enriched soil particles (Barry *et al.*, 1998; Cheung and Kinkle, 2001). Amplification of DNA from soil samples containing differing levels of PAH contamination with mycobacteria-specific primers has revealed the presence of fast growing mycobacteria in several PAH contaminated soils, whereas these organisms could not be detected in non-polluted environments (Leys *et al.*, 2005). Using a similar approach, Cheung and Kinkle (2001) detected novel strains of these bacteria in petroleum-contaminated soils but reported a reduction in diversity, with less mycobacterial phylotypes detected in PAH contaminated soils (Cheung and Kinkle, 2005). A combination of MPN-PCR and FISH revealed that *Mycobacterium* spp. represented a greater proportion of the community in soil fractions where maximum PAH degradation occurred (Uyttebroek *et al.*, 2006). Addition of a previously bioremediated soil (whose culturable portion was dominated by mycobacteria) to a pristine soil artificially contaminated with PAHs had little effect on PAH degradation (Johnsen *et al.*, 2007).

Although a number of bacterial groups appear to be associated with PAH contamination, their function in PAH metabolism *in situ* remains unclear. By feeding a microbial community a ^{13}C labeled substrate and subsequently isolating the heavy labeled fraction of DNA or RNA, organisms involved in the transformation of the substrate can potentially be identified using DNA-stable isotope probing (DNA-SIP) or RNA-SIP, respectively (Manefield *et al.*, 2004). In three separate DNA-SIP based examinations of PAH contaminated soil, *Mycobacterium* spp. were found not to be associated with PAH degradation (Jones *et al.*, 2007; Singleton *et al.*, 2005, 2007). Naphthalene and salicylate-degrading communities were composed mainly of sequences belonging to the *Pseudomonas* and *Ralstonia* genera, whereas phenanthrene-degrading communities were dominated by sequences related to the genus *Acidovorax* (Singleton *et al.*, 2005). Similarly, mycobacteria were not identified as significant pyrene degraders during DNA-SIP of aged PAH-contaminated soil (Jones *et al.*, 2007) or in a bioreactor treating contaminated soil (Singleton *et al.*, 2006). The main pyrene degraders in this bioreactor experiment appeared to be uncultivated low-abundance β and γ proteobacteria, not previously

associated with pyrene degradation, although nondetection of *Mycobacterium* spp. may have been due to an enrichment bias introduced by continuous mixing within the bioreactor that did not favor biofilm-forming bacteria such as mycobacteria. Although SIP methods are very attractive in potentially linking PAH degradation to specific diversity, some caution is required in interpretation of results. Organisms identified by DNA/RNA-SIP may not necessarily be directly involved in PAH degradation but may have incorporated ^{13}C from labeled PAH as a result of cross-feeding (McDonald *et al.*, 2005). Additionally, SIP approaches may introduce selection biases for organisms capable of utilizing test substrates, and often expose communities to substrate concentrations higher than those that exist *in situ* (Jones *et al.*, 2007).

C. Marker genes

Community analysis using specific catabolic genes can be used to study the dynamics of those communities that may potentially be responsible for PAH degradation. In pure culture systems, initial oxidation of PAH and subsequent catechol cleavage are key steps in PAH metabolism and as a result, genes encoding enzymes catalyzing these steps have been proposed as indicators of *in situ* PAH degradation (Hayes and Lovley, 2002). Table 2.5 lists some of the genes used as indicators of PAH degradation. The genes encoding naphthalene biodegradation in *Pseudomonas* spp. are plasmid encoded and located in three operons; the first operon encodes the upper pathway enzymes that transform naphthalene to salicylate; the second encodes the enzymes of the lower pathway which produce TCA intermediates via catechol formation; and the final operon encodes a regulatory protein (Simon *et al.*, 1993; Yen and Gunsalus, 1982, 1985).

The so-called *nah* genes that encode the alpha subunit of the iron–sulphur component of naphthalene dioxygenase (NDO) from strains of pseudomonads are the most widely employed catabolic markers for communities associated with PAH degradation. NDO is a multicomponent dioxygenase, typically consisting of a ferredoxin, a reductase, and an iron–sulphur protein which catalyzes the initial oxidation of PAHs. The iron–sulphur protein is composed of an alpha subunit containing the active site of the enzyme and a smaller beta subunit. The alpha subunit of the iron–sulphur protein of this enzyme is encoded by the *nahAc* gene (Gibson and Parales, 2000; Simon *et al.*, 1993; Yen *et al.*, 1991). This gene has been detected in a range of environments and higher frequencies have been reported in polluted sites (Ghiorse *et al.*, 1995; Langworthy *et al.*, 1998; Laurie and Lloyd-Jones, 2000; Tuomi *et al.*, 2004; Wilson *et al.*, 2003). Copy number of *nahAc* increased over a thousand-fold in a soil exposed to naphthalene, whereas the amount of 16s rRNA remained relatively stable (Moser and Stahl, 2001; Park and Crowley, 2006).

TABLE 2.5 Genes used as indicators of potential PAH degrading communities

Gene (*nah*-like genes)	Protein encoded	Organism	Reference
		Initial dioxygenases	
nah	Napthalene dioxygenase	*Pseudomonas putida* strain G7, *P. putida* NCIB 9816-4	Eaton (1994), Simon *et al.* (1993)
nahAc	α-subunit of naphthalene dioxygenase		
nahAb	Ferrodoxin component of naphthalene dioxygenase		
ndo	Naphthalene dioxygenase	*P. putida* NCIB 9816-4	Kurkela *et al.* (1988)
pah	Naphthalene/phenanthrene dioxygenase	*Pseudomonas* sp. OU582	Takizawa *et al.* (1994)
dox	Naphthalene dioxygenase	*Pseudomonas* sp. C18	Denome *et al.* (1993)
phn phnAc	Phenanthrene dioxygenase α-subunit of phenanthrene dioxygenase	*Burkholderia* sp. strain R007	Laurie and Lloyd–Jones (1999)
dnt	2,4-dinitrotoluene dioxygenase	*Burkholderia* sp. strain DNT	Suen *et al.* (1996)
phd	Phenanthrene dioxygenase	*Nocardioides* sp. strain KP7	Saito *et al.* (1999)
nag	Naphthalene dioxygenase	*Ralstonia* sp. strain U2 *Comamonas testosteroni* GZ 39 and GZ42	Fuenmayor *et al.* (1998), Goyal and Zylstra (1997)
nid	PAH ring cleaving dioxygenase	*Mycobacterium* sp. strain PYR-1	Khan *et al.* (2001)
pdo	Ring hydroxylating dioxygenase	*Mycobacterium* sp. strain 6PY1	Krivobok *et al.* (2003)
nar	Naphthalene dioxygenase	*Rhodococcus* sp. strain NCIMB 12038	Kulakov *et al.* (2000)

bph	Biphenyl and phenanthrene dioxygenase	*Sphingomonas paucimobilis* var. EPA505	Romine *et al.* (1999), Story *et al.* (2000)
		Extradiol dioxygenases	
nahC/pahC	1,2-dihydroxynaphthalene dioxygenase	*P. putida OUS82*	Habe and Omori (2003), Kiyohara *et al.* (1994)
nahH	Catechol 2,3-dioxygenase	*P. putida* G7	Ghosal *et al.* (1987)
nahY	Naphthalene chemotaxis protein	*P. putida G7*	Grimm and Harwood (1999)
xyleE	Catechol 2,3-dioxygenase	*P. putida* mt-2	Nakai *et al.* (1983)
		Other enzymes	
alkB	Alkane monooxygenase	*Pseudomonas oleovorans* ATCC 29347	Beilen *et al.* (1994)
todC1/C2	Toluene dioxygenase	*P. putida* F1	Zylstra and Gibson (1989)

Genes are typically named according to the substrate that the bacterium initially degraded (nah for naphthalene, ndo for naphthalene dioxygenation (equivalent to nah), phe for phenanthrene, dox for dibenzothiophene, dnt for dinitrotoluene). Most strains were subsequently found to grow on a range of PAHs. nag is used to denote genes from organisms that degrade naphthalene via gentisate.

Initial investigations suggested that the *nah* gene was highly conserved among PAH degrading bacteria, but as genes encoding PAH metabolism in a wider range of bacteria were characterized it became clear that the *nah*-like genes from pseudomonads provided a limited view of the gene diversity involved in the initial oxidation of PAHs, even amongst culturable bacteria (Ahn *et al.*, 1999; Dionisi *et al.*, 2004). Only 45% of naphthalene degrading isolates could be detected using probes based on *nah*-like genes and these probes could only detect 15% of the strains that metabolized either phenanthrene or naphthalene (Laurie and Lloyd-Jones, 1999). Even PAH dioxygenases from pseudomonads appeared to differ significantly at the nucleic acid level while still expressing similar substrate specificities (Meyer *et al.*, 1999).

Genes encoding dioxygenases involved in the initial oxidation of low molecular weight PAHs from strains of *Burkholderia*, *Comamonas*, *Ralstonia*, *Mycobacteria Rhodococcus*, and *Sphingomonas* displayed little sequence homology with the archetypal pseudomonad *nah* genes (Allen *et al.*, 1997; Brezna *et al.*, 2003; Goyal and Zylstra, 1996; Khan *et al.*, 2001; Pinyakong *et al.*, 2003). Probes targeted to these genes have been used to screen a number of environments and results indicate that the *nah*-like genes are not typical of the diversity present. *phn*-Like genes, based on the gene encoding the initial oxidation of phenanthrene and naphthalene in *Burkholderia* sp. strain RP007, appeared to be of more ecological significance in New Zealand soils than *nah*-like genes (Laurie and Lloyd-Jones, 1999, 2000). Sequences typical of NDOs from *Pseudomonas* spp. and *Comamonas* spp. were detected in DNA extracted from a sequential reactive barrier treating groundwater from a former gas manufacturing plant, but no sequences representative of *Rhodococci* NDOs were observed (Ferguson *et al.*, 2007).

A number of novel extradiol dioxygenase sequences were observed in soil from PAH contaminated sites in Finland using a suite of primers targeted to genes encoding the catechol cleavage step (Sipila *et al.*, 2006). Similarly, primers targeted to the alpha subunits of Group III *ndo* genes (which includes *phn*, *nah,* and several other *ndo* genes) retrieved mainly sequences representing novel genotypes from three urban mangrove sites (Gomes *et al.*, 2007). Clone libraries, prepared from two contaminated and two pristine soils using a primer set targeted to the conserved amino acid motifs in ring hydroxylating dioxygenases, contained several novel dioxygenase gene fragments. Sequence analysis revealed that these environmental clones clustered into six distinct groups, only one of which included previously described ring hydroxylating dioxygenases (Yeates *et al.*, 2000). Using primers targeting the conserved Rieske center rather than the catalytic domain of dioxygensases, different suites of genes were detected in soil microbial populations degrading a range of PAHs (Ni Chadhain *et al.*, 2006).

PAH contaminated environments typically contain a mixture of low and high molecular weight PAHs. Although the degradation of high molecular weight PAHs is receiving more attention (Kanaly and Harayama, 2000), there is little information concerning the genes involved in degradation of these compounds. Degradation of complex substrates is typically mediated by extensive microbial diversity and designing molecular markers to profile degradative activity in such a community represents a significant challenge.

Although a correlation generally exists between the relative abundance of biodegradative genes and the potential for pollutant degradation (Laurie and Lloyd-Jones, 2000; Meyer *et al.*, 1999; Park and Crowley, 2006), monitoring gene expression (mRNA) in a microbial community in response to PAHs provides more meaningful information concerning the functioning of the community. Wilson *et al.* (1999) demonstrated that *nahAc* genes were actively transcribed in the groundwater from a site contaminated with coal tar. Sequences retrieved were related to dioxygenases from *Pseudomonas putida* NCIB 9816-4 and a *Burkholderia* sp. mRNA based analysis of complex environments is currently limited by difficulties in extracting sufficient mRNA and its short half-life (Pichard and Paul, 1993; Sayler *et al.*, 2001; Sharma *et al.*, 2007). Primer design is critical for any PCR-based analysis and it is clear that existing primers targeting PAH-degradative genes represent only a fraction of *in situ* gene diversity. If microbial populations are to be linked to PAH degradation *in situ*, information is needed regarding the genes expressed by the microbial community in response to PAHs. This should include not only previously uncharacterized degradative genes, but also genes not directly involved in PAH metabolism. The latter genes may provide additional insights into the overall response of the community to the perturbing influence and the factors controlling the rate and extent of degradation.

The construction of a genomic DNA library from a contaminated environment is one potential approach to analyzing environmentally significant genes. Metagenomic analysis involves extracting total genomic DNA from an environmental sample, fragmenting and cloning it into a suitable vector, and transfecting a host bacterium (Rondon *et al.*, 2000). The resulting library is then screened for functional or genetic diversity, allowing the genotype of microorganisms present in the environment to be assessed and providing information on organisms that cannot be cultured. Metagenomic technologies have been used to characterize entire ecological communities, albeit only in environments containing a low level of diversity (Crossman *et al.*, 2004; Handelsman, 2004). Construction of a metagenomic library from soil has traditionally been considered impractical due to the large number of sequences present (Gans *et al.*, 2005). However, the recent development of inexpensive high throughput

pyrosequencing may make the construction of metagenomic libraries from contaminated soil more feasible (Roesch *et al.*, 2007). Although low abundance genomes involved in degradation may be under-represented in a metagenomic library (Eyers *et al.*, 2004), a functional screen would provide significantly more information regarding the true diversity of genes involved in PAH degradation than pure culture studies.

Sequence information obtained from genomic analysis can be used to design DNA microarrays. A DNA microarray typically contains thousands of phylogenetic or functional sequences spotted in a grid on a slide. DNA or mRNA is extracted from samples labeled with fluorescent molecules and hybridized to target DNA on the slide. The resulting hybridization profile is then visualized and digitalized for quantitative analysis. Microarrays based on sequence information retrieved from culturable organisms have been used to monitor genes involved in 2,4-dichlorophenoxyacetic acid (2,4-D) degradation in environmental samples (Dennis *et al.*, 2003). Microarrays containing functional and phylogenetic sequences relevant to PAH degradation would have considerable application in bioremediation, allowing microbial structure, function, and dynamics in a contaminated site to be assessed.

IV. BIOREMEDIATION OF PAH CONTAMINATED ENVIRONMENTS

Bioremediation has been used with varying degrees of success to treat PAH contaminated sites. Bioremediation strategies can involve natural attenuation, bioaugmentation, or biostimulation and can take place *in situ* or *ex situ* (Mueller *et al.*, 1996). Natural attenuation relies solely on the capacity of indigenous microbial communities present to remove contaminating chemicals and may be slow if biodegradation is limited by one or more environmental factors (e.g., lack of nutrients, Fig. 2.1). Although addition of inoculants has been reported in some instances to enhance PAH degradation in soil (Garon *et al.*, 2004; Zheng and Obbard, 2002), bioaugmentation has not been successfully demonstrated for large scale remediation of PAHs (Johnsen *et al.*, 2005). Microorganisms capable of degrading PAHs exist in most environments, and it is thus not surprising that most success to date has been achieved with strategies that stimulate the biodegradative capacity of indigenous microbial communities. PAH concentrations in soil have been successfully reduced using landfarming, composting, and nutrient supplementation (Guerin, 1999; Hansen *et al.*, 2004; Kastner and Mahro, 1996; Kastner *et al.*, 1998; Vinas *et al.*, 2005).

Landfarming is considered an acceptable approach for the remediation of contaminated wood treatment sites (US EPA, 2004). This technology can be carried out *in situ* using traditional agricultural practices

such as tilling and fertilizer application which increases aeration, mixing, and nutrient concentration in the soil. Greater success has been achieved using *ex situ* treatments such as land treatment units (LTUs), composting, and bioreactors. Although these approaches are less cost effective and require excavation of the contaminated materials, they allow greater monitoring and control of environmental parameters and can still take place on-site. 2- and 3-ring PAHs were almost completely removed from a creosote contaminated soil treated in an LTU using a combination of tilling, fertilization with a controlled release of N-P-K fertilizer, and irrigation to maintain soil moisture content at 18% (Guerin, 1999). Higher molecular weight PAHs were not removed to the same extent, <35% of 6-ring PAHs being degraded. Composting of PAH contaminated soil with various wastes including spent mushroom compost, paper, grass, alfalfa, and food waste has been demonstrated to effectively remove PAHs, both as a solid phase treatment and in bioreactors (Joyce *et al.*, 1998; Lau *et al.*, 2003) In general, the rate and extent of PAH degradation is greater in bioreactors as process parameters such as pH, temperature, nutrient levels, and aeration can be more easily controlled (Garon *et al.*, 2004; Woo *et al.*, 2004). Bioreactors also provide the opportunity to add surfactants or inoculants, if appropriate (Kim *et al.*, 2001; Zheng and Obbard, 2002).

Although bioremediation technologies have been successfully applied for PAH removal, processes are often unpredictable and unreliable, and little is understood concerning the complex interrelationships between microbial communities and the associated effects of influencing environmental variables.

A. Factors affecting bioremediation

Typically, microbial communities exposed to PAHs require time to adapt before degradation occurs (Barkay *et al.*, 1995). This acclimation period is of considerable public health and ecological significance as it increases the length of time susceptible species are exposed. If, for example, PAHs have leached into watercourses, wide-scale dissemination may occur from the original source. The duration of the acclimation period depends on a number of environmental variables such as contaminant concentration, bioavailability, pH, temperature, levels of nitrogen and phosphate present, aeration status, and prior exposure of microbial communities to PAHs (Alexander, 1999; Spain and Van Veld, 1983).

Heterogeneous distribution of PAHs in soil combined with their absorption to organic matter and low levels of diffusibility, limits bacterial access to PAHs as substrates (Bosma *et al.*, 1997; Johnsen *et al.*, 2005). Many bacteria produce biosurfactants when grown on hydrocarbons and these can increase PAH solubility (Oberbremer *et al.*, 1990; Van Dyke *et al.*, 1993).

For example, a rhamnolipid producing *P.aeroginosa* strain that did not degrade PAHs, increased the rate of phenanthrene degradation by a PAH metabolizing pseudomonad (Arino *et al.*, 1998). Although surfactant production appears to be an obvious means of overcoming limited bioavailability, there is often no correlation observed between PAH degradation and surfactant production in pure culture (Willumsen and Karlson, 1996). Chemical and biological surfactants have been added to contaminated soils to increase PAH availability, but success has been mixed and in some cases surfactants have inhibited degradation (Laha and Luthy, 1991). Other microbial phenotypes suggested to facilitate growth on hydrophobic compounds include enhanced cell surface hydrophobicity, biofilm formation, motility, and chemotaxis (Johnsen *et al.*, 2005; Parales and Harwood, 2002).

PAH degradation is generally an aerobic process, although anaerobic degradation has been reported (Bianchin *et al.*, 2006; Chang *et al.*, 2005; Holliger and Zehnder, 1996; Zhang *et al.*, 2000). Oxygen levels in PAH contaminated environments such as soils and sediments are typically well below the levels required for aerobic transformation of PAHs. Increasing the aeration status of such environments via tilling of soil, addition of materials that increase the interparticle space within the soil matrix, direct injection of oxygen (sparging), or introduction of oxygen generating species such as H_2O_2 have been shown to increase both the rate and often the extent of PAH degradation (Guerin, 1999; Guerin and Boyd, 1992; Kaplan and Kitts, 2004).

The rate of PAH degradation is generally faster in soil that has a history of PAH contamination. A study by Johnsen and Karlson in 2005 revealed that the rate of ^{14}C-labeled pyrene and phenanthrene mineralization was inversely proportional to the PAH content of 13 different soils. ^{14}C-phenanthrene was removed from all soils tested including two pristine forest soils, but little mineralization of ^{14}C pyrene was observed over 140 days in pristine soils. The dependence of phenanthrene and pyrene removal rates on previous exposure has also been reported in some UK soils (Macleod and Semple, 2006). PAH degradation in environments with no history of previous contamination may result from exposure to PAHs from natural sources such as biogenic synthesis (Grimalt *et al.*, 2004), atmospheric deposition of PAHs from contaminated sites, or the presence of microorganisms that produce broad specificity enzymes such as laccases. *In situ* horizontal transfer of PAH metabolizing genes (*nahAc* and *phnAC)* has been reported between phylogenetically distinct bacteria, and this may also explain the PAH degradative capacity of communities with no known exposure to PAHs (Park and Crowley, 2006; Stuart-Keil *et al.*, 1998; Wilson *et al.*, 2003).

Previous exposure of a microbial community to one PAH has frequently been reported to reduce the acclimation period for degradation of other PAHs (Bauer and Capone, 1988; Beckles *et al.*, 1997).

A *Mycobacterium* sp. isolated from a PAH enrichment of a contaminated river sediment degraded phenanthrene and pyrene following exposure to either compound (Molina *et al.*, 1999), and 4- and 5-ring PAHs disappeared more rapidly from soils amended with complex mixtures of PAHs (Keck *et al.*, 1989). In some instances it would appear that the acclimation period merely reflects the time taken for degrading populations to reach a high enough cell density to effect a detectable change. The density of phenanthrene and pyrene degraders was higher in soils previously exposed to these PAHs (Johnsen and Karlson, 2005; Macleod and Semple, 2006). Any factor stimulating the growth of degrading populations (e.g., addition of nutrients, aeration, etc.) would thus affect degradation rates. For example, addition of phenanthrene increased the initial rate of fluoranthene degradation in soil, but a similar effect was observed if a biosurfactant was added instead of the 3-ring PAH (Hickey *et al.*, 2007).

V. CONCLUSIONS

There have been considerable advances in understanding PAH metabolism in pure culture and identifying microbial diversity in PAH contaminated sites. The real challenge now lies in trying to link these organisms to specific functions associated with *in situ* degradation, and use this information for the rational design of reliable bioremediation strategies.

Technologies such as stable isotope probing, metagenomics, and microarrays offer potential in elucidating *in situ* microbial PAH degradation. Comparison of gene expression in contaminated and uncontaminated environments may reveal key processes associated with *in situ* microbial degradation and adaptation. This information can be used to develop functional and phylogenetic microarrays for the assessment of microbial diversity, degradative potential, and metabolic pathways likely to occur in contaminated sites. Genomic analysis may also assist in culturing previously unculturable PAH degrading organisms, allowing a deeper profiling of microbial activity at the metabolic level. Most studies to date have concentrated on organisms directly involved in PAH degradation and have generally ignored interactions with associated microflora; if factors controlling *in situ* PAH degradation are to be manipulated, these interactions must be understood.

Although significant challenges still exist, bioremediation represents a competitive technology for PAH contaminated sites. Information regarding *in situ* microbial processes can only advance the application of this technology.

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CHAPTER 3

Acid Stress Responses in *Listeria monocytogenes*

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I. INTRODUCTION

The Gram positive intracellular pathogen *Listeria monocytogenes* is the causative agent of listeriosis, a potentially fatal foodborne infection that predominately affects pregnant women and those with underlying immunesuppression. It is significant that the pathogen is regularly exposed to low pH environments during the infectious cycle; in acidic foods, upon passage through the gastric barrier, and subsequently, upon entry to the host cell phagosome. The ability of *L. monocytogenes* to adapt to low pH environments therefore has the potential to significantly influence survival and growth in foods as well as subsequent pathogenesis. Furthermore, the adaptive response to low pH has the capacity to induce cross protection against other stresses including those encountered during intestinal growth (bile salts, elevated osmolarity). It is essential therefore to understand the molecular mechanisms that underpin acid adaptation, pH homeostasis, and stress-hardening in this organism in order to model and control growth of the pathogen in high risk foods and to predict the impact upon ability to cause disease. Specific mechanisms involved in maintenance of intracellular pH homeostasis during acid exposure include the glutamate decarboxylase (GAD) system, the arginine deiminase (ADI) system, the F_0F_1-ATPase, and general stress proteins. The regulatory networks governing control of these systems (including Sigma B and the two-component system LisRK) will also be discussed.

II. LISTERIOSIS

The ubiquitous bacterium *L. monocytogenes* is the etiological agent of listeriosis, a rare but potentially fatal foodborne disease. The pathogen causes disease predominately in immunosuppressed individuals including those with HIV/AIDS and those undergoing immunosuppressive therapies giving rise to meningitis and meningoencephalitis. *L. monocytogenes* also has a propensity to infect pregnant women resulting in infection of the fetus and spontaneous miscarriage or serious neonatal infection in the newborn infant (McCabe-Sellers and Beattie, 2004; McLauchlin *et al.*, 2004; Spyrou *et al.*, 1997; Thom and Forrest, 2006). The pathogen is capable of host cell invasion and intracellular growth and therefore has the ability to cross a number of host barriers to infection, namely the gastrointestinal barrier, the blood-brain barrier, and the placental barrier. Furthermore, although relatively infrequent, cases of febrile gastrointestinal disease suffered by otherwise healthy individuals have also been attributed to infection by *Listeria* (Ooi and Lorber, 2005; Schlech *et al.*, 2005; Sim *et al.*, 2002).

The types of foods associated with transmission of listeriosis include a number of ready-to-eat (RTE) foods including deli meats, hotdogs, soft cheeses, raw milk, and raw vegetables (Hof, 2003; Kathariou, 2002; Tompkin, 2002). The disease may be spread through large common-source epidemics but most commonly occurs as spontaneous independent cases where the source of infection is unclear (Gahan and Hill, 2005; Vazquez-Boland *et al.*, 2001). Significantly, the mortality rate for *L. monocytogenes* infections approaches 30% (Ramaswamy *et al.*, 2007). The serious nature of the infection has resulted in heightened vigilance by the food industry to prevent infections and has led to significant research efforts to identify factors which may influence foodborne growth of the pathogen and subsequent pathogenesis. The molecular analysis of the pathogen has been aided in recent years by the availability of genome sequences of a number of typed isolates of *L. monocytogenes*, including a number of serotype 4b strains associated with large epidemic outbreaks of listeriosis (Doumith *et al.*, 2004; Glaser *et al.*, 2001; Nelson *et al.*, 2004).

III. ACID TOLERANCE RESPONSE (ATR) AND CROSS PROTECTION

It has been observed that exposure to sub-lethal levels of one stress can result in induction of tolerance to the same or other stresses of greater magnitude (Lou and Yousef, 1997). The acid tolerance response (ATR) involves the acquisition of enhanced acid tolerance following a brief exposure to mildly acidic growth conditions (Koutsoumanis *et al.*, 2003) and has been well-documented in many foodborne bacteria, including *Salmonella* spp. (Foster, 1993; Foster and Hall, 1990; Lee *et al.*, 1995), *S. flexneri* (Small *et al.*, 1994), *V. cholerae* (Merrell and Camilli, 1999), and *Listeria* (Cataldo *et al.*, 2007; Davis *et al.*, 1996; O'Driscoll *et al.*, 1996). Acid adaptation, which is normally transient, has become significant with regard to food safety, because within certain foods it can enhance bacterial survival (Cataldo *et al.*, 2007; Gahan *et al.*, 1996; Leyer and Johnson, 1992; Stopforth *et al.*, 2007). Work aimed at uncovering the molecular basis of the ATR is outlined below.

Acid-adapted cultures of pathogens have been shown to exhibit cross-protection against other food-related stresses such as heat and irradiation (Samelis *et al.*, 2003). Indeed, it has been found that many stressors, including those used as preservative methods, such as heat, salt, and low pH, simultaneously induce cross-protection against elevated levels of other stresses, thereby enabling bacteria to survive otherwise lethal challenges (Casey and Condon, 2002; Flahaut *et al.*, 1996). The phenomenon of cross-protection or ''stress hardening'' is clearly seen in *L. monocytogenes* (Faleiro *et al.*, 2003; O'Driscoll *et al.*, 1996; Lou and Yousef, 1997; Skandamis *et al.*, 2008). These effects are most likely mediated by

the induction of global stress-response mechanisms, which confer simultaneous resistance to numerous stresses.

In particular, several studies have shown that acid adaptation in *L. monocytogenes* confers resistance to heat, salt, ethanol, and crystal violet (O'Driscoll *et al.*, 1996). We and others have previously shown that acid adaptation also induces enhanced resistance against bacteriocin activity, a phenomenon possibly linked to an alteration in the fatty acid composition of the membrane (Bonnet and Montville, 2005; van Schaik *et al.*, 1999). Acid adaptation of listeriae has also been shown to engender cross-protection against *in vivo*-associated stresses such as bile (Begley *et al.*, 2002).

IV. ACID RESISTANCE AND LISTERIAL SURVIVAL IN FOODS

As outlined above, listeriosis is primarily transmitted through consumption of contaminated RTE foods (Hof, 2003; McLauchlin *et al.*, 2004; Kathariou, 2002; Tompkin, 2002). In foods, contaminating listeriae frequently encounter sub-optimal environmental conditions and hence their survival relies on the ability to sense and respond to fluctuations in their surroundings. There is great interest in modeling the parameters affecting *L. monocytogenes* growth in foods as recent EU regulations (EC/2073/2005) permit a low level of listerial contamination ($\leq$100 CFU/g) in RTE products provided that the food matrix does not support growth of the pathogen over the shelf-life of the product. The difficulty in controlling listerial growth in foods is compounded by the fact that *L. monocytogenes* is a relatively hardy organism. Analysis of the cardinal temperatures for bacterial proliferation indicates that growth occurs between -1.7 and 45.1 °C under experimental conditions (Membre *et al.*, 2005). The bacterium can also proliferate at highly elevated salt concentrations (up to 10% NaCl; Sleator *et al.*, 2003). Significantly, the bacterium has also been observed to grow at minimal pH values of approximately 4.3 in HCl-acidified media (George *et al.*, 1988; Tienungoon *et al.*, 2000), although lower values have also been noted (Koutsoumanis and Sofos, 2005). Modeling growth limits in foods is extremely complex; however, as a number of other factors (inoculum size, bacterial growth phase, stress hardening, and nutrient availability) will influence bacterial growth in these environments (Koutsoumanis and Sofos, 2005; Skandamis *et al.*, 2008).

The ability to modulate metabolic processes in response to stress has the potential to influence survival of pathogens in acidic foods. Thus the propensity for listerial adaptation to low pH conditions (through the ATR) may have implications for food processors. In this context the ''prior history'' of contaminating bacteria may influence their subsequent survival in the food matrix through ''stress hardening'' (Lou and Yousef, 1997). We have previously demonstrated that *Listeria* cells undergoing an ATR display significantly enhanced survival during storage of low pH foods (cottage cheese,

yoghurt, cheddar cheese, and salad dressing; Gahan *et al.*, 1996). This has recently been extended to Italian-style soft cheeses along with the observation that nonadapted cells become acid adapted during storage of certain cheeses (Cataldo *et al.*, 2007). However, for a number of other food processes and specific food environments acid adaptation did not influence subsequent bacterial survival (Foley *et al.*, 2005; Formato *et al.*, 2007).

V. ACID RESISTANCE AND *L. monocytogenes* PATHOGENESIS

The ability of many pathogens to adapt to and subsequently survive harsh environmental challenges has a direct association with their potential to cause disease. The acid barrier of the stomach is the initial host defence system which must be breached by enteric pathogens in order to subsequently colonize the human host (Audia *et al.*, 2001). The mean stomach pH under fasting conditions in healthy volunteers is approximately 2, a pH that can quickly kill most microorganisms including *L. monocytogenes* (Audia *et al.*, 2001; Richard and Foster, 2004; Smith, 2003). Inherently acid-resistant organisms such as *E. coli* O157 strains have a propensity to survive well in the stomach environment and therefore have low predicted infectious doses (approximately 1000 CFU; Diez-Gonzalez and Karaibrahimoglu, 2004). Other organisms such as *V. cholerae* will not survive well in the gastric environment unless they are pre-adapted (Merrell *et al.*, 2002). The infectious dose of *L. monocytogenes* is considered to be between 10^6 and 10^9 CFU, but is very much dependent upon the nature of the food matrix and host susceptibility (FAO/WHO, 2004; FDA/FSIS, 2003). It is also likely that pre-adaptation and the physiological status of the bacterium will influence infection by *L. monocytogenes* as outlined below.

The importance of maintaining an acidic gastric barrier to foodborne has been highlighted by a number of studies investigating the virulence of pathogens in animal models which have been treated with antacids. Buffering of murine stomach acidity is clearly associated with a significant reduction in the infectious dose of *Salmonella* species (Bearson *et al.*, 1997; Riley *et al.*, 1984). Similarly it was found that buffering of stomach acidity in neutropenic mice via administration of sodium bicarbonate solution prior to intragastric (i.g.) inoculation with *L. monocytogenes* EGD resulted in a significant enhancement in disease severity (Czuprynski and Faith, 2002). It has also been reported that a reduction in gastric acid production following treatment with the H_2-receptor antagonist cimetidine increases the severity of gastrointestinal listeriosis in experimentally inoculated rats, as well as lowering the dose of *L. monocytogenes* necessary to induce an invasive infection (10^2 vs 10^6 CFU for control animals; Schlech, 1993).

A number of retrospective studies have linked the use of acid-suppressive agents such as proton pump inhibitors and H_2-receptor antagonists to an increased likelihood of listeriosis in humans (Cobb *et al.*, 1996; Schuchat *et al.*, 1991). In a food-borne outbreak of hospital-acquired listeriosis in Boston hospitals, patients receiving antacids or H_2-antagonists were found to be more likely than control patients to be infected with *L. monocytogenes* (Ho *et al.*, 1986). Furthermore, in a study by Cobb *et al.* (1996), it was noted that there was an increased prevalence of *L. monocytogenes* fecal carriage documented in female patients receiving long-term treatment with H_2-antagonists (carriage rate of 20% vs 2.1% for control patients). These data suggest that gastric acid is bactericidal to *L. monocytogenes* and that drugs that inhibit or neutralize acid produced in the stomach increase the susceptibility of individuals to infection by this organism (Smith, 2003).

In addition, bacteria with impaired stress responses appear to be less virulent than their fully functional counterparts. Various mutations that confer acid sensitivity have been found to attenuate *S. typhimurium* infection (Bearson *et al.*, 1997). Similarly Marron *et al.* (1997) identified a mutant of *L. monocytogenes* LO28 which is incapable of inducing an ATR and displays reduced virulence potential in mice during systemic infection, suggesting that the ATR contributes in part to the *in vivo* survival of *L. monocytogenes*. Saklani-Jusforgues *et al.* (2000) have shown that prior acid adaptation correlates with increased translocation rates of bacteria to mesenteric lymph nodes in mice inoculated intragastrically. Independent studies also show that acid-adapted or acid-resistant mutant strains of *L. monocytogenes* are more invasive *in vitro* (Conte *et al.*, 2000, 2002) and virulent *in vivo* (O' Driscoll *et al.*, 1996). Whilst the precise locations of the mutations in these acid tolerant mutants have not been identified, their increased pathogenicity may be due to the upregulation of genes involved in pathogenesis. Low pH is an important signal to foodborne pathogens communicating that the bacterium has entered a potential host environment, and triggering the induction of molecular systems which are associated with bacterial virulence (Alpuche Aranda *et al.*, 1992; Audia *et al.*, 2001). It is significant that Listeriolysin O (LLO), a pore-forming toxin that facilitates phagosomal escape by *Listeria* (Portnoy *et al.*, 1992), is distinct from related proteins due to its acidic pH optimum for phagosomal disruption (Kayal and Charbit, 2006). This listerial protein is irreversibly denatured at neutral pH (Schuerch *et al.*, 2005) and only functions at low pH values (Kayal and Charbit, 2006). Furthermore, LLO activity is triggered *in vivo* by a lowering of the pH of the phagosome following cellular infection (Beauregard *et al.*, 1997). Therefore, it appears that *Listeria* has developed a phenotype which not only copes with stressful *in vivo* environments but also is partially dependent on exposure to such conditions.

Researchers have also examined the possibility that acid adaptation in foods may influence survival of gastric acid. It was found that cultures grown in frankfurters which had been treated with acidic antimicrobials showed increased subsequent resistance to the lethal effects of simulated gastric fluid following prolonged storage (Stopforth *et al.*, 2005). Furthermore, studies have shown that prior exposure of *L. monocytogenes* to frankfurter exudate (Wonderling *et al.*, 2004) and turkey meat (Peterson *et al.*, 2007) also results in enhanced resistance to simulated gastric fluid.

VI. LISTERIAL MECHANISMS OF ACID RESISTANCE

A. GAD system

The GAD system provides a mechanism for maintaining pH homeostasis in a number of bacterial genera. The GAD enzyme, usually encoded by *gadA* or *gadB*, irreversibly decarboxylates the acidic intracellular substrate glutamate, producing the neutral compound γ-aminobutyrate (GABA). An intracellular proton is consumed in the reaction, contributing to increased cytoplasmic pH. The GABA produced via the decarboxylation reaction is subsequently exchanged at the cell membrane for a molecule of glutamate by a glutamate:GABA antiporter, which is usually encoded by the *gadC* gene. ATP is also synthesized during the reaction, and this may contribute to pH homeostasis mediated by the F_0F_1-ATPase (Higuchi *et al.*, 1997; see Fig. 3.1).

It is striking that this system of acid resistance has primarily been identified in bacteria which transit the low pH of the gastric environment prior to causing foodborne disease. *L. monocytogenes, S. flexneri*, and *E. coli* have all been shown to require a functional GAD system for survival under low pH conditions (Cotter *et al.*, 2001a,b; Smith *et al.*, 1992; Waterman and Small, 1996). Furthermore, it has been shown in several studies that the level of GAD expression in bacteria correlates with the degree of acid tolerance (Cotter *et al.*, 2001a; Yokoigawa *et al.*, 2003), and it is likely that the presence of this system may facilitate passage through the GIT (Price *et al.*, 2004). Interestingly, factors that are associated with the GI tract (low pH, anaerobiosis, hypo- and hyper-osmotic shock, bile salts, and chloride ions) have been shown to induce expression of the GAD system in a range of bacteria (Blankenhorn *et al.*, 1999; Conte *et al.*, 2002; De Biase *et al.*, 1999; Jydegaard-Axelsen *et al.*, 2004; Bron *et al.*, 2006; Sanders *et al.*, 1998).

The GAD system of *L. monocytogenes* has been investigated in detail and found to play a major role in acid resistance. It appears that *L. monocytogenes* strains differ in their levels of intrinsic acid tolerance (Dykes and Moorhead, 2000), and strain-dependent variations in GAD

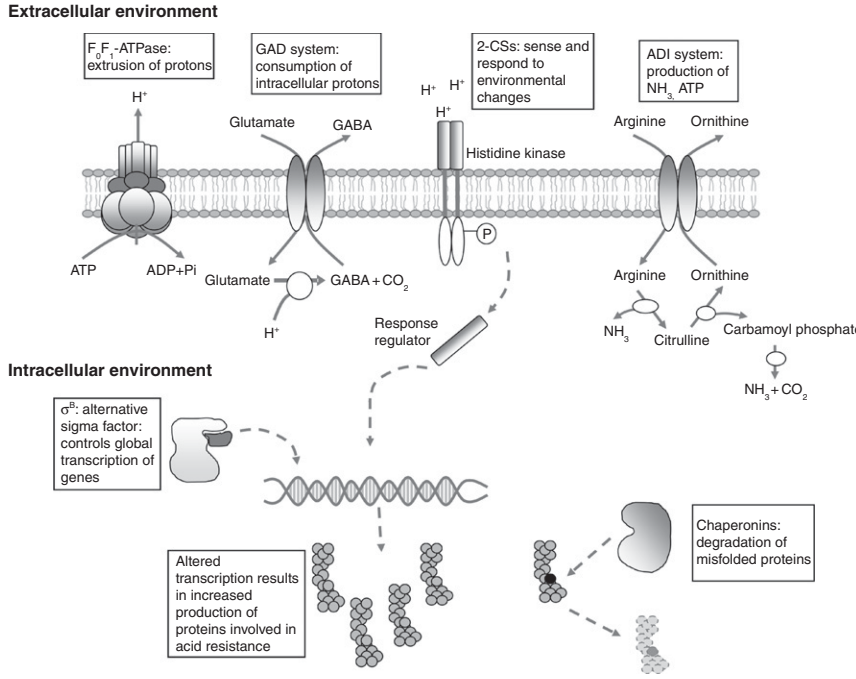

FIGURE 3.1 A graphical representation of selected acid resistance systems and mechanisms employed by the Gram-positive pathogen, *Listeria monocytogenes*.

expression correlate with survival in the hyperacidic environment of gastric fluid (Conte *et al.*, 2002; Cotter *et al.*, 2001a; Olier *et al.*, 2004). Initial characterisation of the listerial system revealed that loss of the genes encoding a key GAD enzyme (now designated GadD2) and a glutamate transporter (now designated GadT2) had a significant effect on the ability of the cell to survive in low pH environments (>5 log reduction relative to the wild-type by 45 min post-exposure at pH 3.5; Cotter *et al.*, 2001a). The GAD system was also shown to be a major component of the ATR as elimination of GAD enzymes significantly reduced acid adaptation in mutant strains. However, some inducible acid tolerance was demonstrated in these strains indicating that other mechanisms may also contribute to the ATR (Cotter *et al.*, 2001a).

Subsequent analysis of the genome of *L. monocytogenes* strain EGDe revealed that this strain has three GAD homologues (now designated *gadD1*, *gadD2*, and *gadD*3), and two antiporters associated with the first two characterized decarboxylase genes (*gadT1* and *gadT2*) (Cotter *et al.*, 2005; Glaser *et al.*, 2001). The GAD genes are arranged in two operons (*gadD1T1* and *gadT2D2*) that are some distance apart in the genome. *gadD3* is located separately from these two operons and is monocistronically

transcribed (Cotter *et al.*, 2005; Joseph *et al.*, 2006). Although the genes encoding the various enzymes are homologous to each other (the *gadD1* and *gadD2* genes share 68.2% identity), they have discrete functions in the listerial acid stress response. We determined that while GadD2 plays a major role in the ability of *Listeria* to survive low pH stress (Cotter *et al.*, 2001a), GadD1 facilitates growth at mildly acidic pH values (Cotter *et al.*, 2005). Significantly, the *gadD1T1* operon is absent from certain strains of *L. monocytogenes* including epidemic strains of serotype 4b (Call *et al.*, 2003; Nelson *et al.*, 2004). Strains which naturally lack this operon were shown to exhibit poorer growth at moderately low pH than strains containing the gene system. Thus the presence of *gadD1T1* is associated with an enhanced growth capacity at low pH and may influence distribution of certain strains in low pH foods and in the environment (Cotter *et al.*, 2005). Whilst we have been unable to generate a clean deletion mutant in *gadD3*, a recent report demonstrated that an insertional mutant could be generated in this locus (Joseph *et al.*, 2006). That study showed that this locus is significantly upregulated during cellular infection and that the *gadD3* mutant was impaired in intracellular growth (Joseph *et al.*, 2006). However, the role of GadD3 in acid tolerance remains to be established.

In *L. monocytogenes* EGDe transcription of *gadD1* occurs primarily in exponential phase, while transcription of *gadT2D2* is comparatively higher and is induced upon entry to stationary phase and in response to acid induction (Conte *et al.*, 2002; Cotter *et al.*, 2001a). It appears that *gadT1* and *gadD3* are induced following acid adaptation (Wemekamp-Kamphuis *et al.*, 2004), with *gadD3* also being induced by entry into stationary phase and osmotic stress (Kazmierczak *et al.*, 2003). In addition, *gadT2D2* and *gadD3* loci are regulated by the alternative sigma factor SigmaB (Wemekamp-Kamphuis *et al.*, 2004). More recent work has indicated that the *gadT2D2* operon is subject to a high degree of regulation, with transcriptional control exerted at multiple points by the regulators CtsR and σ^B (Hu *et al.*, 2007).

Further applied studies have demonstrated an important role for the GAD system in specific food models. Cotter *et al.* (2001b) demonstrated that the GAD system contributes significantly to survival of the pathogen in low pH foods such as fruit juices, yoghurts, salad cream, and mayonnaise. The authors demonstrated that glutamate was in excess in all of these foods, providing a substrate for the GAD system. In diluted skim milk, however, the addition of free glutamate significantly improved survival of the wild-type but not the GAD mutant strain (Cotter *et al.*, 2001b). Jydegaard-Axelsen *et al.* (2004) have demonstrated that expression of the *gad* genes is increased when *L. monocytogenes* is grown in CO_2, conditions known to reduce intracellular pH. Indeed, recent studies have demonstrated that mutation of specific genes within the GAD system can significantly impact upon survival of the pathogen in modified atmosphere-packaged foods (Francis *et al.*, 2007).

B. Arginine and agmatine deiminase systems

Genomic analysis of *L. monocytogenes* EGDe reveals that this bacterium encodes a potential ADI system. This is a 3-enzyme pathway which has been associated with internal pH homeostasis of a number of bacterial genera including *E. faecalis* (Barcelona-Andres *et al.*, 2002), streptococci (Degnan *et al.*, 2000; Dong *et al.*, 2002), *L. plantarum* (Spano *et al.*, 2004), *O. oeni* (Tonon *et al.*, 2001), and *P. aeruginosa* (Bourdineaud *et al.*, 1993). Arginine is transported into the cell in exchange for a molecule of ornithine in an energy-independent fashion by an arginine–ornithine antiporter (encoded by *arcD*), while the enzymes of the pathway catabolize arginine ultimately to ornithine, ammonia, and carbon dioxide (Fig. 3.1). For each mole of arginine catabolized via the ADI system, 2 moles of ammonia (NH_3) are produced, which subsequently combine with intracellular protons to produce ammonium (NH_4^+) ions. This reaction increases the intracellular pH, thereby enabling survival in hostile environments which would otherwise be lethal for the cell. In addition, ATP is generated by the system and this can potentially be used to extrude protons via the F_1F_0-ATPase (Higuchi *et al.*, 1997). We have recently created clean deletion mutants in the putative ADI pathway in *L. monocytogenes* and show that these mutants demonstrate reduced acid tolerance, suggesting that this system plays an active role in mediating pH homeostasis in *Listeria* (submitted for publication).

Analysis of the listerial genome has also revealed the presence of genes encoding a homologous 3-enzyme pathway, the agmatine deiminase system, encoded by the *agu* genes. Agmatine, a decarboxylated derivative of arginine, is deiminated via this system, resulting in the generation of carbamoyl phosphate, putrescine, and ATP (Driessen *et al.*, 1988). The system has been identified in several bacteria and has been characterized as being pH-inducible (Griswold *et al.*, 2006; Llacer *et al.*, 2007; Lucas *et al.*, 2007). Further work is required to determine the functionality of the agmatine deiminase system in *L. monocytogenes* and to determine the regulatory networks that govern expression of these molecular systems.

C. F_0F_1-ATPase

The F_0F_1-ATPase has a fundamental role in the maintenance of pH homeostasis. Functionally, this multisubunit enzyme is organized into two distinct, but physically linked domains; the catalytic portion (F_1) is cytoplasmic and incorporates the α, B, γ, δ, and ε subunits, while the integral membrane domain (F_0), including the a, b, and c subunits, functions as a membranous channel for proton translocation (Sebald *et al.*, 1982). The function of the cytoplasmic domain is to catalyze the synthesis

of ATP when protons move from the outside of cells into the cytoplasm, through the membrane-bound domain, or to hydrolyze ATP when protons are moved out of the cell.

The role of the F_0F_1-ATPase in organisms capable of oxidative phosphorylation is to synthesize ATP aerobically as a result of protons passing into the cell, and to generate a proton motive force (PMF) anaerobically, via the expulsion of protons. As a consequence of the latter mechanism, it is thought that the F_0F_1-ATPase can increase the intracellular pH in situations where it becomes acidified. For many bacteria, activity data demonstrate that a lowering of the intracellular pH is accompanied by induction of the F_0F_1-ATPase (Koebmann *et al.*, 2000; Kuhnert and Quivey, 2003; Kullen and Klaenhammer, 1999), while in a number of bacterial species, including *Lactobacillus acidophilus* (Kullen and Klaenhammer, 1999), *S. mutans* (Kuhnert *et al.*, 2004), and *S. pneumoniae* (Martin-Galiano *et al.*, 2001) transcription of the F_0F_1 operon is induced by exposure to low pH. The inherent acid tolerance of various bacteria is related both to the levels of F_0F_1-ATPase, as well as to the pH optima of the enzymes. It is clear that naturally acid tolerant organisms possess F_0F_1-ATPases with lower pH optima (Sturr and Marquis, 1992; Martin-Galiano *et al.*, 2001).

Analysis of the role of the F_0F_1-ATPase in acid tolerance is limited by the fact that the system is essential for life in many bacterial species (Koebmann *et al.*, 2000). We have been unable to create clean deletion of the genes encoding the system in *L. monocytogenes* (unpubished data). However, 2-dimensional gel electrophoresis studies have shown that the listerial F_0F_1-ATPase subunit is induced as a consequence of exposure to mild acid treatment (Phan-Thanh and Mahouin, 1999). Administration of DCCD, an ATPase inhibitor demonstrated that the ATPase is involved in acid tolerance and the ATR (Datta and Benjamin, 1997). In addition, we have created a partial insertion mutant in the F_0F_1-ATPase in *L. monocytogenes* that is not compromised in fitness. This mutant demonstrated that the F_0F_1-ATPase of *L. monocytogenes* is involved in the induction of the ATR in this bacterium (Cotter *et al.*, 2000).

D. Macromolecular protection and repair

Exposure to environmental stress can result in misfolding of proteins and damage to macromolecules such as DNA, and consequently bacteria require effective systems for repairing or disposing of damaged intracellular macromolecules (Matic *et al.*, 2004). One of the most notable ways in which bacteria respond to damaged DNA is by use of nucleotide excision repair. This process is carried out by UvrA, UvrB, and UvrC proteins, which can recognize and cleave damaged DNA fragments as part of the ''SOS response'' to DNA damage. In *S. mutans* (Hanna *et al.*, 2001) and

L. helveticus (Cappa *et al.*, 2005), it has been established that this process occurs in response to low pH adaptation. Using an in-frame deletion mutant of *uvrA*, Kim *et al.* (2006) have shown that this gene is also required for optimal growth of *L. monocytogenes* in acidic medium, with the Δ*uvrA* mutant exhibiting a significantly increased lag phase relative to the parent in low pH broth.

In addition to the DNA damage induced by exposure to environmental stress, many proteins are synthesized abnormally. Chaperonins are a group of proteins which are responsible for cytoplasmic proteolysis or refolding of these erroneously synthesized proteins, and many of these proteins are induced following acid exposure in *Listeria* (Phan-Thanh and Mahouin, 1999). DnaK is a chaperonin which has been characterized as being necessary for optimal acid resistance in *S. mutans* (Lemos *et al.*, 2007) and *B. suis* (Kohler *et al.*, 2002) as well as *L. monocytogenes*. A *Listeria* mutant lacking a functional *dnaK* gene exhibits reduced survival at low pH values (Hanawa *et al.*, 1999). Another chaperonin, GroESL is induced in response to acid stress in many genera including *S. mutans* (Lemos *et al.*, 2007; Wilkins *et al.*, 2002) and *L. lactis* (Hartke *et al.*, 1997). Exposure of *L. monocytogenes* to low pH environments results in a similar increase in expression of these genes, with a resultant increase in protein detected (Gahan *et al.*, 2001; Phan-Thanh *et al.*, 2000).

Serine proteases act in a similar manner to chaperonin proteins, by degradation of aberrant proteins which are improperly formed as a consequence of exposure to environmental stress. A gene encoding a serine protease, designated *htrA* has been identified and characterized in *Listeria*. The encoded protein has been shown to be involved in the bacterial response to acid exposure (Stack *et al.*, 2005) as well as stress conditions such as heat, ethanol, and osmotic shock (Wonderling *et al.*, 2004). Stack *et al.* (2005) observed that a mutant lacking this gene exhibited reduced growth rates and final optical densities relative to the parent in acidic conditions, and that under conditions of acid stress, transcription of HtrA in *L. monocytogenes* is upregulated. Furthermore, in *L. monocytogenes* *htrA* is regulated by the two component sensor-regulator LisRK (Sleator and Hill, 2005; Stack *et al.*, 2005).

E. Cell membrane changes

In a number of bacterial genera, it has been shown that pH adaptation results in modulation of fatty acid profiles (Fozo and Quivey, 2004a,b; Kim *et al.*, 2005). Similarly, *L. monocytogenes* cells exposed to environmental stresses such as temperature (Annous *et al.*, 1997), osmotic (Chihib *et al.*, 2003), anaerobic stresses (Jydegaard-Axelsen *et al.*, 2004), and low pH stress (Giotis *et al.*, 2007) undergo alterations in membrane fatty acid profiles. Giotis *et al.* (2007) examined the overall content and proportions

of branched-chain fatty acid (BCFA), straight chain fatty acid (SCFA), and unsaturated fatty acids in cultures of *L. monocytogenes* 10403S which were exposed to mild pH stress. This study revealed that the BCFA content as well as the relative proportions of anteiso- and iso- fatty acids in the listerial cell membrane are modified upon cellular exposure to pH stress. It appears that the membrane alterations differ from those documented in other genera, where exposure to moderately acidic conditions results in increased levels of monounsaturated long-chain fatty acids (FAs) in the membrane; in *Listeria*, higher proportions of SCFAs are incorporated into the membrane. van Schaik *et al.* (1999) observed that in acid-adapted cells, production of the SCFAs $C_{14:0}$ and $C_{16:0}$ was increased, with a concomitant decrease in $C_{18:0}$ detected in the membrane. The authors suggested that the cross-protective effects of acid adaptation could potentially be attributed to this phenotype.

F. Sigma B

The ability of *Listeria* to persist and thrive when subjected to rapidly changing environmental conditions suggests that this bacterium is capable of appropriate responses to external stress (Ferreira *et al.*, 2001). The association of alternative sigma factors with the core RNA polymerase provides a mechanism by which bacterial cells can alter global gene expression by redirection of transcription in response to extracellular signals (Wiedmann *et al.*, 1998). Alternative sigma factors target precise recognition sequences and can thereby control the expression of entire regulons dedicated to specific responses (van Schaik and Abee, 2005). The alternative sigma factor σ^B of Gram-positive bacteria is analogous to the well-characterized RpoS of Gram-negatives, including *E. coli*. It appears that SigB and RpoS have parallel roles in the general stress responses of Gram-positive and Gram-negative organisms, respectively (Ferreira *et al.*, 2001; Gertz *et al.*, 2000; Volker *et al.*, 1999).

σ^B is encoded by the *sigB* gene and has been identified and characterized in detail in a number of Gram-positive bacteria, including *L. monocytogenes*, *B. subtilis*, and *S. aureus*. Recent microarray analysis of the σ^B regulon in *L. monocytogenes* revealed 105 σ^B-positively regulated genes and 111 genes which appeared to be under negative control of σ^B at various stages of the growth cycle (Hain *et al.*, 2008). The σ^B regulon therefore represents 7.6% of genes in the *L. monocytogenes* genome. Of the genes positively regulated by σ^B, 75 had homologs in *B. subtilis*, but only 33 had been previously described as σ^B-regulated in *B. subtilis*. The data indicate a divergence of the σ^B regulons between these two bacterial genera, possibly reflecting adaptation to different niches (Hain *et al.*, 2008). A previous partial array study of the σ^B regulon of *L. monocytogenes* identified 55 genes within the σ^B regulon and proved that these were upregulated in a σ^B-dependent

manner following osmotic upshift (Kazmierczak *et al.*, 2003). Collectively these studies have demonstrated that a high proportion of *L. monocytogenes* genes are under the influence of σ^B, including genes involved in pH homeostasis and gastrointestinal persistence.

The response of σ^B to environmental stresses is transcriptionally mediated (Becker *et al.*, 1998) and appears to be extremely rapid; increased expression of σ^B-dependent genes was detected within 5 min of exposure to acid and osmotic stresses in *L. monocytogenes* (Sue *et al.*, 2004). In *L. monocytogenes,* mutants lacking a functional *sigB* gene have been characterized in great detail and exhibit decreased resistance to numerous adverse conditions, including starvation, heat, salt, bile, oxidative stresses, as well as low pH (Begley *et al.*, 2005; Ferreira *et al.*, 2001; Wiedmann *et al.*, 1998).

It appears that σ^B contributes to acid resistance through at least two mechanisms: firstly, a general acid tolerance to which σ^B-regulated systems contribute throughout all growth phases, as well as a pH-inducible ATR mechanism that is at least partially σ^B-dependent in exponential phase cells (Ferreira *et al.*, 2003; Volker *et al.*, 1999). Becker *et al.* (1998) discerned from primer extension analyses that SigB activity in log phase *L. monocytogenes* can be induced from undetectable levels to a level similar to that observed for stationary phase cells following exposure of cells to mildly acidified media (pH 5.3). Given the essential role of GadD2 (formerly GadB) in pH homeostasis and the fact that this locus is σ^B-regulated, we consider it likely that σ^B-mediated induction of GadD2 at low pH is a major contributor to acid adpation and the ATR in *L. monocytogenes* (Cotter *et al.*, 2001a; Kazmierczak *et al.*, 2003).

In *Listeria*, the function of σ^B appears to be both species and strain dependent. While σ^B contributes to both stationary and log phase acid resistance in *L. monocytogenes*, only log phase acid resistance is σ^B-dependent in *L. innocua* (Raengpradub *et al.*, 2008). Furthermore, compelling evidence indicates that the *sigB* gene plays a variable role in stress response in the different genetic lineages of *L. monocytogenes* (Moorhead and Dykes, 2003). It was shown that a serotype 1/2a strain is more reliant upon an intact σ^B regulon than a serotype 4c strain across a range of environmental stresses, including low pH (Moorhead and Dykes, 2003). Such variations in environmental stress resistance across different strains may contribute to disparate survival in foods and therefore indirectly to discrepancies in pathogenicity between different strains (Wiedmann *et al.*, 1998).

It has been shown that the σ^B regulon includes genes encoding the classical virulence factors InlA and InlB (Bischoff *et al.*, 2004; Gertz *et al.*, 2000), and σ^B is required for rapid expression of *L. monocytogenes* genes which are important for survival of gastrointestinal stresses, including bile salts, elevated osmolarity, and reduced pH (Begley *et al.*, 2005; Hain

et al., 2008; Sleator and Hill, 2005; Sue *et al.*, 2004). Indeed, loss of σ^B has been found to result in decreased virulence of *L. monocytogenes* following oral infection in the murine and guinea pig models (Garner *et al.*, 2006; Nadon *et al.*, 2002). However, mutational analysis revealed that σ^B is not required for intracellular spread or for intravenous infection in guinea pigs (Garner *et al.*, 2006). Given the critical role of this alternative sigma factor during the gastrointestinal stage of listeriosis (Garner *et al.*, 2006), and the fact that the σ^B regulon is induced by low pH conditions similar to those encountered during gastrointestinal passage (Sue *et al.*, 2004), it is likely that the relationship between σ^B and the acid resistance of the bacterium is critical for optimal pathogenesis of *Listeria*. Furthermore, this alternative sigma factor has the potential to promote bacterial survival both outside and inside a host, thus contributing to survival at all stages of the infectious cycle (Chaturongakul and Boor, 2004; Gahan and Hill, 2005).

G. LisRK two-component regulatory system

Two-component signal transduction systems (2CSs) are frequently utilized by bacteria as a means by which they sense and respond to fluctuations in their environments. These molecular systems consist of a membrane-associated histidine kinase sensor, which transduces information about the extracellular environment, operating in combination with a cytoplasmic response regulator, which can modify gene transcription, thereby ensuring an adequate response is mounted to external challenges (Parkinson, 1993; Parkinson and Kofoid, 1992). 2CSs have been characterized in various genera and have been frequently associated with bacterial responses to acid stress. Important examples include PhoPS of *E. faecalis* (Teng *et al.*, 2002), PhoPQ and EnvZ/OmpR of *Salmonella* species (Bang *et al.*, 2000; Bearson *et al.*, 1998), and ToxRS of *V. cholerae* (Merrell *et al.*, 2001). In *Listeria*, the LisRK 2CS is required to mount an effective response to numerous stresses, including low pH (Cotter *et al.*, 1999, 2002; Sleator and Hill, 2005). Deletion of *lisK*, encoding the histidine kinase, was found to result in a growth-phase dependent variation in acid tolerance (Cotter *et al.*, 1999), with mutants more resistant to the deleterious effects of lactic acid in stationary phase, but significantly more sensitive in early exponential phase growth (Cotter *et al.*, 1999). Disruption of the response regulator was also found to result in a decreased ability to grow at reduced pH (Kallipolitis and Ingmer, 2001). Mutants lacking a functional LisRK system also exhibit significant sensitivity to cephalosporins (Cotter *et al.*, 2002) and increased ethanol resistance (Cotter *et al.*, 1999), and hence it has been suggested that in the absence of this 2CS, the sensitivity of the cell to low pH may be mediated by changes in the composition of the cell envelope (Hill *et al.*, 2002). We have recently determined that the heat

shock protein HtrA is induced under low pH conditions in *L. monocytogenes* and is regulated by LisRK (Sleator and Hill, 2005; Stack *et al.*, 2005). Further analysis is necessary to determine the components of the LisRK regulon.

VII. CONCLUSION

The ability to respond appropriately and effectively to extracellular signals is a critical feature of pathogenic bacteria. *L. monocytogenes* is routinely exposed to suboptimal low pH environments throughout the infectious cycle. Consequently, the ability of the bacterium to maintain intracellular pH homeostasis is central to the process of disease causation. Whilst the importance of some acid response systems (such as the GAD system) is well established, there are many challenges ahead in terms of understanding the interplay between molecular mechanisms governing stress adaptation in this pathogen. This work will be aided by the availability of genomic information for a number of *Listeria* species and strains and the potential to dissect the transcriptomic and proteomic responses to specific stress conditions. Fundamental to this approach will be the understanding of regulatory networks (including the Sigma B regulon) and how these modulate microbial adaption in complex environments and during infection.

In addition, it is clear that distinct serotypes and lineages of *L. monocytogenes* differ in their ability to respond to acid stress. This has the potential to influence the ability of strains to cross the gastric barrier to infection and may therefore influence the infectious dose of specific strains. An understanding of adaptive responses across a variety of *L. monocytogenes* strains will be an important goal if we are to predict and model the growth of this pathogen in foods and in the host.

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CHAPTER 4

Global Regulators of Transcription in *Escherichia coli*: Mechanisms of Action and Methods for Study

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I. INTRODUCTION

A. An overview of the bacterial multi-subunit RNA polymerase

In bacteria, RNA polymerase exists in two states. One form, known as the core enzyme, can catalyze RNA synthesis but is unable to bind to promoter targets in DNA. The second form of RNA polymerase, the holoenzyme, is capable of both RNA synthesis and promoter recognition. The bacterial RNA polymerase is a multisubunit enzyme and both forms of RNA polymerase posses the α_2, β and β′, and ω subunits. The RNA polymerase holoenzyme contains an additional subunit, σ, and this is the subunit that facilitates DNA recognition. Following σ-mediated DNA binding, transcription initiation occurs, the σ subunit then dissociates from the RNA polymerase–DNA–mRNA complex and the core enzyme completes the process of gene transcription. It is estimated that there are ~5000 copies of RNA polymerase in growing *Escherichia coli* K-12 cells, which must be distributed between ~3000 transcription units. Thus, the cell must carefully regulate the binding of RNA polymerase across its chromosome. RNA polymerase activity can be modulated by DNA sequence elements, transcription factors, nucleoid-associated proteins, small molecules, and RNA polymerase binding proteins (shown in Fig. 4.1).

B. DNA recognition by RNA polymerase

Since the σ subunit of RNA polymerase is responsible for DNA recognition, it plays a pivotal role in managing the chromosome-wide distribution of the transcriptional machinery. Seven σ factors, σ^{70}, σ^{54}, σ^{38}, σ^{32}, σ^{28}, σ^{24}, and σ^{FecI}, have been identified in *E. coli* (Gross *et al.*, 1992, 1998; Helmann and Chamberlin, 1988; Ishihama, 1997) and each σ subunit is required by

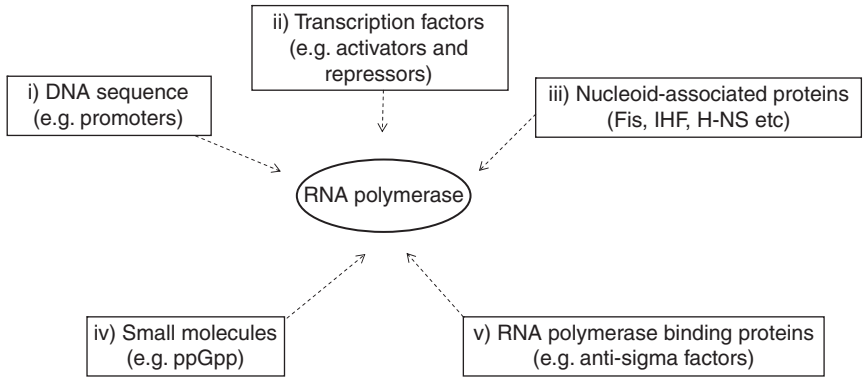

FIGURE 4.1 Factors effecting RNA polymerase activity in *E. coli.*

RNA polymerase in order for a specific set of genes to be transcribed. For instance, DNA sequences upstream of genes expressed during exponential growth are usually recognized by σ^{70}, the housekeeping σ factor. Other σ factors are required for the expression of genes needed for; survival during stationary phase (σ^{38}), growth in low levels of nitrogen (σ^{54}), the heat shock response (σ^{32}), chemotaxis (σ^{28}), the refolding of denatured proteins (σ^{24}), and iron citrate transport (σ^{FecI}).

The DNA elements recognized by the σ subunit of RNA polymerase are ''promoters'' of transcription. Promoters recognized by σ^{70} contain two well-characterized elements, the −10 and −35 hexamer core promoter sequences, which are located 10 and 35 base pairs upstream of the transcription start site (Fig. 4.2). Consensus sequences, deduced by comparing a large number of promoter sequences, have been defined for these elements (Gralla, 1996) and the location and sequence of these elements varies for the alternative σ factors. The degree of identity between the promoter elements at a promoter and the defined consensus sequences determines the basal activity of the promoter. Interestingly, no *E. coli* promoter has −10 and −35 elements that perfectly match the consensus sequence. Consequently, promoter activity can be enhanced by additional DNA sequences. For example, AT-rich ''UP'' elements are found upstream of the −35 hexamer at some promoters (e.g., at the promoters for the seven major ribosomal RNA operons). UP elements are typically around 20 base pairs in length and facilitate binding of αCTD, and hence RNA polymerase, to the promoter. This can increase transcription by 30–70 fold (Rao *et al.*, 1994). A surface of the αCTD, known as the 265 determinant, encompasses residues important for DNA binding by αCTD and is required for maximal activity of promoters containing UP elements. (Gaal *et al.*, 1996; Murakami *et al.*, 1996). The ribosomal RNA promoters are amongst the most active and are estimated to sequester large numbers of RNA polymerase molecules during rapid growth (Grainger *et al.*, 2005). Thus, only a fraction of the 5000 RNA polymerase molecules are available for the transcription of other genes.

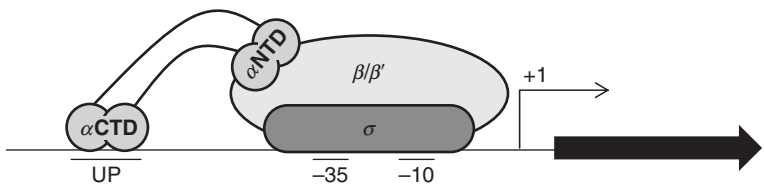

FIGURE 4.2 Promoter DNA recognition by RNA polymerase. RNA polymerase is able to recognize promoter DNA via specific interactions between the σ subunit of the enzyme and the core (−10 and −35) promoter elements. Ancillary promoter elements, such as the UP element that interacts with αCTD, can enhance the affinity of a promoter for RNA polymerase.

II. REGULATION BY TRANSCRIPTION FACTORS

A. An overview of transcription regulators

Some promoters are active in the absence of additional factors and when the genes under their control are not required, they are silenced by transcription repressors. However, most promoters lack a good match to the consensus elements for RNA polymerase binding and require ancillary proteins, known as transcription activators, to function. These transcription activators are sequence specific DNA binding proteins that increase the frequency of transcription initiation. Transcriptional activators and repressors share many properties and often exert their effect in response to specific environmental signals. A small number of transcription factors, termed ''global'' regulators, influence the expression of a large number of transcription units. Conversely, a large number of ''specific'' transcription factors each affect the expression of a small number of transcription units (Martínez-Antonio and Collado-Vides, 2003). The expression of some transcription units, for example the *E. coli melAB* operon, is regulated by a combination of both global and specific transcription factors (Wade *et al.*, 2001). This allows bacteria to regulate the expression of a small number of genes in response to several environmental stimuli.

B. Global transcription factors

The two best characterized global transcriptional regulators in *E. coli* are CRP (*c*AMP *r*eceptor *p*rotein) and FNR (*f*umarate and *n*itrate *r*eductase; reviewed in Browning *et al.*, 2002; Savery *et al.*, 1996). The activity of CRP is regulated by the second messenger cAMP, which is present at high levels in the cell during periods of starvation. Thus, CRP regulates global patterns of transcription in response to carbon availability. FNR directly senses oxygen levels via an iron–sulphur cluster at the N-terminus of the protein and co-ordinates the transcriptional response to oxygen limitation. Much of our understanding of the molecular details of transcription regulation has emerged from detailed studies of CRP and FNR action at a small number of target promoters. Both CRP and FNR predominantly function as activators of transcription and, like most activators, serve to increase levels of promoter occupancy by RNA polymerase. Activators of this type often function by a simple recruitment mechanism and interact specifically with both the promoter and RNA polymerase (Busby and Ebright, 1994; Dove *et al.*, 1997). Promoters regulated by CRP, FNR, and other activators can be grouped into three classes (Busby and Ebright, 1999).

1. Class I activator dependent promoters

At class I activator dependent promoters, the activator protein binds upstream of the core promoter elements. Accordingly, when bound at this location the activator functions by enhancing the binding of the αCTD of RNA polymerase to the promoter, which improves the binding affinity of RNA polymerase (Fig. 4.3A). An example of a class I activator dependent promoter in *E. coli* is the *lac* promoter, which is activated by CRP. The interaction between CRP and αCTD at the *lac* promoter has been investigated extensively; a determinant located between residues 156 and 164 of CRP, Activating Region 1 (AR1), interacts with a region of αCTD close to residue 287, the 287 determinant (Bell *et al.*, 1990; Eschenlauer and Reznikoff, 1991; Niu *et al.*, 1994; Savery *et al.*, 2002; Zhou *et al.*, 1993).

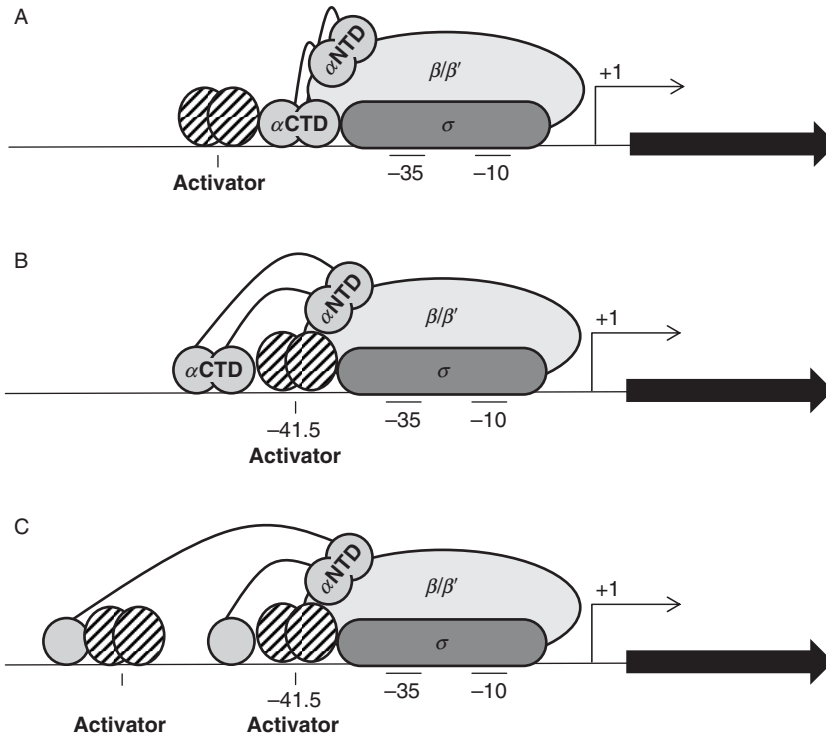

FIGURE 4.3 Class I and class II activator dependent promoters. Sequence specific transcription factors can enhance binding of RNA polymerase to a promoter by making specific contacts with the DNA and with RNA polymerase. At class I promoters, the activator protein binds upstream and contacts the αCTD of RNA polymerase (A). At class II promoters, the activator binds immediately adjacent to the −35 promoter element and can potentially contact all subunits of RNA polymerase (B). Class III promoters contain multiple activator binding sites (C).

Although the precise molecular details of the interaction differ, the FNR protein also has an AR1 that interacts with αCTD at Class I promoters (Lee *et al.*, 2000). Interactions between the promoter proximal αCTD and the σ^{70} subunit of RNA polymerase may further stimulate transcription at some Class I activator dependent promoters (Chen *et al.*, 2003; Ross *et al.*, 2003).

2. Class II activator dependent promoters

At class II activator dependant promoters, the activator protein binds sequences proximal to the core promoter elements, centered at position −41.5. Thus, the activator is close to all subunits of RNA polymerase and there are more opportunities to interact with the transcriptional machinery (Fig. 4.3B). An example is the *E. coli melR* promoter, which has a CRP binding site centered 41.5 base pairs upstream of the transcription start site. Here, CRP utilizes determinants in addition to AR1 (Bell *et al.*, 1990; Rhodius *et al.*, 1997; Williams *et al.*, 1991; Zhou *et al.*, 1994). The key determinant is located on the N-terminal domain of CRP, is known as Activating Region 2 (AR2), and interacts with αNTD (Niu *et al.*, 1996). CRP possesses a third activating region (AR3) that is nonfunctional in the WT protein. Substitution of K52, by a neutral or positively charged residue, resurrects functionality of AR3 (Bell *et al.*, 1990; Williams *et al.*, 1991). When active, AR3 gives rise to a significant increase in transcription at class II, but not class I, CRP dependent promoters (Bell *et al.*, 1990; Williams *et al.*, 1991). AR3 is believed to contact region 4 of the σ^{70} subunit of RNA polymerase, which is a target for many regulators of transcription (Dove *et al.*, 2003; Lonetto *et al.*, 1998). Like CRP, FNR has an AR2 and an AR3 that interact with RNA polymerase at class II promoters although the molecular details of these interactions are less well understood (Li *et al.*, 1998).

3. Class III activator dependent promoters

Some activator dependent promoters contain multiple binding sequences for one or more transcription factors, and this allows several environmental signals to be incorporated into the process of transcription activation (Fig. 4.3C). Though class III activator dependent promoters contain multiple activator target sequences, the positioning of these binding sites is similar to that seen at simple promoters: activator target sites can be located close to the −35 hexamer and/or positioned further upstream. Thus, the mechanism of transcription activation at complex and simple promoters is thought to be the same: RNA polymerase is recruited to the promoter by interactions between the activator protein and subunits of the transcriptional machinery. Synthetic promoters that contain two CRP or FNR binding sites, located in a combination of promoter proximal (class II) and promoter distal (class I) positions, have been created artificially. Experiments of this type have shown that CRP and FNR are

capable of activating transcription synergistically when bound to two distinct sites (Barnard *et al.*, 2003; Law *et al.*, 1999). Though poorly studied, complex promoters are common in *E. coli*.

III. REGULATION OF TRANSCRIPTION BY NUCLEOID-ASSOCIATED PROTEINS

A. The nucleoid-associated proteins

In addition to RNA polymerase and transcription factors, the chromosome of an *E. coli* cell is bound by a battery of proteins involved in DNA repair, replication, protection, and folding. The folded *E. coli* chromosome is called the nucleoid and proteins involved in folding the chromosome are known as nucleoid-associated proteins (reviewed in Dame, 2005; Jin and Cabrera, 2006; Travers and Muskhelishvili, 2005). Nucleoid proteins bind to the DNA with little or no sequence specificity and often induce dramatic bending of the DNA. However, their precise role in regulating chromosome structure is still undetermined. Interestingly, many of the nucleoid-associated proteins can also act as global transcription factors (reviewed in McLeod and Johnson, 2001). With respect to transcription regulation, three of the best studied nucleoid proteins are Fis (Factor for Inversion Stimulation), H-NS (Histone like Nucleoid Structuring protein) and IHF (Integration Host Factor). Although these proteins bind to the DNA with much less specificity than transcription factors like FNR and CRP, they can recognize discrete targets in promoter DNA.

B. Mechanisms of transcription regulation by Fis, H-NS, and IHF

Fis is the best characterized nucleoid-associated protein with respect to transcription regulation. For instance, at the ribosomal RNA promoters, Fis binds at a class I position and makes specific contacts with αCTD to aid recruitment of RNA polymerase to the promoter DNA (Aiyar *et al.*, 2002). Similarly, at the *proP* P2 promoter, Fis binds close to the core promoter elements and activates transcription as a typical class II regulator and again contacts αCTD (McLeod *et al.*, 2002). However, at many other target promoters, Fis regulates transcription via more complicated mechanisms. For example, at the *E. coli dps* promoter Fis acts as a repressor of transcription by binding close to the core promoter elements, together with RNA polymerase, and preventing promoter opening (Fig. 4.4A). Interestingly, whilst the *dps* promoter can be served by RNA polymerase containing σ^{70} or σ^{38}, Fis only represses transcription by σ^{70} associated RNA polymerase (Grainger *et al.*, 2008).

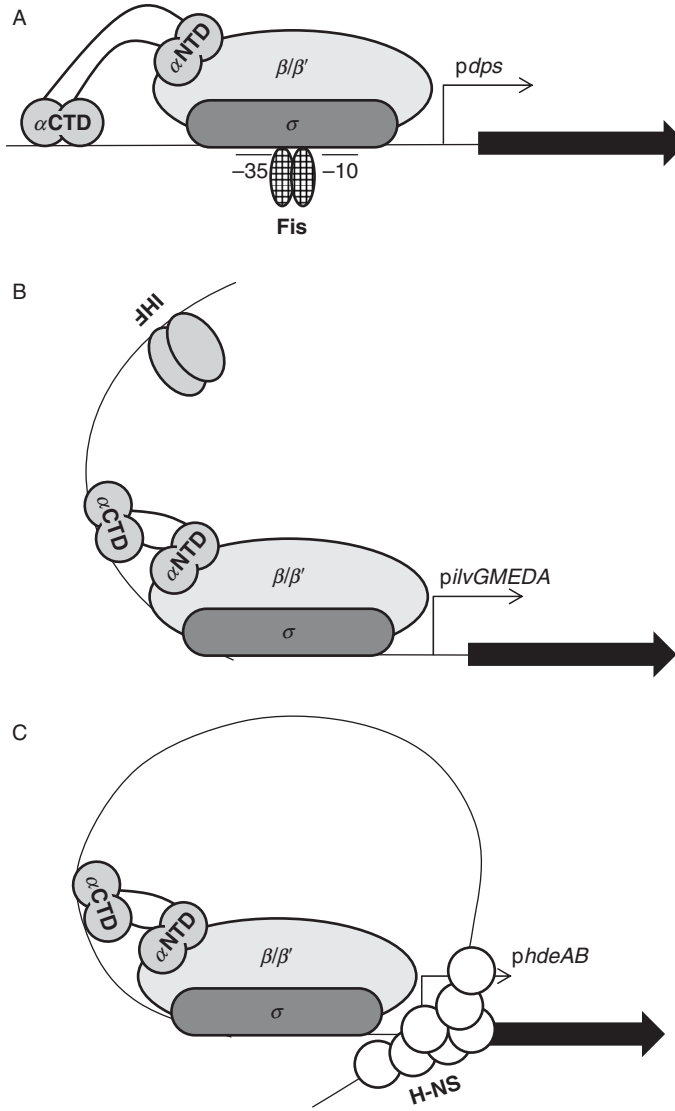

FIGURE 4.4 Complex promoter regulation by nucleoid-associated proteins. Nucleoid-associated proteins often bind to promoters and regulate transcription via complicated mechanisms. For example, the *E. coli dps* promoter (A) is repressed by Fis binding between the core promoter elements and trapping RNA polymerase. At the *ilvGMEDA* promoter (B) IHF can activate transcription by bending the DNA, rather than interacting directly with RNA polymerase. At the *hdeAB* promoter H-NS traps RNA polymerase, and represses transcription, by stabilising a DNA repression loop (C).

As detailed above, activators of transcription often make specific contacts with the transcriptional machinery. However, such an interaction is not always required for transcription activation and a small number of activators can function by bending the promoter DNA. The *E. coli* nucleoid-associated protein IHF is an example of such a protein. At the *ilvGMEDA* operon, IHF binds a DNA target site that is 92 base pairs upstream of the *ilvGMEDA* transcription start point (Fig. 4.4B). The distortion of the DNA that follows IHF binding increases promoter occupancy and open complex formation by RNA polymerase (Parekh and Hatfield, 1996). Like Fis, IHF can act as both an activator and a repressor of transcription. For example, IHF binds to a site at position −88 within the *E. coli nirB* promoter and interferes with class II FNR dependent transcription activation. Thus, in this instance, IHF does not repress transcription by directly hindering the activity of RNA polymerase but instead acts as an ''antiactivator'' of FNR (Browning *et al.*, 2000).

Whilst Fis and IHF can act as both activators and repressors of transcription, H-NS is thought to function solely as a transcriptional repressor. In several instances (for example at the *hdeA* promoter), H-NS stabilizes a loop in the promoter that can trap RNA polymerase and block transcription (Fig. 4.4C) (Shin *et al.*, 2005). At other promoters, such as *proV*, H-NS may simply block DNA binding by RNA polymerase (Nagarajavel *et al.*, 2007). Many promoters regulated by H-NS contain two H-NS binding elements separated by several hundred base pairs. Interaction between H-NS molecules binding at these loci appears to be a key feature of gene regulation by H-NS.

IV. A NOVEL METHOD FOR STUDYING TRANSCRIPTION ON A GLOBAL SCALE

A. Overview of chromatin immunoprecipitation

As outlined above, protein–DNA interactions play a crucial role in transcription and its regulation. Prior to the genomic revolution, biochemical and genetic approaches were the tools of choice for studying protein–DNA interactions, with many studies focusing on a small number of target promoters. Recently, there has been a shift in emphasis to the study of transcription on a global scale. Chromatin immunoprecipitation (ChIP) is a powerful method that permits protein–DNA interactions to be measured directly, *in vivo*, and independently of their consequences (reviewed in Aparicio *et al.*, 2004). In ChIP experiments, cells are fixed with formaldehyde, resulting in proteins being covalently attached to DNA sequences to which they are bound. Following cross-linking, cellular nucleoprotein is extracted and sheared into ~500 base

pair DNA fragments by sonication. The protein of interest is then immunoprecipitated from the cell debris with an appropriate antibody, along with DNA fragments to which it is cross-linked. Protein–DNA cross-links are then reversed and the isolated DNA fragments are analyzed. Segments of the chromosome that were associated with the protein of interest at the moment formaldehyde was added will be enriched in the immunoprecipitated DNA sample.

Several different approaches can be used to analyze the DNA sequences in any sample of immunoprecipitated DNA. For example, if the binding of a protein to a known target is being measured, quantitative PCR can be used to determine the enrichment of the target site. Note that, in ChIP experiments, as well as isolating known targets for a given protein, other targets bound by the factor will also be precipitated. Thus, by defining the composition of the entire immunoprecipitated DNA sample, one can map the binding of a protein across an entire genome. This is most commonly done by labeling the immunoprecipitated DNA and hybridizing it to a suitable DNA microarray, along with a differently labeled control sample (genomic DNA or DNA from a mock immunoprecipitation). This allows sections of the chromosome enriched in the immunoprecipitate to be identified. This approach is often referred to as ChIP-chip and allows measurement of protein–DNA interactions across entire genomes (Fig. 4.5) (Iyer *et al.*, 2001; Ren *et al.*, 2000).

Until recently, ChIP-chip had been applied mainly to eukaryotic systems. However, because of their small genome sizes, bacteria are ideally suited to methodologies involving microarrays. Additionally, developments in chromosome engineering allow rapid epitope tagging of proteins (Cho *et al.*, 2006; Court *et al.*, 2002; Uzzau *et al.*, 2001). Therefore, proteins for which no antibody is available can readily be studied using ChIP-chip. Below we discuss how recent ChIP-chip studies of global regulators in *E. coli* and other bacteria have enhanced our understanding of transcription regulation.

B. Application of ChIP-chip to the study of sequence specific transcription factors

A major goal in the study of gene regulation is to untangle the transcription regulatory networks of *E. coli* and other ''simple'' organisms. To do this, we must catalogue the binding sites of all transcription factors. Initial studies relied on the transcriptomics approach and have been only moderately successful. Such experiments compare the transcriptome (mRNA content) of wild type cells and cells that do not express the transcription factor of interest. The location of transcription factor binding sites is then inferred by identifying transcripts expressed at different levels when the regulator is deleted. While useful, this type of analysis is often

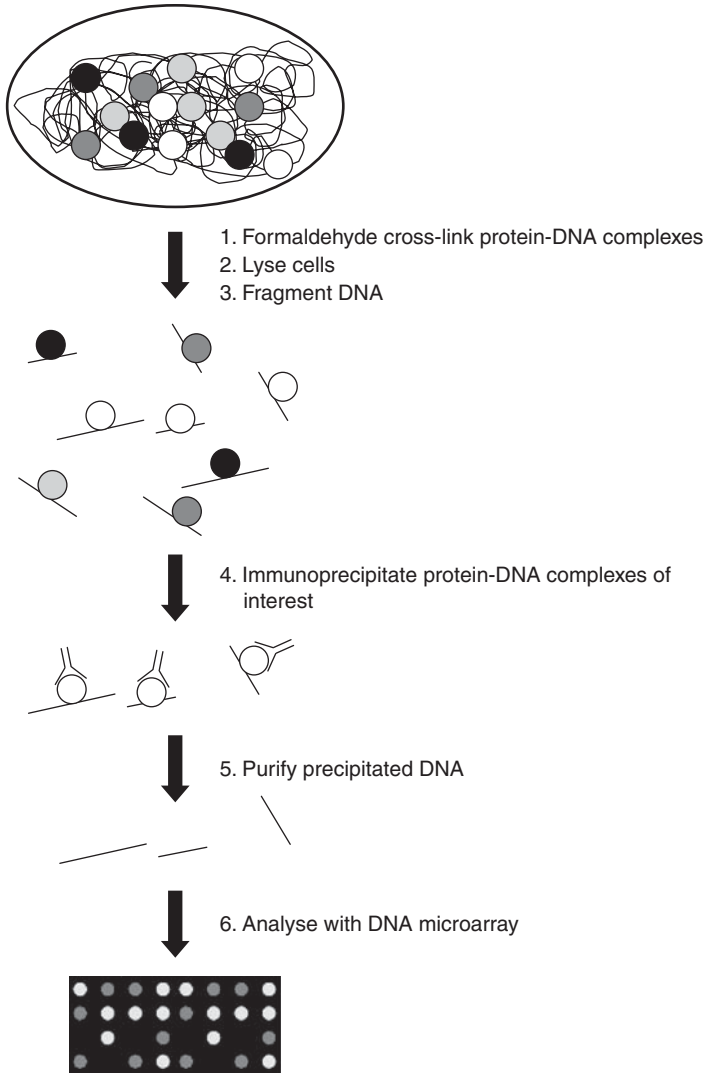

FIGURE 4.5 An overview of chromatin immunoprecipitation and DNA microarray analysis (ChIP-chip). The figure shows and overview of a ChIP-chip experiment. Cells are first fixed with formaldehyde and then lysed before their nucleoprotein content is fragmented by sonication. Formaldehyde cross-linked protein-DNA complexes of interest can then be immunoprecipitated and the isolated DNA fragments purified, labeled and hybridized to a DNA microarray.

complicated because of secondary effects on transcription that result from removal of the transcription factor. Problems are particularly acute if a global regulator is deleted, as this often has an adverse effect on

growth-rate. ChIP does not require mutant cells, eliminating the possibility of indirect effects, and allowing the study of essential proteins.

Laub *et al.* (2002) first demonstrated that ChIP-chip could be used to study DNA binding by bacterial transcription factors in their study of the *Caulobacter crescentus* cell-cycle regulator CtrA. More recent studies have shown that the ChIP-chip approach can be used to measure chromosome-wide DNA binding by other sequence specific transcription factors in other bacteria: Fur in *Helicobacter pylori* (Danielli *et al.*, 2006), CodY (Molle *et al.*, 2003a), Spo0A (Molle *et al.*, 2003b), and SpoIIID (Eichenberger *et al.*, 2004) in *Bacillus subtilis*, and MelR (Grainger *et al.*, 2004), FNR (Grainger *et al.*, 2007), CRP (Grainger *et al.*, 2005), NsrR (Efromovich *et al.*, 2008), and LexA (Wade *et al.*, 2005) in *E. coli*. As well as pinpointing transcription factor binding sites, these ChIP-chip experiments have uncovered unexpected phenomena.

1. The *E. coli* genome is permissive to DNA binding

Studies with LexA have shown that it is able to recognize all matches to the LexA consensus binding site *in vivo* across the chromosome. This shows that there are no sections of the *E. coli* chromosome that are ''closed'' for transcription factor binding. This is in contrast to eukaryotes, where histone proteins sequester large tracts of DNA rendering them unrecognizable to transcription factors (Wade *et al.*, 2005).

2. Transcription factors bind to many nonconsensus sites *in vivo*

ChIP-chip studies have shown that some sequence-specific transcription factors bind at nonconsensus sites *in vivo*. For instance, many CtrA (*C. crescentus*), LexA (*E. coli*) and FNR (*E. coli*) targets identified by ChIP-chip do not contain a good match to the consensus binding site sequence (Grainger *et al.*, 2007; Laub *et al.*, 2002; Wade *et al.*, 2005), and several of the *B. subtilis* Spo0A targets identified by ChIP-chip are not bound in an *in vitro* binding assay (Molle *et al.*, 2003b). A likely explanation for this is that multiple transcription factors may bind co-operatively to adjacent DNA sites, in a manner akin to transcription factors binding to enhancers in mammalian cells. Co-operative interactions between transcription factors could reduce the requirement for consensus sequences, or perhaps alter the sequence preference of individual transcription factors. Alternatively, differences in local DNA topology might influence the sequence preference of transcription factors.

3. Transcription factors bind DNA sites with no known function

Unexpectedly, many transcription factor binding sites identified by ChIP-chip have no obvious role in transcription regulation. For instance, in *E. coli* FNR protein binds to five targets adjacent to genes not involved in the transcriptional response to oxygen availability and expression of these

genes is not altered significantly in cells lacking FNR (Grainger *et al.*, 2007). Similarly, many genes adjacent to LexA targets do not display altered activity following the exposure of cells to UV light, which induces rapid degradation of LexA (Wade *et al.*, 2005). There are numerous possible explanations for these observations. For example, the transcription factor may function only in specific contexts. This is probable since, many transcription factors bind to their DNA targets under both activating and nonactivating conditions. Alternatively, the protein may simply act only to ''tweak'' levels of promoter activity. Another explanation is that some transcription factor target sites simply have no functional relevance and are by-products of evolution.

C. Application of ChIP-chip to the study of nucleoid-associated proteins

ChIP-chip has already been used to study the chromosome-wide DNA binding patterns of several nucleoid-associated proteins; H-NS in *Salmonella typhimurium* LT2 (Lucchini *et al.*, 2006; Navarre *et al.*, 2006) and Fis, H-NS, and IHF in *E. coli* (Grainger *et al.*, 2006; Oshima *et al.*, 2006). As for studies with sequence specific transcription factors, this work has not only identified lists of targets but has also highlighted unexpected properties of the nucleoid proteins.

1. Fis, IHF, and H-NS are biased towards noncoding sections of DNA

In eukaryotes, chromosome folding histone proteins saturate the DNA and have to be removed in order for genes to be transcribed. Although all of the bacterial nucleoid proteins that have been studied by ChIP-chip bind to both coding and noncoding DNA they are all bias towards noncoding sections of the genome, consistent with their proposed ancillary role in transcription regulation (Grainger *et al.*, 2006).

2. H-NS silences "Foreign" DNA

Studies of global H-NS binding in both *S. typhimurium* and *E. coli* have shown that sections of the chromosome that have been laterally acquired are rich in H-NS and silenced by binding (Lucchini *et al.*, 2006; Navarre *et al.*, 2006; Oshima *et al.*, 2006). For example, in *S. typhimurium*, H-NS coats the pathogenicity island SPI1 and occludes the binding of RNA polymerase to render the DNA transcriptionaly silent. It is thought that H-NS repression if relived by the binding of an activator protein that can displace the bound H-NS. Interestingly, in *E. coli*, it has been found that many locations where H-NS is bound to the DNA also contain RNA polymerase (Grainger *et al.*, 2006; Oshima *et al.*, 2006) perhaps suggesting that trapping of RNA polymerase (as shown in Fig. 4.3Ci) may be a common mechanism of transcription regulation by H-NS.

3. Many promoters are bound by several nucleoid-associated proteins

The frequency at which the nucleoid proteins are found to bind at promoter sites suggests that most *E. coli* promoters are likely to be bound by at least one of these factors. Thus it seems that there are few naturally occurring simple promoters; most promoters are likely to be regulated by a combination of sequence specific transcription factors and nucleoid proteins.

D. RNA polymerase-omics

For those studying global patterns of transcription it is important to measure binding, and changes in the binding, of the transcriptional machinery, and RNA polymerase. Typically, the location of RNA polymerase is inferred by studying global mRNA levels using DNA microarray analysis. Although useful, there are several drawbacks to this approach. For example, if RNA polymerase is bound to the DNA but not actively transcribing a gene then this will be undetectable by measuring mRNA. Moreover, mRNA stability is not uniform and this can affect the apparent levels of any given transcript. A useful alternative to transcriptomics is to measure chromosome-wide DNA binding by RNA polymerase in ChIP-chip experiments (Graniger *et al.*, 2005).

E. Protocols for ChIP-chip experiments with *E. coli*

In this section, we describe protocols for a typical *E. coli* ChIP-chip study: RNA polymerase redistribution induced by an environmental stress (sodium salicylate treatment). Note that, with modifications, these protocols could be used to study DNA binding by any DNA associated protein under any environmental condition.

1. Immunoprecipitation of DNA targets associated with RNA polymerase

1. Grow two 25 ml cultures of *E. coli* to an OD_{650} of 0.4 in LB medium with aeration at 37 °C.
2. Add Sodium Salicylate to one of the cultures, to a final concentration of 5 mM, and let the cultures continue to grow for a further 40 min. Formaldehyde is then added to a final concentration of 1% for 20 min before Glycine is added to a final concentration of 450 mM for a further 5 min. Note that when these reagents are added to cultures care should be taken to ensure that cells are not stressed by temperature shifts (caused by removing cultures from shaking water baths, etc.) and so on.
3. Collect the cells from each culture by centrifugation and wash twice with 10 ml of Tris-buffered saline (TBS), pH 7.5 by centrifugation and resuspension of the cells. After the second wash, resuspend cells in

0.5 ml of lysis buffer (10 mM Tris–HCl, pH 8.0, 20% sucrose, 50 mM NaCl, 10 mM EDTA, 10 mg/ml lysozyme) and incubate for 30 min at 37 °C. Note that lysis buffer without lysozyme should be prepared in advance and stored at room temperature. Aliquots of this buffer can then be supplemented with lysozyme prior to use.

4. After lysozyme treatment, lysis is completed by the addition of 4.5 ml of immunoprecipitation buffer (50 mM HEPES–KOH, pH 7.5,150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS). PMSF is then added to a final concentration of 1 mM and the sample is chilled on ice in preparation for sonication.
5. Sonication is used to fragment the DNA prior to immunoprecipitation of cross-linked protein-DNA complexes. Ideally, the DNA fragments should be between 500 and 1000 base pairs in length.
6. Remove cell debris from the sample by centrifugation.
7. Wash a suitably sized aliquot of Ultralink protein A/G beads (Pierce) three times with TBS prior to use by centrifugation and resuspension of the beads. Note that the beads are delicate and should be centrifuged at a low speed in a bench top microfuge. After the final wash, resuspend the beads to form a 50% slurry. To immunoprecipitate cross-linked RNA polymerase-DNA complexes, add an 800 µl aliquot of the sonicated chromatin to 20 µl of washed protein A/G beads and then 4 µl of monoclonal anti-RNA polymerase (β-subunit) antibody (Neoclone). Mix at room temperature for 90 min on a rotating wheel.
8. After immunoprecipitation, the protein A/G beads are collected and washed in Spin-X columns (Sigma, Cat. No. CLS 8160). The beads are washed twice with 0.5 ml of immunoprecipitation buffer, once in 0.5 ml of immunoprecipitation buffer containing 500 mM NaCl, once with 0.5 ml wash buffer (10 mM Tris–HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% Na deoxycholate), and once with 0.5 ml Tris–EDTA, pH 7.5.
9. To remove cross-linked protein-DNA complexes from the beads, resuspend the beads in 100 µl of elution buffer (50 mM Tris–HCl, pH 7.5, 10 mM EDTA, 1% SDS) and incubate the sample at 65 °C for 10 min. After centrifugation the flow through from the spin-X column should be retained and the beads can be discarded.
10. Add 10 µl of 40 mg/ml Pronase (Roche) to the flow through and incubate at 42 °C for 2 h and 65 °C for 6 h. This step can be done overnight using a PCR machine with the sample being cooled to 4 °C until they are collected the following day.
11. Purify the DNA fragments using a Qiagen PCR purification kit according to the manufacturer's protocol. Elute the DNA from the Qiagen column in 30 µl of water and determine the DNA concentration. A single immunoprecipitation usually yields about 100 ng of DNA. Several equivalent immunoprecipitations may set up to increase the final amount of DNA isolated.

2. DNA labelling, microarray hybridization, and processing

In our experiments, we have used high-density *E. coli* MG1655 microarrays produced by Oxford Gene Technology (www.ogt.co.uk). Similar arrays are available from other suppliers and should be suitable for analysis of immunoprecipitated DNA produced by these experiments. The arrays that we used comprise 21,321 60-base-long oligonucleotides, with an average spacing between them of 160 base pairs. The probes are not in genome order on the array, which provides a control against array visualisation artifacts. We labeled DNA immunoprecipitated from the ''+salicylic acid'' culture with Cy5 and DNA from the ''−Salicylic acid'' culture with Cy3. We then compared the two samples by simultaneous hybridization to the array. Note that there is no amplification of the precipitated DNA in this protocol, which avoids possible artifacts because of amplification bias.

1. Ensure that DNA samples are in a volume of 20 µl and at a minimum concentration of 5 µg/ml. This may mean that DNA isolated from several equivalent immunoprecipitations has to be combined and concentrated using a vacuum centrifuge. Mix the DNA with 20 µl of 2.5x random primer (Bioprime kit, Invitrogen) and 0.25 µl of sterile water and denature by heating to 94 °C for 3 min.
2. After denaturation, add 5 µl of dNTP mix (2 mM dATP, 2 mM dGTP, 2 mM dTTP, 0.5 mM dCTP), 3.75 µl of Cy5- or Cy3-labeled dCTP (1 mM; GE Healthcare), and 1 µl of the Klenow fragment of DNA polymerase (Bioprime kit), mix gently, and incubate at 37 °C for 2 h.
3. Add a further 0.5 µl of DNA polymerase to each tube and incubate at 37 °C for a further 2 h.
4. Purify DNA fragments from the labeling reaction using Qiagen PCR purification columns according to the manufacturer's instructions. The labeled DNA should be eluted from the column using 50 µl of Qiagen elution buffer.
5. The labeled DNA samples can now be mixed and hybridized to the array according to the manufacturer's instructions (freely available at www.ogt.co.uk). Oxford Gene Technology arrays are manufactured on an Agilent platform, and hybridizations are best performed in an Agilent SureHyb apparatus.

3. Visualization and analysis of DNA microarray data

After scanning the microarray, a list of Cy5 and Cy3 signal intensities is generated for each probe on the array and plotted against the genomic coordinate of the probe. This generates a genome-wide DNA-binding profile for RNA polymerase. This analysis can be done in a Microsoft Excel spreadsheet, and binding sites are localized approximately by comparison of peak heights with a suitable *E. coli* database. Alternatively,

the profile can be scrutinized using a ''genome browser,'' in which the fluorescence ratios are superimposed on the genetic map of *E. coli*. Oxford Gene Technology's ChIP Browser software (www.ogt.co.uk) facilitates this type of analysis. An overview of data generated from this type of experiment is shown in Fig. 4.6A. Note that by generating lists of genes up and down regulated by salicylic acid shock (as would be done with a ''transcriptomics'' experiment), one can view the binding profile of RNA polymerase across transcription units and investigate the subunit composition of the transcriptional machinery at different parts of the chromosome as well (Fig. 4.6B).

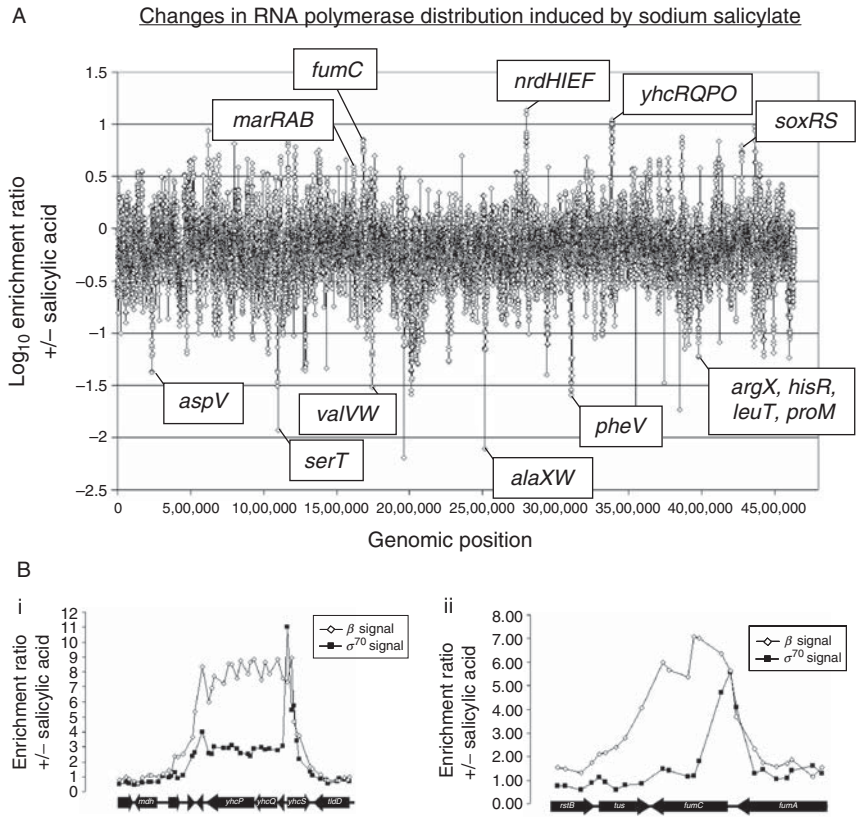

FIGURE 4.6 Changes in chromosome-wide RNA polymerase binding induced by treatment of *E. coli* cells with Sodium salicylate. The figure shows data from ChIP-chip experiments investigating the redistribution of RNA polymerase across the *E. coli* chromosome that occurs when cells are treated with Sodium salicylate. In addition to measuring the global redistribution of the transcriptional machinery and identifying up/down regulated genes (panel A) the data at specific loci can reveal the local distribution and subunit composition of the transcriptional machinery (panels B and C).

V. CONCLUDING REMARKS

New genomic technologies mean that cataloguing all targets for global regulators of transcription is now an achievable goal. As well as determining the regulatory role of each transcription factor, the data generated from such experiments will be particularly useful systems biologists who are modeling the transcription networks of *E. coli* and other bacteria. It is important to note that, despite our increasing tendency to study transcription on a global scale, we still do not understand the molecular details of transcription regulation by most factors at most promoters in *E. coli*. Moreover, in less well studied bacteria, and many higher organisms, there is a complete lack of such information. Thus, future research on the function of transcriptional regulators is likely to be targeted at both their global role is well as the fine molecular details of their action.

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CHAPTER 5

The Role of Sigma B (σ^B) in the Stress Adaptations of *Listeria monocytogenes*: Overlaps Between Stress Adaptation and Virulence

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Contents

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I. *LISTERIA MONOCYTOGENES*: AN ADAPTABLE PATHOGEN

The genus *Listeria* consists of a group of nonsporulating, low G + C, facultatively anaerobic, Gram-positive bacteria that includes six species. Of these only one, *L. monocytogenes*, is pathogenic for humans. This pathogen can be isolated from a wide variety of different environments including soil, water, decaying vegetation, and animal faeces, as well as a range of foods destined for human consumption. Its almost ubiquitous nature is underpinned by a remarkable ability to adapt to and thrive in comparatively harsh environmental conditions (reviewed in Gandhi and Chikindas, 2007). It is one of the few bacterial pathogens, capable of growth at refrigeration temperatures and is even capable of growth at temperatures as low as −0.4 °C (Walker *et al.*, 1990). It can tolerate environments with high salt concentrations, being capable of growth in the presence of 2 M NaCl and capable of surviving for extended periods in the presence of 3 M NaCl (Cole *et al.*, 1990). *L. monocytogenes* is extremely tolerant to bile salts and can even persist and grow within the murine gall bladder (Hardy *et al.*, 2004). It can grow over a pH range of 4.3–11.0 (Farber, 1989; Vasseur *et al.*, 1999), and it displays a strong adaptive acid tolerance response (ATR) that confers significant protection against acidic challenges as low as pH 2.5 (Davis *et al.*, 1996; O′ Driscoll *et al.*, 1997). Efficient transcriptional responses play a key role in allowing this pathogen to successfully inhabit such a diverse range of niches. Transcriptional reprogramming is achieved through the use of a plethora of signalling pathways and transcriptional regulators, but it is the availability of sigma factors that determines which sets of genes get transcribed at any given time, by virtue of their ability to target RNA polymerase to specific promoter sequences upstream from genes and operons.

II. THE SIGMA FACTORS OF *L. MONOCYTOGENES*

The genome of *L. monocytogenes* encodes a total of five sigma factors (Glaser *et al.*, 2001) (Table 5.1), although some phylogenetic lineages encode only four (Zhang *et al.*, 2003). The principal sigma subunit of RNA polymerase in *L. monocytogenes* is RpoD (σ^A) encoded by the *rpoD*

TABLE 5.1 The sigma factors of *L. monocytogenes*

Sigma factor	Gene	Alternative gene name	*B. subtilis* homologue	Phenotye of *L. monocytogenes* null mutant	First described in references:
σ^A	*rpoD*	*lmo1454*	*sigA*	n/a	Metzger *et al.*, 1994
σ^B	*sigB*	*lmo0895*	*sigB*	Stress sensitive, reduced invasiveness	Wiedmann *et al.*, 1998
σ^C	*sigC*	*sigV*, *lmo0423*	*sigV*	Heat sensitive	Zhang *et al.*, 2005
σ^H	*sigH*	*lmo0243*	*sigH*	Sensitive to alkaline pH	Phan-Thanh and Mahouin, 1999; Rea *et al.*, 2004
σ^{54}	*sigL*	*rpoN*, *lmo2461*	*sigL*	Resistant to mesentericin Y105	Robichon *et al.*, 1997

gene (*lmo1454*). It is a member of the sigma 43 subgroup of the sigma 70 family of sigma factors and is very closely related to SigA, the major housekeeping sigma factor of *Bacillus subtilis* (Metzger *et al.*, 1994). Sigma 54 (σ^{54}) is encoded by the *sigL* gene (*lmo2461* or *rpoN*) and it was first identified in a screen for *L. monocytogenes* mutations that conferred resistance to the bacteriocin mesentericin Y105. The σ^{54} protein shares 38% sequence identity with σ^{54} from *B. subtilis* (Robichon *et al.*, 1997). A transcriptomic and proteomic study identified 77 genes under the control of σ^{54}, many with functions in carbohydrate and amino acid metabolism (Arous *et al.*, 2004). Two reports suggest that σ^{54} might play a role in stress tolerance in *L. monocytogenes*. Firstly, mutants lacking σ^{54} are defective for the ability to utilise the osmoprotectant carnitine, although this finding is independent of effects on the transcription of the known carnitine uptake genes (Okada *et al.*, 2006). Secondly, mRNA corresponding to the *sigL* gene is known to be present at increased levels when cells are grown at 10 °C (Liu *et al.*, 2002). The *sigH* gene (*lmo0243*) encodes sigma H (σ^{H}), a sigma factor that may be involved in responding to changes in environmental pH, since mutants lacking it grow poorly at alkaline pH (Rea *et al.*, 2004) and its expression is induced in response to acidic pH (Phan-Thanh and Mahouin, 1999). The *sigC* gene (*lmo0423* or *sigV*) encodes sigma C (σ^{C}), a sigma factor that belongs to the extracytoplasmic function (ECF) family of sigma factors (Zhang *et al.*, 2005). The *sigC* gene is present only in strains of *L. monocytogenes* that belong to the phylogenetic lineage II of the species (Zhang *et al.*, 2003). Mutants that lack *sigC* are found to exhibit a temperature sensitive phenotype (Zhang *et al.*, 2005). Finally, sigma B (σ^{B}), encoded by the *sigB* gene (*lmo0895*), was first identified based on its homology to *sigB* from *B. subtilis.* Early studies demonstrated an involvement in both acid tolerance (Wiedmann *et al.*, 1998) and osmoregulation (Becker *et al.*, 1998). Considerable research attention has focused on this sigma factor and this will be the principal focus of this review.

III. SIGMA B (σ^{B})

A. Complex protein–protein interactions control σ^{B} activity

At present little is known about how the activity or the expression of σ^{B} in *L. monocytogenes* is modulated in response to changing environmental conditions. However, the high degree of conservation between the *sigB* operons in *L. monocytogenes* and *B. subtilis* (Fig. 5.1) suggests that similar regulatory mechanisms will apply (Becker *et al.*, 1998; Wiedmann *et al.*, 1998). In *B. subtilis* the activity of σ^{B} is regulated primarily at the post-translational level by modulating its interaction with an anti-sigma factor, called RsbW (recently reviewed in Hecker *et al.*, 2007). Free RsbW can

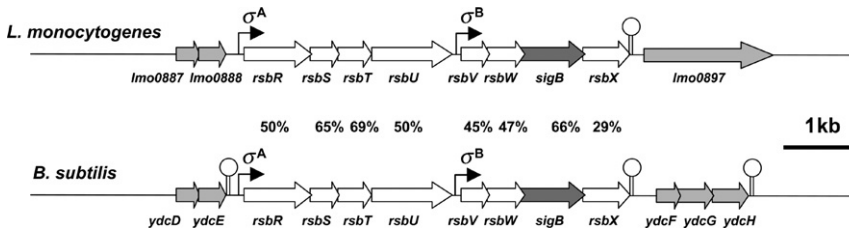

FIGURE 5.1 The conserved *sigB* operon in *L. monocytogenes* and *B. subtilis.* Genes belonging to the *sigB* operon are indicated as open arrowheads, with *sigB* itself shown in dark grey. Genes in light grey lie outside of the *sigB* operon. Promoters are indicated as angled arrowheads and putative transcriptional terminators are indicated as stem-loop structures. The percentages of amino acid sequence identity between homologues are indicated. The schematic is drawn to scale.

interact with σ^B and this association prevents productive interactions between σ^B and RNA polymerase core enzyme. An antagonist protein (or anti-anti-sigma factor) called RsbV can bind to RsbW and this interaction liberates σ^B to participate in the initiation of transcription. The capacity of RsbV to associate with RsbW is determined by the phosphorylation state of RsbV. In the unphosphorylated form it interacts with RsbW, thereby releasing σ^B for participation in transcription, whereas in the phosphorylated form it dissociates from RsbW, allowing the σ^B-RsbW interaction to occur. This regulatory mechanism is called ''partner switching'' since it involves RsbW switching between two binding partners, RsbV and σ^B (Fig. 5.2). The overall phosphorylation state of RsbV is regulated by the actions of two dedicated phosphatases, called RsbU and RsbP, and the kinase activity of RsbW. In rapidly growing cells that are not experiencing significant stresses RsbV mainly exists in the phosphorylated form, which allows the anti-sigma factor RsbW to prevent the association of σ^B with RNA polymerase. Under these conditions the kinase activity of RsbW out-competes the phosphatase activities of RsbU and RsbP. However when the cells experience stress the phosphatase activities increase, resulting in a more stable interaction between RsbV and RsbW, thereby releasing σ^B and triggering the induction of the σ^B regulon. Thus, it appears that the phosphorylation state of RsbV is the principal determinant of whether the σ^B regulon is expressed or not (Fig. 5.2).

The σ^B regulon in *B. subtilis* is induced primarily in response to two classes of stress, broadly classified as environmental stress (e.g., elevated temperature or osmolarity) and energy stress (e.g., carbon starvation and ATP depletion). Environmental stress triggers the activation of σ^B via the RsbU phosphatase (Yang *et al.*, 1996), whereas the energy stress appears to influence σ^B via RsbP (Vijay *et al.*, 2000). In *L. monocytogenes*, there is no

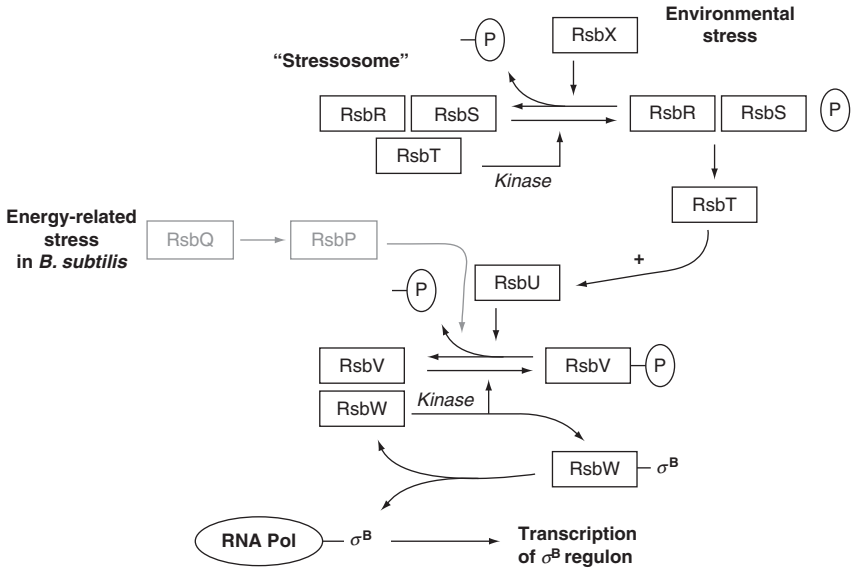

FIGURE 5.2 Model for the regulation of σ^B activity. This model for the regulation of σ^B activity is based on extensive research performed with *B. subtilis*, cited throughout the text, but it has not yet been validated in *L. monocytogenes*. The model proposes that the availability of σ^B for transcription is regulated by modulating the phosphorylation state of RsbV. In the dephosphorylated state RsbV binds to RsbW and this interaction prevents RsbW from interacting with σ^B, thereby allowing σ^B to participate in transcription. The dephosphorylation of RsbV is catalysed by the protein phosphatase RsbU. The activity of this phosphatase is in-turn regulated by modulating the phosphorylation state of upstream proteins, RsbR and RsbS. The "stressosome" is a large 25S protein complex that includes multiple copies of RsbR, RsbS, RsbT as well as homologues of RsbR. In *L. monocytogenes* energy-related stresses are thought to be transmitted via RsbT, since no RsbP homologue is encoded in the genome. How the initial stress-related signal is sensed by the "stressosome" remains to be determined.

close homologue of RsbP and the available evidence suggests that both energy-related and environmental stresses are sensed through the RsbU phosphatase (Chaturongakul and Boor, 2004). Interestingly, it appears that cold stress can be sensed directly by σ^B in *B. subtilis*, independently of either RsbU or RsbP. Indeed this activation can also occur independently of RsbV (Brigulla *et al.*, 2003). The mechanism of phosphatase-independent activation of σ^B in *B. subtilis* has not yet been elucidated, nor is it known if this pathway exists in *L. monocytogenes*.

The initial sensory input that triggers the downstream activation of σ^B in *B. subtilis* is still not clear. Upstream from RsbU there is another partner switching module that liberates to RsbT from a complex with RsbR and RsbS. RsbT then interacts with RsbU and this interaction promotes the

phosphatase activity of RsbU. The RsbRST complex is a large multisubunit complex that also includes a number of paralogues of RsbR (RsbRB, RsbRC, RsbRD, and YtvA; Delumeau *et al.*, 2006). The large 25S complex formed by these proteins has been described as a ''stressosome'' (Delumeau *et al.*, 2006). It is not yet clear whether the RsbS, RsbT, and RsbR proteins from *L. monocytogenes* are associated with a stressosome-like complex. However, the genome of *L. monocytogenes* does encode a number of RsbR paralogues, including Lmo0161, Lmo1642, and Lmo1842, as well as the YtvA paralogue, Lmo0799.

Although post-translational regulation of σ^B activity is likely to be critical for the control of σ^B in *L. monocytogenes*, it is clear that regulation can also take place at the transcriptional level. Transcription of *sigB*, which lies in an 8-gene operon comprising *rsbR*, *rsbS*, *rsbT*, *rsbU*, *rsbV*, *rsbW*, *sigB*, and *rsbX* (Fig. 5.1), is initiated upstream from *rsbV* (Becker *et al.*, 1998; Rauch *et al.*, 2005). Transcription of this operon is primarily dependent on the presence of σ^B (Becker *et al.*, 1998), suggesting that a stimulation of σ^B activity will rapidly result in an increase in *sigB* transcript. A variety of different stresses are each known to induce transcription from the σ^B promoter upstream from *rsbV*, including salt, ethanol, organic acid, heat shock, and entry into stationary phase (Becker *et al.*, 1998). Growth at cold temperatures also leads to increased transcription from the σ^B promoter upstream from *rsbV* (Becker *et al.*, 2000). Of these stresses, osmotic shock (with 4% NaCl) gives rise to the biggest increase in transcription (Becker *et al.*, 1998).

B. Elucidation of the σ^B regulon by proteomics and transcriptomics

In order to understand the role that σ^B plays in the overall biology of *L. monocytogenes*, a number of groups have used proteomic and transcriptomic studies to define the full extent of the σ^B regulon. It is now clear that σ^B regulates a large and diverse set of genes (probably approaching 200 genes) that are predicted to function in stress tolerance (see Section IV), carbohydrate metabolism (see Section VI), transport and cell envelope processes, and virulence (see Section VII). A proteomic study, aimed at identifying components of the σ^B regulon that contribute to acid tolerance, found nine proteins whose expression was acid inducible in a σ^B-dependent manner (Wemekamp-Kamphuis *et al.*, 2004b). Recently, we reported the results of a proteomic study of the σ^B regulon using both conventional two-dimensional gel electrophoresis (2DGE) and a novel approach, based on tandem mass spectrometry, called Isobaric Tags for Relative and Absolute Quantitation (iTRAQ). In this study, 38 proteins expressed in a σ^B-dependent manner were identified, 17 of which are under positive control by σ^B (Abram *et al.*, 2008). Promoter mapping of

several of σ^B–dependent genes facilitated the definition of a refined σ^B promoter consensus sequence for *L. monocytogenes*. The proposed consensus sequence is GTTTNW-N13/14-GGGWADW, where N is any base, W is A or T, and D is A, T, or G (Abram *et al.*, 2008).

The availability of the genome sequence for *L. monocytogenes* (Glaser *et al.*, 2001) has allowed global transcriptional studies to be undertaken. Using a consensus sequence-based search of the *L. monocytogenes* genome it is possible to identify genes with putative σ^B promoter sequences. This approach has been used to generate a limited set gene array, carrying 166 putative σ^B-dependent genes. This gene array allowed the identification of 55 genes that were differentially expressed in a *sigB* deletion mutant (Kazmierczak *et al.*, 2003). Genes identified in this study included several with stress-related functions, but also several known virulence genes, including members of the internalin family *inlA, inlB, inlC2, inlD*, and *inlE*. This study also highlighted the involvement of σ^B in regulating the expression of many genes with cell wall and/or transport-related functions. A more recent study using a whole genome microarray found 168 genes to be under positive σ^B control, with 145 of these preceded by a potential σ^B promoter sequence (Raengpradub *et al.*, 2008). Many of the genes identified have functions in metabolism or transport, while many have undetermined functions. Interestingly, only 65 genes in the non-pathogenic species *Listeria innocua* are found to be under positive σ^B control, a result that points to a possible role of σ^B in adaptating to the host environment (Raengpradub *et al.*, 2008). It is interesting to note that a significant subset of PrfA-regulated genes have potential σ^B promoter sequences (Milohanic *et al.*, 2003). PrfA is the principal regulator of virulence gene transcription in *L. monocytogenes* (Chakraborty *et al.*, 1992; reviewed in Scortti *et al.*, 2007; see Section VII). This finding highlights the overlap that exists between the control of the general stress response and the control of virulence-related functions.

IV. A CENTRAL ROLE FOR σ^B IN ADAPTATION TO STRESS

A. σ^B and osmoregulation

L. monocytogenes is one of only a small number of bacterial pathogens that can tolerate low water activities. It is capable of growth at NaCl concentrations as high as 2 M (~12% w/v) and can survive extended periods in the presence of 3 M (18% w/v) NaCl (Cole *et al.*, 1990). Central to its osmotolerance are three transport systems that enable the efficient uptake of osmoprotectants, such as glycine betaine (betaine) and carnitine (Angelidis and Smith, 2003; Fraser *et al.*, 2000; Sleator *et al.*, 2003). Two transporters are responsible for betaine uptake; BetL and Gbu (Ko and

Smith, 1999; Sleator *et al.*, 1999). BetL is a sodium-dependent secondary transporter that shares significant homology to the well-studied solute transporters OpuD and BetP, from *B. subtilis* and *Corynebacterium glutamicum*, respectively. Mutants lacking BetL display a slow growth phenotype in complex growth media with elevated osmolarity (Sleator *et al.*, 1999). Gbu is an ATP-dependent betaine transporter and is comprised of three distinct subunits, GbuA, GbuB, and GbuC. Mutants lacking this uptake system are unable to use betaine efficiently as an osmoprotectant and display a cold-sensitive phenotype (Ko and Smith, 1999), suggesting that betaine accumulation is also an important component of cryotolerance. Carnitine uptake occurs predominantly through the activity of OpuC, another ATP-dependent transporter that belongs to the binding protein-dependent subgroup of the ABC transporter superfamily (Fraser *et al.*, 2000, 2003). The OpuC transporter is encoded by a four-gene operon that consists of the *opuCA*, *opuCB*, *opuCC*, and *opuCD* genes (Fraser *et al.*, 2000). Mutants lacking OpuC are defective for carnitine uptake (Fraser *et al.*, 2000) but some carnitine transport still occurs suggesting the presence of alternative route for the transport of this compatible solute (Fraser and O' Byrne, 2002; Sleator *et al.*, 2001).

The transcription of the *gbu*, *betL*, and *opuC* operons is known to be inducible in response to osmotic shock (Cetin *et al.*, 2004; Fraser *et al.*, 2003; Sleator *et al.*, 2003). Indeed, all three transport systems are also induced at the transcriptional level in response to low temperature growth (Wemekamp-Kamphuis *et al.*, 2004a), a finding that highlights the overlap between osmoregulation and cryotolerance in *L. monocytogenes*. Putative σ^B promoters were identified upstream from *opuCA* (Fraser *et al.*, 2000; Sleator *et al.*, 1999), but not upstream from *gbuA* (Ko and Smith, 1999). Subsequently, the transcription of *opuCA* was confirmed to be dependent on σ^B (Fraser *et al.*, 2003; Sue *et al.*, 2003) and a σ^B promoter has been mapped 58 bp upstream from *opuCA* (Kazmierczak *et al.*, 2003). A second study confirmed the σ^B-dependence of *opuCA* transcription but identified a somewhat unusual sequence, 47 bp further upstream, as the σ^B promoter (Cetin *et al.*, 2004). The role of σ^B in transcribing *betL* and *gbuA* is less clear. The osmotic induction of *gbuA* transcription in *L. monocytogenes* 10403S was found to occur even in a strain lacking σ^B (Fraser *et al.*, 2003). However, in the same strain Cetin *et al.* (2004) identified two promoters upstream from *gbuA* and confirmed that one of these (*gbuAP2*) is dependent on σ^B. Although *betL* is preceded by a putative σ^B promoter sequence (Sleator *et al.*, 1999) it appears that its transcription does not depend on this sigma factor (Cetin *et al.*, 2004; Fraser *et al.*, 2003). Therefore, the finding that betaine accumulation is impaired in a Δ*sigB* mutant (Becker *et al.*, 1998) is probably accounted for by reduced Gbu expression rather than an effect on BetL.

B. σ^B and acid resistance

L. monocytogenes can modulate its acid tolerance in response to growth phase and to changes in environmental pH (Davis *et al.*, 1996; O'Driscoll *et al.*, 1997). Within the cell a number of different systems contribute to acid tolerance including, the glutamate decarboxylase (GAD) system (Cotter *et al.*, 2001b, 2005), a variety of general shock proteins with protein repair and chaperone activities, such as DnaK, GroEL, HtrA, the Clp ATPases (reviewed in Cotter and Hill, 2003), as well as a possible role for the F_1F_0-ATPase (Cotter *et al.*, 2000). The role of σ^B in acid tolerance was first demonstrated when a Δ*sigB* mutant was found to survive poorly at pH 2.5 compared to the wild type strain (Wiedmann *et al.*, 1998). In addition, σ^B plays a role in the ATR of *L. monocytogenes* (Ferreira *et al.*, 2003), a response that is induced during exposure to mildly acidic pHs, conferring high levels of protection against extreme low pH. However, the ATR is not abolished in a strain lacking σ^B, suggesting that other regulatory mechanisms must be employed. σ^B does not appear to contribute to the growth phase-dependent acid resistance mechanism(s) that are responsible for increased acid resistance in stationary phase (Ferreira *et al.*, 2003).

σ^B primarily influences acid resistance through the activation of the GAD acid resistance system, which is known to play an important role in survival of *L. monocytogenes* in low pH environments, such as acidified foods (Cotter *et al.*, 2001b) or gastric fluid (Cotter *et al.*, 2001a). Depending on the strain, *L. monocytogenes* can possess three decarboxylases (*gadD1*, *gadD2*, and *gadD3*) and two glutamate/γ-aminobutyrate (GABA) antiporters (*gadT1* and *gadT2*) which are organized in three operons, *gadD1T1*, *gadT2D2*, and *gadD3* (Cotter *et al.*, 2005). GadD2/T2 are important for survival in severe acid challenges (pH 2.8) and their expression is induced during stationary phase, while GadD1 plays a role enabling growth in mildly acidic conditions (pH 5.1) and it is induced during exponential phase (Cotter *et al.*, 2005). However, the role of GadD3 is still unknown since mutants lacking this decarboxylase have not been possible to generate (Cotter *et al.*, 2005). In a Δ*sigB* mutant, the acid-induced transcription of *gadD2*, *gadT2*, and *gadD3* is essentially eliminated, highlighting the role for σ^B in acid adaptation (Wemekamp-Kamphuis *et al.*, 2004b). The *gadT1* transcript levels are reduced in a *sigB* mutant, but transcription still appears to be acid inducible, while *gadD1* levels actually increase in a *sigB* background, perhaps to compensate for the reduced expression of the other GAD systems (Wemekamp-Kamphuis *et al.*, 2004b). Potential σ^B promoter sequences are found upstream of the *gadT2D2* and *gadD3* operons (Wemekamp-Kamphuis *et al.*, 2004b), but only the *gadD3* σ^B promoter has been confirmed (Kazmierczak *et al.*, 2003).

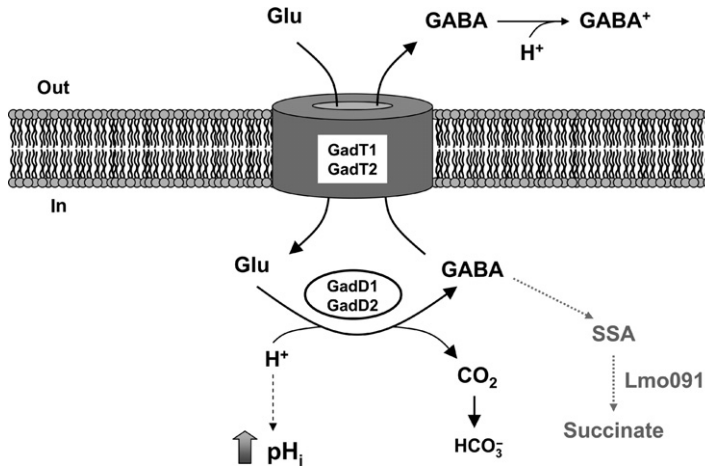

FIGURE 5.3 Overview of the Gad system conferring acid tolerance (*E. coli*). The antiporters GadT1 or GadT2 exchange a glutamate molecule for a molecule of GABA. The glutamate is converted to GABA through a decarboxylation reaction, catalyzed by either GadD1, GadD2 (the role of a third decarboxylase, GadD3, remains unclear). This reaction consumes a proton, which raises the intracellular pH and thereby contributes to the acid resistance of the cell. The exported GABA is also predicted to become protonated when the extracellular pH is low, thereby helping to alkalanise the environment in the vicinity of the cell. A possible alternative fate for GABA within the cell is shown in grey. The presence of a potential succinate semialdehyde dehydrogenase in the *L. monocytogenes* genome (*lmo0913*) suggests that GABA may be metabolized to succinate via succinate semialdehyde (SSA).

The present model accounting for GAD-mediated acid tolerance (Fig. 5.3) is based on the observations in *Escherichia coli* (Castanie-Cornet *et al.*, 1999; Richard and Foster, 2004). In *L. monocytogenes* this system is proposed to transport glutamate through glutamate/GABA antiporters (GadT1 and GadT2) into the cell, and subsequently to convert it to GABA by the action of GAD (Cotter *et al.*, 2001a, 2005). This decarboxylation reaction is catalyzed by either GadD1 and GadD2 (or possibly GadD3) and involves the consumption of a proton, which raises the intracellular pH and thereby contributes to the acid resistance of the cell (Cotter *et al.*, 2001a). Subsequently, the glutamate/GABA antiporter removes the GABA from the cell in exchange for another glutamate molecule (Cotter *et al.*, 2001a; Wemekamp-Kamphuis *et al.*, 2004b). Interestingly, Abram *et al.* (2008) have demonstrated that levels of Lmo0913 are reduced in the Δ*sigB* mutant while GadT2 is over expressed. Lmo0913 is homologous to succinate semialdehyde dehydrogenase, an enzyme that participates in the catabolism of the intermediate GABA, which is known to be toxic to *B. subtilis* (Belitsky and Sonenshein, 2002) and other microorganisms

(Arst, 1976). It is suggested that GadT2 could prevent GABA accumulation that might result from the lack of Lmo0913 in the Δ*sigB* mutant strain (Abram *et al.*, 2008). Similarly, Cotter *et al.* (2005) have suggested that Lmo0913 could potentially compensate for the absence of an antiporter such as GadT2 by ensuring the removal of GABA.

Several other proteins that might play a role in acid adaptation (Pfk, GalE, ClpP, and Lmo1580) have been suggested to be regulated by σ^B as they were not induced in the Δ*sigB* mutant in response to sub-lethal acidification (Wemekamp-Kamphuis *et al.*, 2004b). The promoter regions of the corresponding genes contain potential σ^B recognition sequences, but these have not yet been confirmed experimentally (Wemekamp-Kamphuis *et al.*, 2004b).

C. σ^B is involved in cryotolerance

L. monocytogenes is known to be able to grow at temperatures as low as −0.4 °C (Walker *et al.*, 1990). This characteristic is at least partly due to its ability to accumulate cryoprotective compounds like carnitine or betaine (Bayles and Wilkinson, 2000; see Section IV.A). Efficient uptake of the compatible solutes betaine and carnitine at low temperatures is σ^B-dependent and consequently mutants lacking σ^B grow poorly at low temperatures (Bayles and Wilkinson, 2000). Carnitine is transported via the OpuC uptake system and the transcription of the genes encoding this transporter is under σ^B control (Fraser *et al.*, 2003; see Section IV.A). However, σ^B does not seem to play an active role in *betL* transcription or in betaine utilization (Fraser *et al.*, 2003). A role for σ^B in cryotolerance has also been suggested by Wemekamp-Kamphuis *et al.* (2004b), who showed that a Δ*sigB* mutant is sensitive to freeze-thaw cycles. However, the role of σ^B in cryotolerance remains somewhat uncertain since a recent study shows that the transcription of *opuC* occurs independently of σ^B at 4°C and also finds that a *sigB* deletion mutant grows normally at low temperatures (Chan *et al.*, 2007). Whether these apparently conflicting observations reflect strain variations or methodological differences between laboratories remains unclear at present. The transcription of the cold stress genes *ltrC* (Zheng and Kathariou, 1995) and *fri* (Hebraud and Guzzo, 2000; Olsen *et al.*, 2005) is known to be at least partially σ^B-dependent (Chan *et al.*, 2007), but a role for these genes in cryotolerance hasn't yet been established.

D. σ^B affects piezotolerance

σ^B plays an important role in the piezotolerance (tolerance to High Hydrostatic Pressure, HHP) of *L. monocytogenes* as mutants lacking this sigma factor are sensitive to various pressures (Wemekamp-Kamphuis

et al., 2004b). A possible explanation for this is the activation of the *clpP* gene which encodes the proteolytic subunit of the Clp ATP-dependent protease (Nair *et al.*, 2000), and is σ^B-regulated (Wemekamp-Kamphuis *et al.*, 2004b). Work by Karatzas and Bennik, (2002) and Karatzas *et al.*, (2003) has shown that a multiple stress-tolerant mutant in which ClpP is over expressed, is resistant to HHP. In addition, *clpP* is induced following HHP treatment suggesting that it contributes to piezotolerance (Bowman *et al.* 2008).

Another possible explanation for the involvement of σ^B in piezotolerance is through the induction of cold shock proteins (CSPs). Two CSPs, detectable on polyacrylamide gels but whose identity is uncertain, are induced during high pressure treatment (Wemekamp-Kamphuis *et al.*, 2002a). Pre-exposure of *L. monocytogenes* cells to a low temperature results in increased piezotolerance (more than 100-fold increased survival after treatment at 300 MPa) (Wemekamp-Kamphuis *et al.*, 2002a). The CspL cold shock protein might be responsible for the pressure-sensitive phenotype of a Δ*sigB* mutant, since the gene encoding this protein (*cspL*) is preceded by a potential σ^B promoter sequence (Wemekamp-Kamphuis *et al.*, 2004b). However, further work is required to prove that the transcription of this gene is σ^B-dependent and to genetically identify the σ^B-regulated components that contribute to piezotolerance.

E. Antimicrobial resistance and σ^B

The resistance of *L. monocytogenes* to the bacteriocins nisin and lacticin 3147, and the antibiotics ampicillin and penicillin G and V is also under σ^B influence (Begley *et al.*, 2005, 2006). These compounds act on the cell envelope, suggesting that σ^B may play a role in controlling membrane integrity. The PVA protein, which is also involved in resistance to bile (see Section IV.F), confers resistance to penicillin V, but not penicillin G (Begley *et al.*, 2005). The corresponding *pva* gene is homologous to a penicillin V amidase and probably hydrolyses penicillin V to 6-aminopenicillinic acid. Consensus σ^B binding sites were found in the promoter regions of *htrA* (*lmo0292*) and *mdrL* (*lmo1409*), suggesting possible regulation by σ^B (Begley *et al.*, 2006). *htrA* encodes a putative serine protease, which is required for tolerance of penicillin G (Stack *et al.*, 2005) and for optimal growth at elevated temperatures or in the presence of sub-lethal concentrations of hydrogen peroxide (Wonderling *et al.*, 2004). Furthermore, *mdrL* which encodes an antibiotic efflux pump could be responsible for efficient efflux of antibiotics (Mata *et al.*, 2000). However, despite the presence of consensus σ^B binding sites upstream of these genes, no evidence has shown σ^B-dependent transcription.

F. The role of σ^B in resistance to bile

Bile is one of the most important defence systems of the body against intestinal pathogens (Hofmann and Eckmann, 2006). Bile acts as a detergent that can solubilize and emulsify lipids contained in the bacterial membranes (Begley *et al.*, 2005; Hofmann, 1999; Hofmann and Eckmann, 2006). Therefore, a tolerance of bile by intestinal pathogens is essential for their ability to colonize the host (see Section VII.B.2). In *L. monocytogenes* bile tolerance is dependent upon expression of the *bilE* operon, comprising the *bilEA* and *bilEB* genes, which encodes a bile exclusion system (Sleator *et al.*, 2005). The BilE system has similarities with multidrug efflux pumps of Gram negative bacteria as well as bile efflux pumps from higher organisms. Deletion of *bilE* results in the accumulation of bile intracellularly, suggesting that BilE probably acts to transport bile out of the cell (Sleator *et al.*, 2005). The transcription of *bilE* is significantly reduced in a Δ*sigB* mutant, a finding that is consistent with the presence of a consensus σ^B–dependent promoter binding site upstream of the *bilE* start codon (Fraser *et al.*, 2003; Sleator *et al.*, 2005).

Apart from bile removal mechanisms, several bacteria also possess bile modification mechanisms. *L. monocytogenes* possesses one bile salt hydrolase gene (*bsh*). The *bsh* gene is known to be regulated by both PrfA (Dussurget *et al.*, 2002) and σ^B (Sue *et al.*, 2003), although the latter appears to play a more significant role in its regulation (Begley *et al.*, 2005). Both *bsh* transcript and bile salt hydrolase activity are detectable in the stationary phase of growth in a Δ*prfA* mutant, but they are eliminated completely in a Δ*sigB* mutant. Furthermore, a Δ*sigB* mutant is exquisitely sensitive to bile salts *in vitro* (Begley *et al.*, 2005). Another σ^B-regulated gene, *pva*, also plays an important role in bile tolerance in *L. monocytogenes* (Begley *et al.*, 2005). However, PVA does not affect the hydrolysis of bile salt and it still remains to be clarified how it influences bile tolerance (Begley *et al.*, 2005).

V. DOES COMPETITION BETWEEN SIGMA FACTORS INFLUENCE GROWTH RATE IN *L. MONOCYTOGENES?*

One of the somewhat unexpected phenotypes displayed by mutants lacking the *sigB* gene is that they can grow faster than the wild-type under certain culture conditions. In media with limiting glucose mutants lacking σ^B grow significantly faster than the parent strain. The same is true for mutants lacking either of the two positively acting regulators of σ^B, RsbT or RsbV (Chaturongakul and Boor, 2004). In complex medium at 3°C, mutants of *L. monocytogenes* lacking *sigB* are also found to grow faster than the parent (Brondsted *et al.*, 2003). We have found that a Δ*sigB* mutant grows faster than the wild-type in chemically defined

media supplemented with NaCl (Abram and O'Byrne, unpublished data). In *B. subtilis* a similar phenomenon has been described; cells lacking *sigB* have a selective growth advantage in glucose-limited continuous cultures (Schweder *et al.*, 1999). At present there is no clear explanation for these observations, but it is interesting to speculate that sigma factor competition might play a role. In *E. coli* mutations arise in the *rpoS* gene, encoding the stress-inducible sigma factor σ^S, when cells are grown under nutrient limiting conditions (King *et al.*, 2006; Notley-Mcrobb *et al.*, 2002). This finding has been suggested to occur because of the competition that arises between the sigma factors involved in growth-related functions (σ^{70}) and in protection or maintenance functions (σ^S). In this model, σ^S provides the bacterium with protection against adverse conditions, but at the expense of diverting resources away from growth-related functions. Thus, in the absence of σ^S more efficient transcription of housekeeping genes allows the cell to grow at a faster rate (Nystrom, 2004). This model represents a plausible explanation for the unexpectedly fast growth of *L. monocytogenes* mutants lacking *sigB* under some conditions, but experimentation will be required to rigorously test this idea.

VI. ROLE OF σ^B IN METABOLISM

Apart from its important roles in stress resistance, σ^B also plays a significant role in regulating metabolism-related functions (Kazmierczak *et al.*, 2003). In *B. subtilis* and *Staphylococcus aureus*, σ^B affects expression of genes involved in metabolic functions related to carbon metabolism, envelope function and turnover (Pane-Farre *et al.*, 2006; Price *et al.*, 2001). In *L. monocytogenes* genes with metabolism-related functions are very highly represented in the set of genes found to be differentially regulated in a Δ*sigB* mutant using microarrays (Kazmierczak *et al.*, 2003). In a more recent microarray study, approximately 30% of the σ^B-dependent genes detected (47 out of 168) had metabolism-related functions, the largest single category of genes apart from genes of unknown function (Raengpradub *et al.*, 2008). Similarly, a proteomics approach has shown that out of 38 differentially expressed proteins in a Δ*sigB* mutant, 22 have functions related to metabolism (Abram *et al.*, 2008). These proteins appear to be involved in the metabolism of alternative carbon sources like glycerol, tagatose, *N*-acetyl-D-glucosamine, 6-phospho-β-D glucosyl-1,4-D-glucose, and fructose. This role is exemplified by the inability of a Δ*sigB* mutant to utilize glycerol efficiently, which is attributed to the lower levels of three proteins that encode subunits of a dihydroxyacetone kinase (Lmo2695, Lmo2696, and Lmo2697) involved in glycerol metabolism (Abram *et al.*, 2008). In addition, σ^B is involved in the regulation of genes whose products are likely to catalyse reactions of the pentose phosphate

and glycolytic pathways (Abram *et al.*, 2008). However, it remains unclear whether σ^B has a dedicated role in regulating metabolism in the cell or whether it merely optimises metabolism in response to perturbations in environmental conditions.

VII. σ^B PLAYS IMPORTANT ROLE IN VIRULENCE

A. PrfA and the intracellular stages of infection

The transcription of the majority of virulence-related genes in *L. monocytogenes* is regulated by the PrfA protein (positive regulatory factor A), a CRP-like DNA-binding protein that binds to its target sequence—a PrfA box—as a homodimer (recently reviewed in Scortti *et al.*, 2007). Two promoters (*prfAP1* and *prfAP2*) are located directly upstream of the *prfA* gene (Fig. 5.4). *prfAP1* is σ^A–dependent, while *prfAP2*, which is responsible for the majority of the *prfA* transcript levels intra- and extracellularly, actually consists of two overlapping promoters that are under the control of σ^B and σ^A (Kazmierczak *et al.*, 2006; Rauch *et al.*, 2005). However, haemolysis, which is important for escape from the phagocytic vacuole and thus important for virulence, was similar to that of the wild-type strain for both Δ*sigB* and Δ*prfAP2* mutants. The Δ*prfAP1* mutant showed nearly a threefold reduction in hemolytic activity compared to the wild type accompanied by intracellular growth attenuation (Nadon *et al.*, 2002). In addition, elimination of either *prfAP1* or *prfAP2* resulted in fully virulent mutants in an animal model and only deletion of both promoters resulted in lower virulence (Freitag and Portnoy, 1994). Thus it appears that although σ^B regulates transcription through *prfAP2*, it does not contribute to PrfA expression in *L. monocytogenes* intracellularly (Kazmierczak *et al.*, 2006). This dual regulation of *prfA* transcription by both σ^B and σ^A ensures efficient transcription under a wide variety of

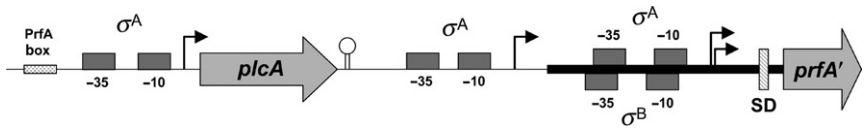

FIGURE 5.4 σ^A and σ^B-dependent promoters upstream from the *prfA* gene. A schematic illustration of the region encompassing the four known promoters driving *prfA* transcription is shown. The thermosensor element (Johansson *et al.*, 2002) is indicated in black; the promoter elements are indicated as grey boxes; the transcription start sites are indicated by angled arrowheads; the PrfA box and Shine-Dalgarno (SD) elements are labeled; and the *plcA* gene, which encodes phophatidylinositol-specific phopholipase C, is indicated as a grey arrow. The illustration is not to scale.

conditions even in the absence of one of the two regulators. In addition, PrfA directly or indirectly activates several genes in association with σ^B (Milohanic *et al.*, 2003). Sequences similar to the σ^B consensus promoter sequence are contained upstream from 22 genes whose transcription is PrfA-dependent (Milohanic *et al.*, 2003). Several of these genes (*lmo2391*, *lmo2748*, *lmo0913*, and *opuCA*) are known to be regulated by σ^B and this dual regulation may reflect an effort by the bacterium to balance between the expression of virulence and stress-related functions, depending on the prevailing environmental conditions.

B. Early and extracellular stages of infection

1. Survival in the stomach

Although σ^B does not seem to play a major role intracellularly (see Section VII.A), it is important for the early stages of infection within the host (Fig. 5.5). This is exemplified by the normal virulence of a Δ*sigB* mutant injected intravenously into guinea pigs, while the same mutant is significantly attenuated compared to the wild-type when introduced intragastrically to guinea pigs (Garner *et al.*, 2006). Once the contaminated food is ingested, the cells of *L. monocytogenes* are found in the stomach, which is the first important host defense barrier against food-borne bacterial pathogens (Audia *et al.*, 2001). In the stomach, several mechanisms conferring acid resistance are activated including those regulated by σ^B (e.g., Gad system). A Δ*sigB* mutant is sensitive to synthetic gastric fluid, highlighting the importance of this regulator in surviving the acidic conditions of the stomach (Ferreira *et al.*, 2003; see Section IV.B). This is at least partly due to the activation of the Gad system by σ^B, which is important for survival in gastric fluid (Cotter *et al.*, 2001; see Section IV.B).

2. Survival in the duodenum and the gall bladder

Once the bacterium passes the stomach, it is found in the duodenum which is the first part of the small intestine, into which bile is excreted from the gall bladder (Fig. 5.5). At this stage several mechanisms of resistance to bile (*bilE*, *bsh*, *pva*, and *btlB*), some of which are σ^B-dependent, act to chance the survival in this part of the intestinal tract (see Section IV.F). From the duodenum the cells of *L. monocytogenes* can pass to the small intestine or the gall bladder. Due to their tolerance to bile cells can replicate extracellularly in the gall bladder as shown in Balb/c mice (Hardy *et al.*, 2004). It has also been proposed that growth in this organ might have an impact on pathogenesis or spread of the organism contributing to listeriosis (Hardy *et al.*, 2006). Hardy *et al.* (2006) showed that transit through the murine bile duct into the intestine can occur within 5 min of induction of gall bladder contraction by food and that

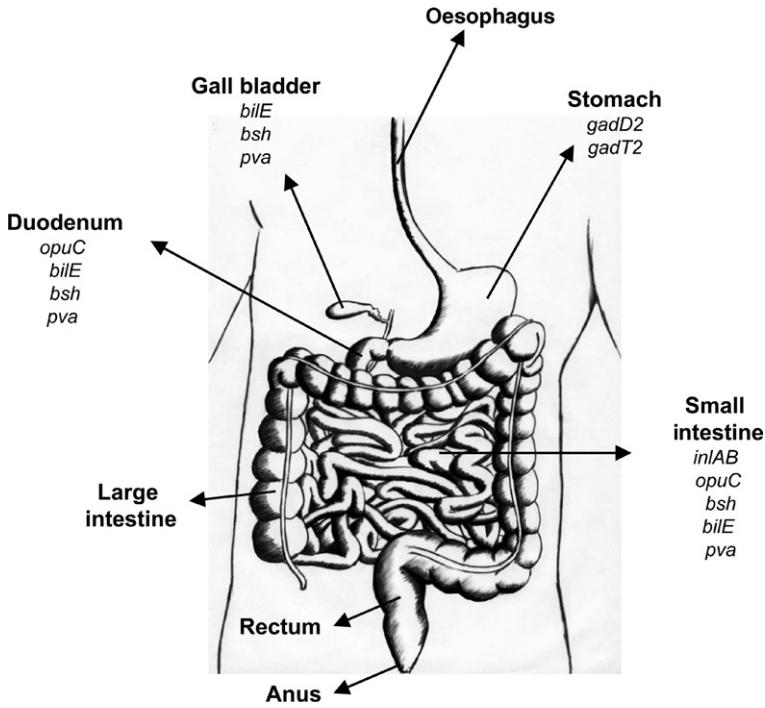

FIGURE 5.5 σ^B-dependent genes required for survival and colonisation of the host. *L. monocytogenes* cells are ingested together with contaminated food and after passage through the oesophagus they enter the stomach where the *gadD2*, *gadT2* genes play a role in surviving the acidic conditions (Cotter *et al.*, 2001a). In the duodenum, the combination of high osmotic stress and bile lead to activation of *opuCA*, *bsh* (Sue *et al.*, 2004), and *bilE* (Sleator *et al.*, 2005). In the gall bladder the *bsh* (Sue *et al.*, 2004), *bilE* (Sleator *et al.*, 2005), and *pva* (Begley *et al.*, 2005) genes are important for survival. When the cells are in the small intestine the genes activated are *opuCA*, *bsh* (Sue *et al.*, 2004), *bilE* (Sleator *et al.*, 2005), and finally *inlA* and *inlB* (Lecuit *et al.*, 2004) which help internalization in the intestinal epithelial cells initiating the intracellular stages of the infection. PrfA, the activator of virulence gene transcription, appears to be largely dispensable for survival within the gastrointestinal tract (Faith *et al.*, 2005).

movement of bacteria through the intestinal lumen can occur very rapidly in the absence of fecal material. This is the first time that the gall bladder is shown to be a site of growth for any bacterium in an animal model (Hardy *et al.*, 2006).

3. High osmotic pressure in the small intestine as a signal

Once out of the stomach and during the passage through the duodenum and the small intestine, the cells are confronted with the increased osmotic pressure encountered in the lumen of the small intestine and

the duodenum (Chowdhury *et al.*, 1996). *L. monocytogenes* is able to adapt to these conditions through efficient use of compatible solutes (Chowdhury *et al.*, 1996; see Section IV.A). One such system regulated by σ^B is OpuC, which is responsible for the transport uptake of the compatible solutes glycine betaine and carnitine (Fraser *et al.*, 2003; Sue *et al.*, 2003). The uptake of these compatible solutes helps the bacterium to counteract the negative effects of the increased osmotic pressure. It has been demonstrated that deletion of *opuC* in *L. monocytogenes* results in lower virulence highlighting the role of osmotolerance in pathogenesis (Wemekamp-Kamphuis *et al.*, 2002b). In addition, loss of OpuC results in a significant reduction in the ability of *Listeria* to colonize the upper small intestine and cause subsequent systemic infection following peroral inoculation (Sleator *et al.*, 2001). However, deletion of other genes important for osmotolerance, like *gbu* and *betL* do not seem to affect virulence (Wemekamp-Kamphuis *et al.*, 2002b). Pre-exposure to osmotic stress can protect against bile, whereas pre-exposure to bile fails to protect against osmotic or bile stress (Sleator *et al.*, 2007). Based on this observation, it has been proposed that increased osmolarity, which is firstly encountered in the duodenum after leaving the stomach might act as a key signal for *L. monocytogenes*, leading to the induction of a bile protective response (Sleator *et al.*, 2007).

4. Invasion of intestinal epithelium

σ^B affects the invasiveness of *L. monocytogenes* in human intestinal epithelial cell lines (Kim *et al.*, 2004, 2005). This effect seems to be primarily due to the σ^B-dependent regulation of internalins, which are cell wall-anchored proteins that play important roles in pathogenesis of *L. monocytogenes*. Internalin genes under σ^B control include *inlA, inlB* (Kim *et al.*, 2004, 2005), *inlC2* and *inlD* (McGann *et al.*, 2007), and *inlD* (McGann *et al.*, 2007). InlA is important for invasion of nonprofessional phagocytes such as epithelial cells. It binds to human E-cadherin as a receptor and this contributes to the passage through the human intestinal and placental barrier (Lecuit *et al.*, 2004). PrfA also has some influence on the expression of InlA and InlB (McGann *et al.*, 2007).

However, deletion of *prfA* does not affect dramatically the levels of InlA and InlB, unless it is accompanied by a deletion of *sigB*, or by growth of the cells in BHI supplemented with charcoal (McGann *et al.*, 2007). In contrast, deletion of *sigB* has a dramatic effect on the levels of InlA and InlB (McGann *et al.*, 2007) and on the cell invasion mediated by these internalins (Kim *et al.*, 2005). These results suggest that PrfA plays a relatively minor role in cell invasion when σ^B is present and they highlight the important role that σ^B plays in the ability of *L. monocytogenes* to invade cells within the host.

VIII. CONCLUSIONS

It is clear that σ^B is not a dedicated virulence sigma factor, since non pathogenic species of *Listeria* also use this sigma factor. Indeed, many of the genes under σ^B control are the same in *L. monocytogenes* and in the non pathogenic *L. innocua* (Raengpradub *et al.*, 2008). However, it appears that σ^B has been co-opted to participate in virulence-related functions in *L. monocytogenes*. Perhaps, it is not that surprising that σ^B, whose principal role is in controlling the general response to stress, should play a role in adapting to the often harsh conditions encountered within the host (low pH, elevated osmolarity, bile salts, etc.). More surprising is its role in regulating the transcription of internalin genes and its partial role in regulating the transcription of *prfA*. It may be that *L. monocytogenes* has evolved to sense the harsh conditions present within the gastrointerstinal tract and to coordinate the regulation of the protective functions required in this environment with those necessary for the early stages of establishing an infection. Indeed, to some extent the invasion of host cells can be viewed as a stress response, since it involves an escape from the stressful conditions found in the lumen of the ileum to the comparatively safe environment of the host cell cytoplasm.

Although σ^B appears to play a variety of roles within the cell, the data available suggest that its principal role is in coordinating the general stress response in *L. monocytogenes*. The various stress-sensitive phenotypes associated with the loss of the *sigB* gene highlight its importance in protecting the cell against environmental stresses, both outside and within the host. In some cases, the molecular basis for this protection is now well understood (e.g., osmoregulation via Gbu and OpuC), while in others we do not yet understand the molecular basis of the protective mechanisms (e.g., resistance to bacteriocins). Many of the genes identified as being under σ^B control, in several different studies, have no known functions. Some of these genes are likely to encode novel protective mechanisms and therefore characterizing their molecular functions will give new insights into molecular basis for stress tolerance in *L. monocytogenes*. Ultimately, it is to be hoped that these insights can be exploited to prevent this important pathogen from establishing life-threatening infections in humans.

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CHAPTER 6

Protein Secretion and Membrane Insertion Systems in Bacteria and Eukaryotic Organelles

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I. INTRODUCTION: TRANSPORT PROTEIN CLASSIFICATION

For several years, our laboratory has been concerned with the phylogenetic characterization and classification of transmembrane transport systems (Saier, 1998). Transporters generally consist of channels or carriers (Saier, 1999), and carriers can function by uniport, cation:solute symport, cation: solute antiport, or solute:solute antiport (Saier, 2000a). Additionally, cytoplasmic protein domains and/or subunits can be superimposed upon these integral membrane channel and carrier proteins to allow the direct coupling of chemical energy to transport, as can be provided, for example, by ATP hydrolysis (Saier, 2000b; Saier and Tseng, 1999). The transporter classification (TC) system (Busch and Saier, 2002) involves transport protein categorization (Table 6.1) in five steps as follows: First, permeases are grouped according to transporter type as follows: category 1, channels; category 2, secondary carriers; category 3, primary active transporters; category 4, group translocators; category 5, transmembrane electron flow carriers; category 8, accessory transport proteins; and category 9, transporters or putative transporters of unknown mechanism of action.

Second, these major divisions of transporters are subdivided according to protein structural type or energy coupling mechanism. Third, the resultant permease types are divided into recognizable families. Fourth, these families are subdivided into phylogenetic clusters or subfamilies, and last, within each phylogenetic cluster, all functionally characterized transporters that catalyze different transport processes and/or exhibit different substrate specificities are separately tabulated (Busch and Saier, 2002). The TC system is therefore based on both function and phylogeny. This classification system has been adopted by the International Union of Biochemistry and Molecular Biology (IUBMB). The system is described briefly on the IUBMB website and in more detail in the TC database (TCDB, www.tcdb.org; Saier *et al.*, 2006). It includes all systems currently recognized to catalyze protein secretion and membrane insertion.

TABLE 6.1 The TC system of transport protein classification[a]

1	Channels/pores
	[a] A. α-Type channels
	[a] B. β-Barrel porins
	[a] C. Pore-forming toxins (proteins and peptides)
	D. Non-ribosomally synthesized channels
	[a] E. Holins
	F. Vesicle fusion pore
	G. Paracellular channels
2	Electrochemical potential-driven transporters
	[a] A. Porters (uniporters, symporters, and antiporters)
	B. Non-ribosomally synthesized porters
	[b] C. Ion-gradient-driven energizers
3	Primary active transporters
	[a] A. P–P-bond hydrolysis-driven transporters
	B. Decarboxylation-driven transporters
	C. Methyltransfer-driven transporters
	D. Oxidoreduction-driven transporters
	E. Light absorption-driven transporters
4	Group translocators
	A. Phosphotransfer-driven group translocators
	B. The nicotinamide ribonucleoside uptake transporters
	C. The acyl-CoA ligase-coupled transporters
5	Transmembrane electron transfer carriers
	A. Two-electron carriers
	B. One-electron carriers
8	Accessory factors involved in transport
	[c] A. Auxiliary transport proteins
	B. Ribosomally synthesized protein/peptide toxins that target channels and carriers
	C. Non-ribosomally synthesized toxins that target channels and carriers
9	Incompletely characterized transport systems
	[d] A. Recognized transporters of unknown biochemical mechanism
	[d] B. Putative uncharacterized transport proteins
	C. Functionally characterized transporters lacking identified sequences

[a] Representatives of these types of systems are known to be capable of exporting proteins and other cell surface macromolecules.
[b] These systems can energize proteins translocation, but in the inward direction.
[c] These proteins can facilitate protein translocation.
[d] Systems included in the 9-category translocate proteins by unknown or poorly defined mechanisms.

II. THE DIVERSITY OF PROTEIN TRANSLOCASES IN BACTERIA AND EUKARYOTIC ORGANELLES

Protein export systems are present in all living organisms. Protein export/membrane insertion systems currently recognized in living organisms fall into TC categories 1A, 1B, 1C, 1E, 2A, 3A, 9A, and 9B of the TC system (Table 6.2; www.tcdb.org; Saier, 2000a,b; Saier *et al.*, 2006). Table 6.2 lists the organismal distributions and the energy sources of most types of protein secretory pathway (SP) systems that are currently well characterized in living organisms. References cited in this review and in TCDB provide descriptions of the protein families as well as mechanistic information.

Currently characterized type I (ATP-binding cassette or ABC-type) protein secretory pathway (ISP) systems (Fig. 6.1) are restricted to some bacteria, especially Gram-negative bacteria, as well as archaea, and a few eukaryotes, although members of the ABC superfamily are found in essentially all living organisms (Davidson and Maloney, 2007; Schmitt *et al.*, 2003; Yamane *et al.*, 2004). The Gram-negative bacterial export systems transport their protein substrates across both membranes of the cell envelope. These systems include ABC efflux pumps (TC #3.A.1.109 and 110) complexed with membrane fusion proteins (MFPs; TC# 8.A.1) and outer membrane factors (OMFs; TC# 1.B.17). ABC-type protein exporters in Gram-positive bacteria often depend upon an MFP for activity but lack an OMF (Harley *et al.*, 2000). This suggests that at least some ABC systems depend on an MFP for activity, but the molecular basis for this observation is not known. In agreement with these suggestions, MFPs, but not OMFs, coevolved with the primary transporters (Dinh *et al.*, 1994).

By contrast, general SP (GSP or Sec) systems for protein export across the cytoplasmic membrane are found ubiquitously in all living organisms (Fig. 6.2A; Cao and Saier, 2003). Moreover, the principal channel-forming constituents follow the organismal phylogenies (Fig. 6.2B), suggesting vertical transmission from a common ancestor (Cao and Saier, 2003). The twin arginine targeting translocases (Tat), also for export specifically across the cytoplasmic membrane, are by no means ubiquitous, but they are widely distributed. By contrast, main terminal branch (MTB) systems, responsible for transport of many proteins across the outer membranes of Gram-negative bacteria, are exclusively restricted to these organisms (Sandkvist, 2001). In this article, we refer to the Sec or Tat plus MTB systems as type IISP systems, analogously to the type ISP, type IIISP, and type IVSP systems which translocate their protein substrates across both membranes.

Well-characterized type III (flagellar [fla]- and pathogenesis [path]-related) and type IV (conjugation [conj]- and virulence [vir]-related) systems have been best characterized in Gram-negative bacteria (Baron and Coombes, 2007). However, flagellar export systems, related to but

TABLE 6.2 Protein secretory pathways (PSP) in Gram-negative bacteria and derived eukaryotic organelles

	Type (Abbreviation)	Name	TC #[a]	Bacteria	Archaea	Eukarya	# Proteins/ system	Energy	References
IMPs—Gram-negative bacterial inner membrane channel-forming translocases									
1	ABC (ISP)	ATP-binding cassette translocase	3.A.1	+	+	+	3–4	ATP	Holland *et al.* (2005)
2	Sec (IISP)	General secretory translocase	3.A.5	+	+	+	~12	GTP or ATP+PMF	Cao and Saier (2003)
3	Fla/Path (IIISP)	Flagellum/virulence-related translocase	3.A.6	+	–	–	>10	ATP	Nguyen *et al.* (2000)
4	Conj/Vir (IVSP)	Conjugation-related translocase	3.A.7	+	–	–	>10	ATP	Cao and Saier (2001)
5	Tat (IISP)	Twin arginine targeting translocase	2.A.64	+	+	+ (chloroplasts)	2–4	PMF	Yen *et al.* (2002b)
6	Oxa1 (YidC)	Cytochrome oxidase biogenesis family	2.A.9	+	+	+ (mitochondria; chloroplasts)	1	None or PMF	Yen *et al.* (2001); Yi and Dalbey (2005)
7	MscL	Large conductance mechanosensitive channel family	1.A.22	+	–	–	1	None	Pivetti *et al.* (2003)
8	Holins	Holin functional superfamily	1.E.1-21	+	–	–	1	None	Young (2002)
Eukaryotic organelles									
9	MPT	Mitochondrial protein translocase	3.A.8	–	–	+ (mitochondria)	~20	ATP	Lister *et al.* (2005)
10	CEPT	Chloroplast envelope protein translocase	3.A.9	(+)	–	+ (chloroplasts)	≥3	GTP	Steiner *et al.* (2005); Robinson *et al.* (2000)

(*continued*)

TABLE 6.2 (*continued*)

	Type (Abbreviation)	Name	TC #[a]	Bacteria	Archaea	Eukarya	# Proteins/ system	Energy	References
11	Bcl-2	Eukaryotic Bcl-2 family (programmed cell death)	1.A.21	–	–	+	1?	None	Crompton *et al.* (2002)
Gram-negative bacterial outer membrane channel-forming translocases									
1	MTB (IISP)	Main terminal branch of the general secretory translocase	3.A.15	+[b]	–	–	~14	ATP; PMF	Peabody *et al.* (2003)
2	FUP	Fimbrial usher protein	1.B.11	+[b]	–	–	1	None	Yen *et al.* (2002a)
3	AT	Autotransporter-1	1.B.12	+[b]	–	–	1	None	Loveless and Saier (1997)
4	AT-2	Autotransporter-2	1.B.40	+[b]	–	–	1	None	Kim *et al.* (2006)
5	OMF (ISP)	Outer-membrane factor	1.B.17	+[b]	–	+(?)	1	None	Yen *et al.* (2002a)
6	TPS	Two partner secreton	1.B.20	+	–	+	1	None	Yen *et al.* (2002a)
7	Secretin (II (MTB) and III SP)	Secretin	1.B.22	+[b]	–	–	1	None	Yen *et al.* (2002a)
8	OmpIP	Outer membrane protein insertional porin	1.B.33	+	–	+ (mitochondria; chloroplasts)	≥5	None?	Wu *et al.* (2005)

[a] See Saier (2000a) and Saier *et al.* (2006).
[b] Only identified in Gram-negative bacteria.

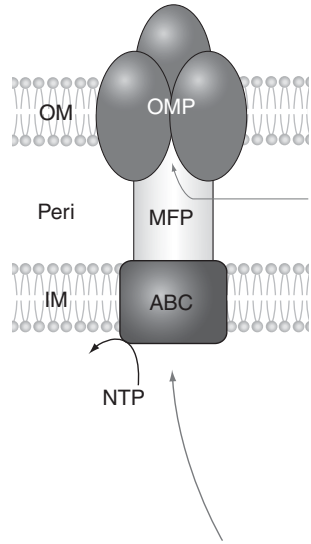

FIGURE 6.1 Schematic depiction of type I (ABC) protein secretion systems. In all schematic figures, the periplasm (Peri) separates the inner membrane (IM) from the outer membrane (OM) of the Gram-negative bacterial cell envelope. Abbreviations: NTP, nucleoside triphosphate; ABC, an ATP-binding cassette transporter; MFP, a membrane fusion protein; OMP, an outer membrane pore-forming factor.

structurally and phylogenetically distinct from the pathogenesis-type systems, are prevalent in both Gram-negative and Gram-positive bacteria (Christie, 2001; Plano *et al.*, 2001). Type IV conjugation systems are also present in Gram-positive bacteria (Dubnau, 1999; Grynberg *et al.*, 2007; see TCDB), although these systems are still relatively poorly characterized. All proteins of these secretory systems, ISP, IISP, IIISP, and IVSP, as referred here, export proteins across both membranes of Gram-negative bacterial envelopes. They are probably energized by ATP, although GTP and the proton motive force (pmf) may contribute to protein export via the Sec, IIISP, and MTB systems (Economou, 2002). An additional type of system, often termed VISP, has been described, but it is not clear if it is actually a distinct type of system. It may merely be a sequence divergent form of type IV (Pukatzki *et al.*, 2006; Zheng and Leung, 2007).

Twin arginine targeting (Tat) translocases, which usually translocate fully folded redox enzymes and other protein complexes into the periplasm of Gram-negative bacteria, are energized by the pmf exclusively. In Gram-negative bacteria, when protein substrates of the Sec and Tat systems are exported from the periplasm to the external milieu, they often use the MTB for final secretion across the outer membrane (Johnson *et al.*, 2006). However, other protein-specific systems can also catalyze this process (Yen *et al.*, 2002b).

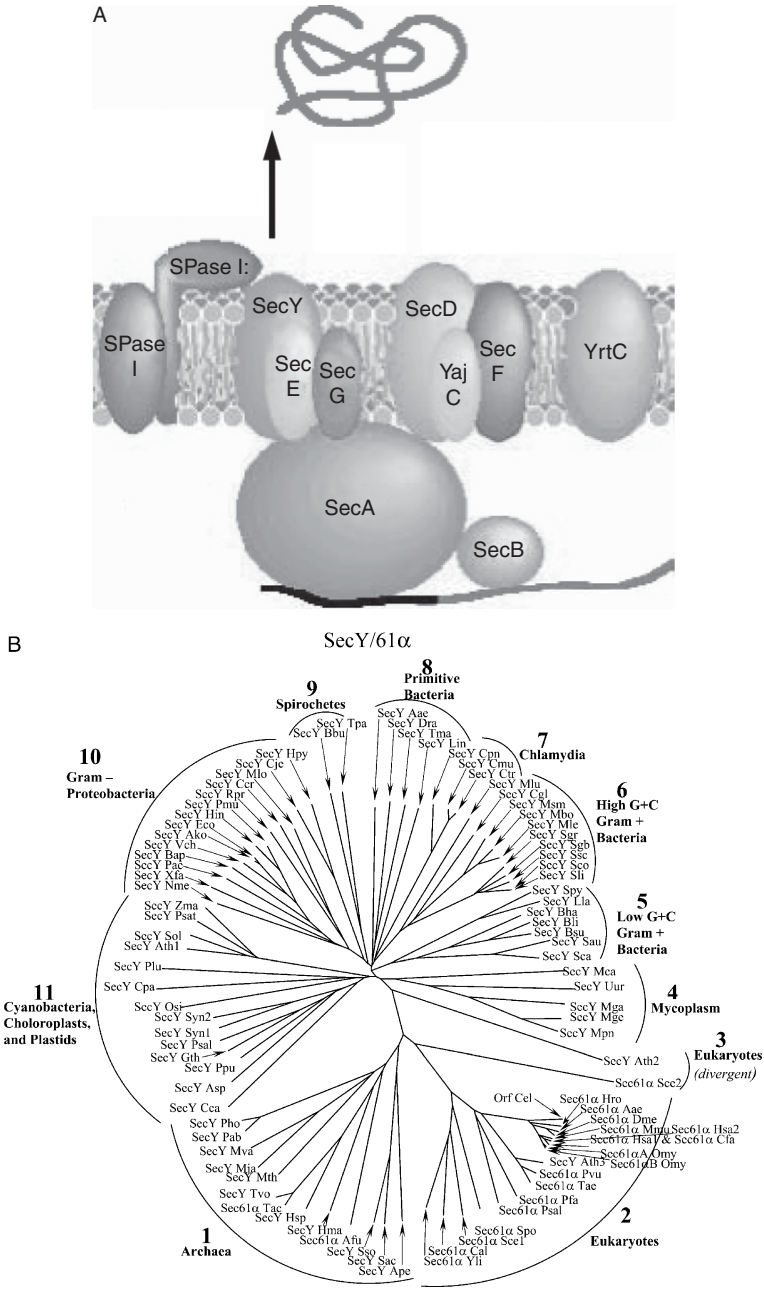

FIGURE 6.2 (A) Schematic depiction of the *Escherichia coli* general secretory (Sec) system. SPase I and II are signal peptidases. SecA and SecB are preprotein export chaperone proteins. SecA is the ATPase that energize translocation across the SecYEG

Mitochondrial protein translocases (MPTs; TC #3.A.8), possibly derived from primitive protein translocation systems in α-proteobacteria, appear to have evolved to their current level of complexity after the degeneration of endosymbiotic bacteria into mitochondria (Paschen *et al.*, 2005). Their evolution probably followed transfer of much of their genetic material to the nucleus of the host eukaryotic cell, requiring extensive import (Wickner and Schekman, 2005). Although there are only a few prokaryotic homologues of constituents of these mitochondrial import systems (see also TCDB, TC #1.B.33; Lister *et al.*, 2005), several chloroplast envelope protein translocase constituents of the CEPT family (TC #3.A.9; Schleiff *et al.*, 2003a,b) have bacterial homologues, suggesting an origin in the primordial cyanobacterial cell (Ertel *et al.*, 2005; Paschen *et al.*, 2005; Steiner *et al.*, 2005).

The cytochrome oxidase biogenesis (Oxal) family is still poorly characterized. Functional data were first available for mitochondria, and subsequently for plant chloroplasts and bacteria (Luirink *et al.*, 2001; Pohlschroder *et al.*, 2005; Yi and Dalbey, 2005). In both eukaryotic organelles as well as *Escherichia coli*, these proteins seem to function primarily (but not exclusively) to effect integral membrane protein insertion (Luirink *et al.*, 2005).

The large conductance mechanosensitive channels (MscL) possibly have the capability to export small proteins such as thioredoxin as well as osmolytes from the cytoplasm of the bacterial cell following osmotic downshift (see Pivetti *et al.*, 2003 for a review). However, osmotic adaptation rather than protein export is clearly their primary function.

Small holin proteins, of which there are more than two-dozen recognized families, form oligomeric pores in membranes. They function to export autolytic enzymes that hydrolyze the peptidoglycan layer of the bacterial cell wall. Export is from the cell cytoplasm to an extracytoplasmic locale where these enzymes can promote cell death (Ramanculov and Young, 2001; Wang *et al.*, 2000; Young, 2002; Young and Bläsi, 1995). The holin/autolysin pair is usually encoded either by phage genes, in which

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transmembrane pore. SecDF/YajC is a nonessential complex that facilitates insertion of integral inner membrane proteins into the membrane, either in conjunction with SecYEG or independently. YidC facilitates insertion of integral membrane proteins into the membrane with or without the SecY complex. (B) Phylogenetic tree of sequenced homologues of the SecY protein of *E. coli* and the Sec61α protein of *S. cerevisiae*. Reproduced from Cao and Saier (2003), with permission, wherein protein and organismal abbreviations can be found. The tree was generated with the CLUSTAL X and TREEVIEW programs (Thompson *et al.*, 1997; Zhai *et al.*, 2002). Note that the tree reveals one eukaryotic and one archaeal cluster (clusters 2 and 1, respectively) plus "splinter" group members from yeast and plants. The other eight phylogenetic clusters are exclusively of bacterial origin, where the proteins cluster according to the phylogenies of their source organisms (16S rRNAs).

case they promote cell lysis and phage release, or by bacterial chromosomal genes, in which case they mediate programmed cell death (Srividhya and Krishnaswamy, 2007). Cell death inducing systems of animal cells include the Bcl-2 family proteins (TC#1.A.21), which interact with voltage-dependent anion channel proteins (VDAC; TC# 1.B.8) in the outer membranes and the ATP/ADP exchanger of the mitochondrial carrier family (MC; TC #2.A.29) in the inner membranes of mitochondria (Adams and Cory, 1998; Willis *et al.*, 2005, 2007).

Finally, bacteria synthesize and secrete numerous toxins such as diphtheria and tetanus toxins which insert into a host animal cell membrane, forming pores that transport cytoplasmic toxic proteins into the cell. All such toxins are included in TC classification 1.C. Descriptions of these systems as well as references to research concerning them can be found at the TCDB database (www.tcdb.org). These proteins will not be the focus of this review. However, TCDB provides a useful tool that can be used together with this review.

A number of systems in addition to the MTB cited above are known to function exclusively in export across the lipopolysaccharide (LPS)-containing outer membranes of Gram-negative bacteria (Table 6.2). These systems include: (1) the fimbrial usher porins (FUP) that translocate fimbrial subunits across the outer membranes and assemble these subunits into intact fimbriae (Yen *et al.*, 2002a), (2 and 3) two independently evolving families of autotransporters (AT-1 and AT-2) that exhibit C-terminal, oligomeric, pore-forming, β-barrel domains that are thought to translocate their N-terminal virulence-related protein domains to the external surface of the membrane (Cotter *et al.*, 2005; Kim *et al.*, 2006; Roggenkamp *et al.*, 2003; Thanassi *et al.*, 2005), (4) the two partner secretons (TPS) that include members, most of which translocate and sometimes modify toxins and other exported proteins (Jacob-Dubuisson *et al.*, 2001; Yen *et al.*, 2003a), (5) secretins, oligomeric pore-forming constituents of types II (MTB) and III (Path) systems (Collins and Derrick, 2007; Thanassi, 2002), and (6) OMFs that are pore-forming constituents that function with type I (ABC) protein exporters to allow protein transport across the outer Gram-negative bacterial membrane in a process coupled to ATP hydrolysis catalyzed by the cytoplasmic membrane ABC exporter (Federici *et al.*, 2005). Finally, (7) the outer membrane protein insertion porin (OmpIP) is a multicomponent system that appears to facilitate insertion of periplasmic β-structured OMPs from the periplasm into this membranous structure (Doerrler and Raetz, 2005; Voulhoux *et al.*, 2003).

There are thus eight known, independently functioning systems that specifically effect protein export across or insertion into the inner (cytoplasmic) membranes of Gram-negative bacteria and eight that effect export across or insertion into the outer (LPS) membranes (Table 6.2; Saier, 2006, 2007; Yen *et al.*, 2002a). Interestingly, the inner membrane

protein export systems can function by a threading mechanism (ABC and Sec), by translocating fully or partially folded subunits (IIISP, IVSP, MscL, and possibly holins), or by translocating fully folded and assembled multisubunit protein complexes (Tat). Strictly outer membrane translocases usually (but not always) function by energy-independent diffusion-type mechanisms that may translocate partially or fully folded substrate proteins. The nature of the substrates of and the translocation mechanisms utilized by the outer membrane FUP, AT-1, AT-2, and TPS family channels are still ill defined, but they probably all translocate unfolded proteins; by contrast, the MTB probably transports folded proteins. In the following sections, we will discuss the functions and phylogenies of these different types of protein secretion systems in Gram-negative bacteria. For recent surveys of protein secretory systems encoded within the genomes of select Gram-negative bacteria (see Barabote *et al.*, 2007; Ma *et al*, 2003; Yen *et al.*, 2008).

III. COMPLEX INNER MEMBRANE SECRETORY SYSTEMS IN BACTERIA

A. Type I (ABC–MFP–OMF-type) protein exporters (Fig. 6.1)

Type I ABC macromolecular export systems are widespread in nature. Of the 77 currently recognized families of these strictly ATP-dependent systems, two families are specific for large proteins (TC# 3.A.1.109-110), both from Gram-negative bacteria. Members of four other families export peptides or small proteins (TC# 3.A.1.111-113, 3.A.1.123), and exporters of six other families are specific for complex carbohydrates. These ABC exporters generally consist of two integral membrane domains and two cytoplasmic ''energizer'' domains that hydrolyze ATP. The systems may recognize a C-terminal targeting sequence in the transported substrate protein, but transport seems to be limited by the size and ease of unfolding of the substrate protein. ABC-type protein export systems, several of which can be present in a single bacterial cell (Ma *et al.*, 2003), can associate with two auxiliary proteins, the MFPs and the OMFs that allow transport across both membranes of the Gram-negative bacterial envelope in a single step as noted above (Holland *et al.*, 2005; see Fig. 6.1).

ABC transporters usually exhibit substrate specificities that reflect the phylogenies of these systems (Bouige *et al.*, 2002; Dassa *et al.*, 1999; Saurin *et al.*, 1999). In cases that have been studied, the constituents of these systems seem to have rarely, if at all, undergone shuffling during their evolutionary histories (Kuan *et al.*, 1995; Paulsen *et al.*, 1997a; Tam and Saier, 1993). It is presumed that this restriction reflects a need for strict protein:protein interactions for optimal function (Cao and Saier, 2001; Nguyen *et al.*, 2000; Peabody *et al.*, 2003).

Comparable studies with the two auxiliary constituents, the MFPs and the OMFs, have revealed that while the MFPs have evolved in parallel with their primary permeases (Dinh *et al.*, 1994), the OMFs have not (Paulsen *et al.*, 1997b). The discovery that MFP homologues are present in Gram-positive bacteria (Harley *et al.*, 2000), and the demonstration that at least some of these proteins essential for transport activity (Axelsson and Holck, 1995; Quadri *et al.*, 1997; Venema *et al.*, 1996) are in agreement with their close connections with the permeases.

Recently, high-resolution 3-dimensional structures of both uptake- and efflux-type ABC systems and their accessory proteins have been determined (Hollenstein *et al.*, 2007; Hvorup *et al.*, 2007; Oldham *et al.*, 2007; Ward *et al.*, 2007). MsbA of *E. coli* is one such efflux system specific for drugs and lipids (Ward *et al.*, 2007) while the solved BtuCDF system, specific for vitamin B_{12}, is an example of an uptake system (Hvorup *et al.*, 2007). Their 3-dimensional structures reveal marked differences between systems catalyzing uptake and efflux particularly in the transmembrane domains, although the ATP hydrolyzing constituents are strikingly similar (Davidson and Maloney, 2007).

TolC of *E. coli* is an OMF that functions with several types of transporters. Its structure has also been solved (Eswaran *et al.*, 2004; Higgins and Linton, 2004; Higgins *et al.*, 2004a,b; Koronakis, 2003; Koronakis *et al.*, 2000, 2004; Touze *et al.*, 2004). The TolC protein exhibits a 3-dimensional fold unlike any previously characterized protein. It forms a trimeric, outer membrane β-barrel pore structure (12 β-strands, 4 per subunit) as well as a transperiplasmic, trimeric, α-helical conduit (12 α-helices: 6 continuous, 6 discontinuous; 4 per subunit) which probably connects the inner membrane permease to the outer membrane pore. The OMF by itself provides the transperiplasmic channel. The MFP probably interlinks the inner and outer membrane transport pathways. However, it may serve other functions (Delepelaire, 2004). This last postulate is consistent with the occurrence of MFP homologues in Gram-positive bacteria that lack outer membranes (Harley *et al.*, 2000). Some of these MFP proteins have been shown to be essential for transport function as noted above.

B. General secretory translocases (Sec systems; Fig. 6.2)

Type II GSP (Sec) systems in Gram-negative bacteria consist of essential and auxiliary protein subunits (Rusch and Kendall, 2007; Wickner and Schekman, 2005). Every living organism that has been examined has a Sec system, and most have only one (Cao and Saier, 2003). Nevertheless, only some of the constituents are found universally. The essential *E. coli* translocase constituents include the universal, heterotrimeric, integral, inner membrane protein complex, SecYEG, the prokaryotic-specific cytoplasmic ATPase, SecA, and several additional proteins, all of which follow the

phylogenies of the host organisms with few exceptions (Cao and Saier, 2003; see Fig. 6.2A and B). SecA may recruit SecYEG prokaryotic-specific complexes (or vice versa) to form reversible active translocation complexes (Benach *et al.*, 2003). The active assembly includes a SecA homodimer and a SecYEG homodimeric or homotetrameric complex (Scheuring *et al.*, 2005; Tziatzios *et al.*, 2004). SecA is apparently not found in eukaryotes. SecY of *E. coli* is a 10 TMS protein of about 450 amino acyl residues that is believed to form the protein-translocating channel when complexed with the two small integral membrane proteins, SecE and SecG, each maximally of about 140 amino acyl residues in length (Ito, 1992). The SecYEG complex is ubiquitous, being present in every bacterium, archaeon, and eukaryote with a fully sequenced genome (Kinch *et al.*, 2002).

Two auxiliary proteins, SecD and SecF, in *E. coli* are homologous to members of the RND superfamily (TC #2.A.6; Tseng *et al.*, 1999). They are not present in many organisms, including slowly growing bacteria and all eukaryotes. Another protein, YajC of *E. coli*, forms a complex with SecD–SecF, both independent of and in complexation with SecYEG (Müller *et al.*, 2001). The SecDF–YajC complex is not essential for secretion, but it stimulates secretion up to 10-fold, particularly at lower temperatures. The mechanistic role of this auxiliary prokaryotic-specific complex is not clearly defined, but it is not required for maintenance of the pmf (Nouwen *et al.*, 2001) as had been proposed earlier.

Although Sec-dependent protein export and integral membrane protein insertion are driven by ATP/GTP hydrolysis, the pmf is stimulatory and may function solely in translocating the C-terminal parts of the unfolded substrate proteins. Thus, it is possible that both energy sources are required for efficient translocation with each acting at different steps (Driessen and Nouwen, 2007; Geller, 1991; Rapoport *et al.*, 1996). Point mutations in SecY have been described that abolish the pmf-dependence of the translocation process, but nucleoside triphosphate hydrolysis appears to be essential under all conditions.

Insertion of integral inner membrane proteins in bacteria is dependent on a complex resembling the eukaryotic signal recognition particle (SRP) protein–RNA complex which functions as an essential constituent for protein membrane insertion (Müller *et al.*, 2001). It has also been shown to play a role in the export of some secretory proteins such as DsbA, β-lactamase, and some autotransporters in *E. coli* (Sijbrandi *et al.*, 2003; Takamatsu *et al.*, 1997). The primary protein constituents of the bacterial complex, Ffh (an SRP54-like protein) and FtsY (an SRP receptor [subunit α]-like protein), probably act as GTP-dependent chaperones, feeding substrate proteins into the SecYEG complex (Driessen and Nouwen, 2007; Rapoport, 2007; Scotti *et al.*, 1999). Insertion of most polytopic inner membrane proteins shows a dependency on Ffh and FtsY as well as the SecYEG channel complex, although in some cases, the Oxa1 homologue in *E. coli*, YidC, may replace

this complex (Fröderberg *et al.*, 2003; van der Laan *et al.*, 2005). How the SecYEG channel may facilitate membrane protein insertion, based on the high-resolution X-ray structure of the SecYEG channel complex, has been discussed (Collinson, 2005; Rapoport *et al.*, 2004; van den Berg *et al.*, 2004).

C. Type III flagellar and pathogenicity-related systems (Fig. 6.3)

Type III secretion systems are typically employed by pathogenic Gram-negative bacteria to deliver virulence proteins, termed effectors, directly into the cytosol of host cells (Ghosh, 2004; Hueck, 1998). The battery of effectors delivered varies widely in support of diverse host–pathogen

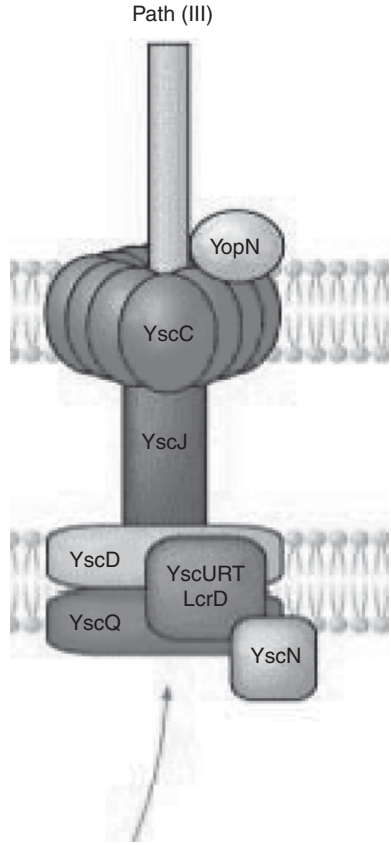

FIGURE 6.3 Generalized schematic structure of the Type III/Pathogenicity (flagellar-like) protein secretion system of *Yersinia* species. YscC is the dodecameric outer membrane secretin while YopN may serve as both YscC anchor and Ca^{2+} sensor for regulations of Yop synthesis. YscN is the energizing ATPase. Other constituents shown comprise the basic structure of the "injectisome."

relationships, and these generally function to disable host immunological defenses. In contrast to this effector diversity, roughly half of the proteins comprising the syringe-like type III secretion apparatus, or ''injectosome,'' are conserved between bacterial species. Most of these conserved proteins are similar to those of the basal body of the flagella, which secrete subunits during assembly and are capable of secreting virulence factor (Young *et al.*, 1999). Protein transport by both types of related systems is dependent on the pmf (Galperin *et al.*, 1982; Minamino and Namba, 2008; Paul *et al.*, 2008; Wilharm *et al.*, 2004), and ATP hydrolysis at the inner membrane (Eichelberg *et al.*, 1994; Emerson *et al.*, 1970; Iino, 1969; Tamano *et al.*, 2000; Woestyn *et al.*, 1994). The structural and functional similarities exhibited by the flagellar and pathogenic type III secretion systems have been explored through phylogenetic analyses, and it has been concluded that either type III systems evolved from flagellar systems or that both systems evolved separately from a common ancestor (Gophna *et al.*, 2003; McCann and Guttman, 2008; Nguyen *et al.*, 2000; Saier, 2004).

Genes encoding these proteins are sometimes in the chromosome, in which case they are most frequently found within ''pathogenicity islands'' which are inserted DNA segments derived from foreign organismal sources (Coburn *et al.*, 2007; Hansen-Wester and Hensel, 2001; Yip *et al.*, 2005). However, they may also be plasmid-encoded. As noted above, while the flagellar proteins have been acquired largely by vertical transmission, horizontal transmission of the pathogenicity systems appears to have dominated their distribution (Nguyen *et al.*, 2000; Saier, 2004). These two related types of systems are thus functionally and structurally equivalent, although their constituents generally cluster separately on a phylogenetic tree (Nguyen *et al.*, 2000).

The type III secretion apparatus is composed of 20–25 distinct proteins, most of which are required for the translocation of effectors into host cells. As noted above, genes encoding the injectosome components are typically present within one or more virulence plasmids, although some effector genes can be found clustered within pathogenicity islands on the bacterial chromosome (Hansen-Wester and Hensel, 2001). *Yersinia* spp. harbor well-characterized type III secretion systems and export effectors termed Yops. They disable the host defenses by injecting effectors which modulate the inflammatory response and affect cell survival. Effectors include Rho-GTP binding proteins and focal adhesion proteins (Aepfelbacher *et al.*, 2007).

Prior to translocation, most effectors are bound to specialized chaperone proteins known as ''Syc.'' These proteins are required for the efficient translocation of their cognate effectors and are thus necessary for bacterial virulence. Chaperones are postulated to function by presenting effectors to the secretion apparatus (Birtalan *et al.*, 2002; Lee and Galán, 2004; Lee and Schneewind, 2002) and an additional translocation signal is known to reside within the first 15 amino acids of some of the effector

proteins, the equivalent mRNA codons, or both (Karavolos *et al.*, 2005; Sorg *et al.*, 2005). In spite of much evidence describing the necessity of this region, a receptor for such a signal remains unidentified.

While assembly of the bacterial flagellar filament requires a type III export pathway for ordered delivery of structural subunits from the cytosol to the cell surface, transient interactions with chaperones seem to protect subunits and pilot them to dock at the membrane export ATPase complex. An essential flagellar export protein, FliJ, has a novel chaperone escort function in the pathway. It recruits unladen chaperones for the minor filament-class subunits of the filament cap and hook-filament junctional substructures. FliJ does not recognize unchaperoned subunits or chaperone-subunit complexes, and it associates with the membrane ATPase complex, suggesting a postdocking function (Evans *et al.*, 2006). Empty chaperones that are recruited by FliJ *in vitro* are efficiently captured from FliJ–chaperone complexes by cognate subunits. FliJ with subunit bound to the target chaperone has a much greater affinity for chaperone than does FliJ alone (Evans *et al.*, 2006). Thus, FliJ recruits chaperones and transfers them to subunits driven by competition for a common binding site. This escort mechanism provides a means by which free export chaperones can be cycled after subunit release, establishing a new facet of the secretion process. As FliJ does not escort the chaperone for the major filament subunit, cycling may offer a mechanism for export selectivity and thus promote assembly of the junction and cap substructures required for initiation of flagellin polymerization.

As for the flagellar system, one of the constituents of the IIISP system, YscN of *Yersinia* species, is the ATPase that is believed to couple ATP hydrolysis to protein export. Six integral inner membrane proteins (LcrD and YscD, R, S, T, and U) may form a complex that provides the transport pathway. The YscC protein, an outer membrane secretin, forms dodecameric pores through which the injectisome penetrates the outer membrane (Burghout *et al.*, 2004a,b; Nguyen *et al.*, 2000).

IIISP systems can secrete proteins directly into the host cell cytoplasm without exposure to the extracellular milieu (Coombes and Finlay, 2005). This fact implies the existence of a pore complex that spans the host cell cytoplasmic membrane and is contiguous with the bacterial secretion apparatus. This becomes possible by virtue of the ''needle complex'' that transports proteins through itself (See Fig. 6.3; Cornelis, 2006; Mota, 2006). The *Yersinia* proteins that are believed to provide this function are YopB and YopD that span the host cell cytoplasmic membrane and form oligomeric pore complexes (Olsson *et al.*, 2004; Ryndak *et al.*, 2005; Viboud and Bliska, 2005). These putative host cell membrane pore proteins comprise the bacterial type III-target cell pore (IIITCP) family (TC #1.C.36).

Flagella and injectosomes possess exquisite and similar mechanisms for exclusively secreting hook or needle proteins during assembly, and then

switching to filament or Yop secretion upon completion of the respective structure. Injectosome length is determind by YscP, which acts as a molecular ruler to regulate polymerization of the needle subunit YscF (Journet *et al.*, 2003). YscP recognizes the completion of elongation and signals the event to the basal body component YscU which is proteolytically activated to halt the secretion of YscF and begin secretion of LcrV, YopB, and YopD upon contact with a host cell (Agrain *et al.*, 2005; Sorg *et al.*, 2007). These ''translocator Yops'' localize at the distal tip of the needle and form a pore to penetrate the target cell membrane (Broz *et al.*, 2007; Coombes and Finlay, 2005; Francis and Wolf-Watz, 1998; Hakansson *et al.*, 1996; Hoiczyk and Blobel, 2001; Mueller *et al.*, 2005; Olsson *et al.*, 2004; Ryndak *et al.*, 2005). Based on homology to proteins in *Salmonella* and *E. coli*, the *Yersinia* ATPase YscN (Crepin *et al.*, 2005) is thought to unfold effectors at the base of the needle. They subsequently transverse the extracellular space within the conduit formed by YscF (Crepin *et al.*, 2005). Finally, entry to the host cell cytoplasm is likely achieved via the pore formed by YopB and YopD.

D. Type IV conjugation- and virulence-related (IVSP) systems (Fig. 6.4)

Protein complexes of the IVSP family consist of multiple subunits that span the two membranes and the peptidoglycan cell wall of the Gram-negative bacterial cell envelope or the single membrane plus wall of the Gram-positive bacterial cell envelope (Fig. 6.4). In acid fast bacteria (Mycobacteria, Nocadia, and Corynebacteria), they may penetrate the outer mycolic acid-containing membrane as well. They export proteins and DNA–protein complexes out of the cell and into the cytoplasm of a recipient cell (Christie and Cascales, 2005; Winans *et al.*, 1996). These systems are very promiscuous, being capable of transporting various DNA–protein complexes into cells of other bacteria, yeast, animals, and plants, and also into the external medium (Hamilton *et al.*, 2005; Li *et al.*, 2005). For example, the VirB systems of agrobacterial species are specifically designed to transfer T-DNA into plant cells causing cancerous growth, but they can also transfer the IncQ plasmid RSF1010 into both plant and bacterial cells (Bohne *et al.*, 1998). Further, the various Inc IVSP systems are designed to mediate plasmid transfer from the donor bacterium to a recipient bacterium, but cross-specificity has been demonstrated (Lybarger and Sandkvist, 2004). C-terminal sequences of proteins covalently or noncovalently linked to the DNA may be recognized as a prelude to nucleoprotein transport (Christie and Cascales, 2005). While proteins in addition to the VirB2-B11 proteins may be involved in the transfer process, the VirB proteins appear to be the primary ones involved in export from the cytoplasm across the two membranes of the agrobacterial envelope. Schematic models of type IVSP systems such as that shown in Fig. 6.4 have been presented, each

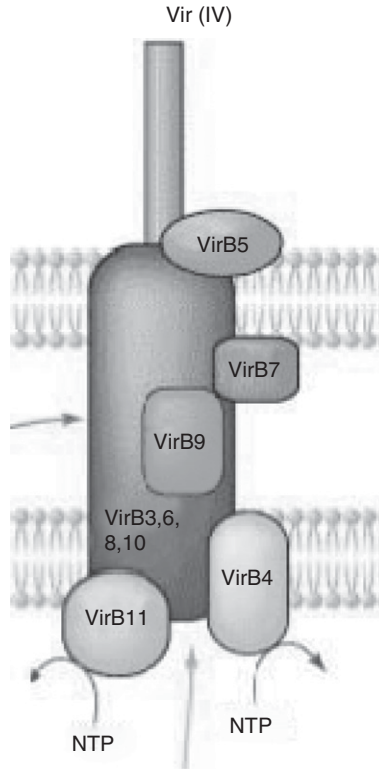

FIGURE 6.4 Generalized schematic view of the type IV conjugation/virulence protein secretion system of *Agrobacterium* species. Two ATPases (VirB4 and VirB11) serve as ATPases. Other proteins comprise the structural elements of the secretion apparatus. Different type IV systems differ strikingly in their protein compositions.

emphasizing different aspects of these highly variable systems (Cao and Saier, 2001; Chen *et al.*, 2005; Christie and Cascales, 2005).

The VirB system of *A. tumefaciens* is related to (1) a natural competence (CAG; ComB) system of *Helicobacter pylori* which may also be involved in transfer of virulence factors including the CagA antigen into host animal cells by a type IV secretion-related mechanism; (2) the Ptl system, involved in secretion of pertussis toxin from the *Bordetella pertussis* cell; (3) the TraS/TraB system of the *Pseudomonas aeruginosa* conjugative plasmid, RP1; (4) the Trb system of plasmid pTiC58 of *Agrobacterium*, one of three genetically encoded systems for conjugal transfer of this Ti plasmid; (5) the Tra system of plasmid F in *E. coli*; and (6) the Dot conjugative transfer/virulence system of *Legionella pneumophila* (Chen *et al.*, 2005; Christie *et al.*, 2005; Ninio and Roy, 2007). There are also more distantly related putative IVSP proteins in Gram-positive bacteria.

Although members of the type IV secretion family share many characteristics, not all systems contain the same sets of genes. Thus, the *virB* systems of Ti plasmids and the *trb* system of RP4 have only six recognized genes in common. The distantly related CAG system of *H. pylori* contains at least eight known constituents with little sequence similiarity to the Vir system (Karnholz *et al.*, 2006), and the *dot* system of *L. pneumophila* contains only two easily recognizable *virB* homologues. Homologues of only one VirB protein, VirB10 (TrbI) are demonstrably present in all known type IV secretion systems characterized (Cao and Saier, 2001). Structural and functional models for the conjugative transfer of DNA–protein complexes through the *A. tumefaciens* VirB–VirD4 system have been presented (Chen *et al.*, 2005; see Fig. 6.4).

Transport of proteins and DNA–protein complexes has been thought to occur in a single energy-coupled step. However, this hypothesis has been challenged by the observation of periplasmic intermediates of IVSP substrates (Pantoja *et al.*, 2002). It is possible that, as originally proposed, transport across both membranes is normally linked, but that some periplasmic leakage can occur. Alternatively, high affinity capture following periplasmic release could account for these observations.

E. The putative type VI symbiosis/virulence secretory systems (TC #9.A.34)

Some *Vibrio cholerae* strains do not cause cholera and instead cause other human infections by poorly understood mechanisms. One such strain possesses on its small chromosome a 15 cistron operon. The encoded 15 proteins have been reported to catalyze protein secretion by a mechanism that does not require the presence of a hydrophobic N-terminal sequence (Pukatzki *et al.*, 2006). The protein substrates can be secreted into the extracellular medium and possibly into eukaryotic cells. The genes have been named *vasA-L* (virulence-associated secretion) or the type VI secretion system (T6SS; Pukatzki *et al.*, 2006). At least some of the *vas* genes are required for cytotoxicity by a contact-dependent mechanism.

Many Gram-negative bacteria have sets of homologous genes as well as potential effector-encoding genes. The *V. cholerae* Vas system may secrete proteins called Hep, VrgG1, VrgG2, and VrgG3. VasK (1181 aas) resembles IcmF (973 aas; AAU26555) of the *L. pneumophila* Dot system throughout most of its length. Similarly, VasF (257 aas) resembles IcmH (261 aas; AAU26556) of the Dot system throughout most of its length, and like the VasF protein, IcmH shows four peaks of hydrophobicity with the first and last being most hydrophobic. VasF and IcmH also show some sequence similarity with portions of OmpA/MotB proteins, possibly reflecting possession of a peptidoglycan binding domain. While 15 cistrons are present in the *vas* operon, it is unlikely that they all function directly in transport.

For example, VasG resembles the *E. coli* ClpB ATP-dependent, stress-induced, heat shock chaperone protein that uses ATP hydrolysis to unfold denatured proteins in conjunction with DnaJK and GrpE in *E. coli.* Moreover, VasH resembles a sigma-54 dependent transcriptional activator, and a sigma-54 homologue is encoded downstream of the *vas* operon. Only VasA, VasF and VasK have been shown to promote virulence. The energy-coupling mechanism is unknown (Pukatzki *et al.*, 2006).

In *Edwardsiella tarda*, an *E. tarda* virulent protein (EVP) gene cluster has recently been implicated in protein secretion (Zheng and Leung, 2007). The entire EVP cluster resembles the T6SS of *V. cholerae*. All of the 16 *evp* operon genes were mutagenized; the secretion of EvpP appeared to be dependent on 13 of the proteins including EvpC (a homologue of Hcp) and EvpI but not EvpD and EvpJ. The 16 EVP proteins could be grouped according to their functions and cellular locations. The first group comprises 11 nonsecreted and possibly intracellular apparatus proteins. Among them, EvpO, a putative ATPase which contains a Walker A motif, shows possible interactions with three EVP proteins (EvpA, EvpL, and EvpN). The second group includes three secreted proteins (EvpC, EvpI, and EvpP). Secretion of EvpC and EvpI may be mutually dependent, and these two proteins are apparently required for the secretion of EvpP. An interaction between EvpC and EvpP was demonstrated. However, as noted above, two proteins (EvpD and EvpJ) are not required for the T6SS-dependent secretion (Zheng and Leung, 2007).

The resemblance between constituents of the VISP and IVSP systems noted above leads to the possibility that type VI systems are sequence divergent type IV systems. Indeed, the extensive component sequence divergence of the latter systems further substantiates this proposal. Also in Gram-positive bacteria, the putative type IV secretion systems are surprisingly complex and diverse. Further studies will be required to put these observations into a clear, evolutionary framework.

F. Twin arginine translocation (Tat) systems (Fig. 6.5)

The TatABCE system of *E. coli*, has been extensively characterized (Brüser, 2007; Sargent *et al.*, 2006; Yen *et al.*, 2002b). This system forms a large (~600 kDa) complex which interacts with fully folded substrate redox proteins that have N-terminal (S/T)RRXFLK ''twin arginine'' leader motifs (Müller, 2005). It translocates several redox enzymes to the *E. coli* periplasm including nitrate reductase (NapA), formate dehydrogenase (FdnGHI), dimethylsulfoxide reductase (DmsABC), and trimethylamine *N*-oxide reductase (TorA), all of which have this leader motif (Gralnick *et al.*, 2006; Sambasivarao *et al.*, 2000, 2001, 2002; Stanley *et al.*, 2002). Hydrogenases, formate dehydrogenases, and several other proteins, including non-redox proteins and some integral membrane proteins (several dozen altogether in

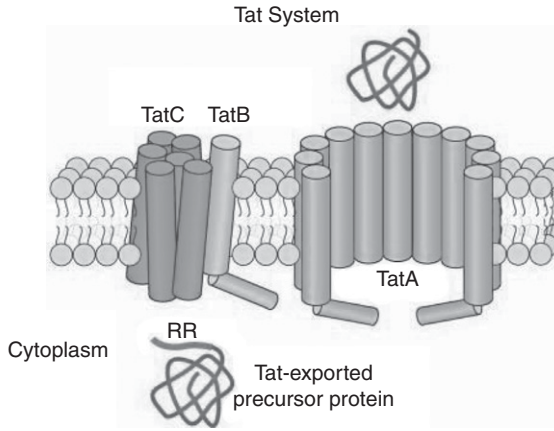

FIGURE 6.5 Schematic diagram of the *E. coli* twin arginine targeting translocase (Tat) system. This system uses the proton motive force (pmf) to drive secretion. TatC may serve as the cytoplasmic receptor as well as the intramembrane proton channel. TatA/E monomers assemble to form the channel of variable sizes that can accommodate fully folded and assembled substrate protein complexes. The N-terminal twin arginine signal sequence of the substrate protein is removed (proteolytically cleaved) following secretion through the cytoplasmic membrane.

E. coli), use this pathway (Berks *et al.*, 2005). These proteins associate with their cofactors in the cell cytoplasm before translocation.

Tat systems apparently function independently of other types of protein secretory systems present in *E. coli* (Palmer *et al.*, 2005). Only one TatA/B/E homologue and TatC are absolutely required for function (Bogsch *et al.*, 1998; Hicks *et al.*, 2005; Sargent *et al.*, 1999). TatA, TatB, and TatE are paralogues of each other and exhibit a single TMS each (Müller and Klösgen, 2005). TatA and TatE exhibit much more similarity in sequence to each other than they do to TatB (Yen *et al.*, 2002b), and they can functionally substitute for each other. TatA (the major, more conserved constituent) and TatB (the minor, less conserved constituent) may together comprise large cylindrical channel-forming complexes of variable diameters that may serve as the channel for protein translocation (see Fig. 6.5; Gohlke *et al.*, 2001; Hicks *et al.*, 2005). TatE is absent in many species such as *P. aeruginosa* (Yen *et al.*, 2002b).

Homologues of representative *E. coli* Tat proteins are found in a variety of Gram-negative and Gram-positive bacteria, archaea, and thylakoid membranes of plant chloroplasts (van Dijl *et al.*, 2002; Yen *et al.*, 2002b). TatC, with six putative TMSs, may serve as a specificity determining receptor for the complex (Jongbloed *et al.*, 2000). Substrate proteins bind to the receptor complex, inducing formation of the protein-translocating channel (Berks *et al.*, 2003, 2005; Dabney-Smith *et al.*, 2006;

Gerard and Cline, 2006). An organism may encode within its genome 1, 2, or 3 TatA homologues, and 1, 2, or 3 TatC homologues, but no organism with a completely sequenced genome examined for these proteins encodes one but not the other (Yen *et al.*, 2002b). Homologues are not demonstrable in yeast, in animals, or in many prokaryotes, particularly those with small genomes and a fermentative lifestyle. Thus, these systems are not ubiquitous as are GSP (Sec) systems (Cao and Saier, 2003), but they are wide spread. Energy coupling for transport involves the pmf both in chloroplasts and in *E. coli* (Müller, 2005; Theg *et al.*, 2005). A protein:proton antiport mechanism is inferred (Berks *et al.*, 2003, 2005; Theg *et al.*, 2005). Possibly the TatC receptor provides the H^+ channel function, TatB provides interconnecting interfaces between TatC and the TatA complex, and the large multi subunit TatA complex, of variable sizes and numbers of subunits, depending on the size of the protein substrate complex, comprise the protein translocating channel (see Fig. 6.5).

A TatC phylogenetic tree revealed tremendous diversity in the sequences of these proteins (Yen *et al.*, 2002b). All of the low G + C Gram-positive bacterial homologues proved to cluster together as did the high G + C Gram-positive bacterial homologues, and most of the Gram-negative bacterial proteins formed two distinct but adjacent clusters. However, the archaeal homologues were found in multiple clusters while the plant proteins localized to two clusters. It is possible that a few gene duplication events that occurred early during the evolution of Tat family constituents were responsible for these unexpected phylogenetic characteristics (van Dijl *et al.*, 2002; Yen *et al.*, 2002b).

IV. OMP TRANSLOCASES OF GRAM-NEGATIVE BACTERIA

A. The MTB (Fig. 6.6)

The MTB consists of a complex, spanning the two membranes of the Gram-negative bacterial envelope, of 12–15 different proteins that somehow function in the energized transport of folded exoproteins from the periplasm across the outer membrane to the external milieu (Peabody *et al.*, 2003). One of the proteins of the *Klebsiella* MTB complex, the PulD secretin (TC #1.B.22), is homologous to one of the constituents of the IIISP system (TC #3.A.6; Fig. 6.6; Peabody *et al.*, 2003). PulD and its homologues form dodecameric ring structures with large, central, gated pores (internal diameters of 50–100 Å; Chami *et al.*, 2005; Collins *et al.*, 2001; Schmidt *et al.*, 2001). Another constituent of the MTB (the PulE ATPase) is homologous to an ATPase (VirB11) of the IVSP complex (TC #3.A.7). Otherwise, homology of the distinct protein constituents of these two translocases has not been established. Their constituents share few structural and functional features and probably evolved independently of each other.

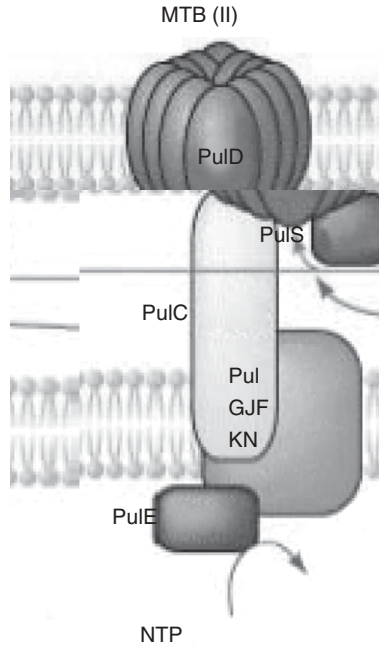

FIGURE 6.6 MTB, a type II outer membrane SP in *Klebsiella* species. PulE is an ATPase; PulD comprises the outer membrane dodecameric secretin complex with PulS serving as a lipoprotein anchor. PulC spans the periplasm and interacts with the inner membrane complex, the outer membrane secretin, and the substrate protein as it passes from the periplasm to its external destination.

However, they sometimes use common constituents as noted above, and parallels with type IV protein secretion systems have been noted (Filloux, 2004).

The other constituents of the MTB are either integral constituents of the inner membrane (PulC, F, G, H, I, J, K, L, M, N, and O), a peripheral constituent of the inner membrane (PulE) or in one case, a peripheral outer membrane lipoprotein which probably functions as a secretin-specific chaperone/anchor protein (PulS; Nouwen *et al.*, 1999). One of the inner membrane proteins (PulO) is a peptidase/*N*-methyl transferase that processes the pilin-like precursors of PulG, H, I, J, and K. The PulE ATP-binding ATPase/kinase exhibits an essential zinc-finger motif. Another protein, PulL, is required for PulE to associate with the membrane. These proteins probably form a trans-periplasmic complex called a ''secreton'' that (1) recognizes the substrate proteins in the periplasm, (2) energizes transport across the outer membrane, and (3) controls opening of the PulD secretin pore. Retraction and extension of the periplasmic pseudopilus, consisting in part of the PulG protein, may energize

transport (Burrows, 2005; Vignon *et al.*, 2003). Other proteins may be involved in secreton assembly. Substrate proteins fold in the periplasm prior to transport across the outer membrane. The secretion signal may be contained in the tertiary conformation of the native protein, or multiple signals may be present (Francetic and Pugsley, 2005).

One of the *V. cholerae* integral MTB membrane constituents, EpsC (homologous to PulC of *K. pneumoniae*), has been examined both structurally and functionally (Korotkov *et al.*, 2006). While anchored in the inner membrane, the largest part of EpsC is periplasmic, containing a so-called homology region (HR) domain and a PDZ domain. The PDZ domain probably interacts with exoproteins to be secreted, while the HR domain plays a key role in linking the inner-membrane sub-complex of the MTB to the outer membrane secretin (Korotkov *et al.*, 2006). Thus, it is possible to visualize the mulitcomponent complex where EpsC (PulC) plays an important bridging function between the inner and outer membrane (Fig. 6.6).

B. FUP systems

Fimbriae of Gram-negative and Gram-positive bacteria fall into several distinct classes, and mutiple mechanisms for their assembly have been identified (Anantha *et al.*, 2004; Zavialov *et al.*, 2007). Among these are the FUP systems (TC#1.B.ll), which are responsible for the biogenesis of numerous fimbriae (pili) in Gram-negative bacteria, cyanobacteria, and *Deinococcus radiodurans*. *D. radiodurans* is designated a Gram-positive bacterium, but it has an unusual dual membrane envelope (Yen *et al.*, 2002a). Frimbriae often function as adhesins that recognize specific macromolecules, usually glycoproteins and glycolipids (Zavialov *et al.*, 2007).

The operon encoding the structural proteins of each fimbrium also encodes a fimbrium-specific periplasmic chaperone protein and a fimbrium-specific outer membrane usher protein. The latter constituents fall into one of six phylogenetic classes (Nuccio and Bäumler, 2007). However, the chaperone and usher proteins, in general, appear to have evolved in parallel from their evolutionary precursor proteins (Van Rosmalen and Saier, 1993).

The usher proteins contain a large central domain that spans the outer membrane 24 times as β-strands, presumbaly forming a β-barrel structure (Mol and Oudega, 1996). Following translocation across the inner membrane by the Sec system, the pilus subunits are bound to the chaperone protein, preventing the self-assembly of pili in the periplasm. Interactions between the chaperone and the periplasmic domains of the usher protein release the pilus subunits, which are subsequently exported through the usher protein across the outer membrane as a prelude to pilus assembly on the outer surface of the outer membrane (Sauer *et al.*, 2000; Thanassi and Hultgren, 2000). The mechanism by which the assembled fimbrial

structures are exported through the usher proteins is not well understood. However, assembly involves the external surfaces of the usher proteins. Thus, these proteins are multifunctional, serving as chaperones, porins, and assembly apparatuses.

C. Autotransporter-1 (AT-1) systems

Autotransporters of the AT-1 (or AT) family (1.B.12) each consists of a single protein with an N-terminal Sec-type signal peptide, a central passenger domain, and a C-terminal β-domain of 250–300 amino acyl residues (Dautin and Bernstein, 2007). Although the β-domains of different autotransporters of the AT-1 family are homologous, they are extremely diverse in sequence. Moreover, the passenger domains, which determine the functions of the exoproteins, vary tremendously in sequence and size (Loveless and Saier, 1997). They can serve as adhesins, toxins, proteases, heme receptors, and other virulence factors. These proteins are found primarily in proteobacteria, but the chlamydial kingdom and a few other Gram-negative bacterial kingdoms also have recognizable AT-1-type autotransporters (Yen *et al.*, 2002a). Following secretion across the inner membrane via the Sec system, and cleavage of the signal peptide, multiple β-domains form oligomeric ring-shaped complexes of ~500 kDa in the outer membrane, thought to allow passage of the folded N-terminal domains through the channel (Veiga *et al.*, 2002). The passenger domains, most of which are virulence factors in Gram-negative pathogenic bacteria, are either released to the environment or remain attached to the cell surface. There is still substantial controversy about the outer membrane transport mechanism (Dautin and Bernstein, 2007).

D. Autotransporter-2 (AT-2) systems

A novel type of autotransporter (TC # 1.B.40) has been identified in proteobacteria and a few more distantly related Gram-negative bacteria. The prototype is the adhesin protein YadA of *Yersinia enterocolitica* (El Tahir and Skurnik, 2001; Grosskinsky *et al.*, 2007; Roggenkamp *et al.*, 2003). Rather than having 14 or 15 amphipathic β-strands as is true for the better characterized AT-1 autotransporters described above, YadA contains a C-terminal putative transport domain of only about 70 residues consisting of four amphipathic β-strands. This ''transporter'' domain is joined to the N-terminal passenger domain by a coiled-coil linker (Cotter *et al.*, 2005). This linker is essential for stability and translocation of the passenger domain through the outer membrane.

The structures of the transporter domains and the nature of the passenger domains are not yet fully defined. However, the available evidence suggests that the passenger domains, the linker, and possibly the AT-2

domains, arose by intragenic duplication of segments of defined size, creating multiple repeat units in the proteins. The repeat units vary in size from 7 to about 50 amino acyl residues (Kim *et al.*, in press). A single AT-2 protein may have multiple copies of as many as three different types of repeat units, where the largest repeats are near the N-termini of the passenger domain and the smallest is near the C-termini of this domain, overlapping the linker region connecting the passenger domain and the AT-2 domain (Kim *et al.*, in press). In spite of their sequence diversity, most of these proteins may serve as adhesins (Scarselli *et al.*, 2006; Tang *et al.*, 2007). Secondary functions include host cell invasion, immunoglobulin binding, serum resistance, and hemeagglutination.They are found in a large range of Gram-negative bacteria and their phage. As for AT-1 family members, AT-2 proteins are exported to the outer bacterial surface by a still controversial mechanism.

E. The Intimin/Invasin or Autotransporter-3 Systems

The Intimin/Invasin (Int/Inv) or Autotransporter-3 (AT-3) family (TC # 1.B.54) of adhesins are outer membrane (OM) proteins found in strains of *Yersinia* spp. (Inv), pathogenic *E. coli* (Int), and other proteobacteria. These homologous proteins mediate attachment to and invasion into their host cells. Intimins/Invasins are translocated from the cytoplasm across the IM via the Sec-translocase (TC # 3.A.5), and are related to each other both in terms of sequence and structure (Gal-Mor *et al.*, 2008; Adams *et al.*, 2004; Wentzel *et al.*, 2001).

Both intimins and invasins expose structurally similar domains on the bacterial cell surface resembling an extended rigid rod made of domains similar to eukaryotic members of the immunoglobulin superfamily. The carboxy-termini have a folding topology related to C-type lectin-like receptor-binding domains, which are separated from a membrane-embedded N-terminal domain by several tandem Ig-like repeats, four in invasins and three in intimins. With >36% identity existing within the first 500 amino acids of most of these proteins, the conserved N-terminal domains are believed to form porin-like beta-barrels in the OM. These domains are probably used to export the C-terminal passenger domains across the outer bacterial cell membrane (Adams *et al.*, 2004; Batchelor *et al.*, 2000; Gal-Mor *et al.*, 2008).

The extracellular C-terminus of an Int/Inv is responsible for receptor binding to Tir (translocated intimin receptor) and b1 integrin, respectively. Intimins are surface proteins of enteropathogenic and enterohemorrhagic *E. coli* that promote intimate bacterial adhesion associated with attaching and effacing lesion formation (Adams *et al.*, 2004; Gal-Mor *et al.*, 2008). The Tir binding sites of intimins are located at the opposite side of the C-terminal lectin-like domain. Invasins lack the short alpha-helix

(residues 904–909 of intimin) involved in Tir binding (Luo *et al.*, 2000). Invasins bind to high-affinity members of the beta1 family of integrins to mediate bacterial entry into eukaryotic cells (Adams *et al.*, 2004).

Intimin-mediated adhesion of bacterial cells to eukaryotic target cells can be mimicked by surface display of a short fibrinogen receptor binding peptide. Intimate bacterial adhesion associated with attaching and effacing lesion formation is promoted by intimin. Intimin targets the translocated intimin receptor (Tir) which is exported by the bacterium and integrated into the host cell membrane. Tir is introduced into the host cell membrane via a type III protein secretion/translocation system. For both Intimins and Invasins, a C-terminal fragment of ~190aa is sufficient for function, although no significant sequence similarity is observed. At least five different subtypes of intimins have been described. They are integrated into the *E. coli* OM by their amino-terminal regions, while the C-terminal 280 amino acids are surface exposed (Batchelor *et al.*, 2000; Wentzel *et al.*, 2001).

The EaeA intimin from *E. coli* strain EHEC O157:H7 is 939 amino acyl residues long. It contains an N-terminal transporter domain, which resides in the bacterial cell OM and promotes translocation of the four C-terminally attached passenger domains. The cell binding activity of EaeA has been localized to its C-terminal 280 residues. It is assumed that the N-terminal 550 residues of intimins form porin-like structures and are folded into antiparallel beta-barrels. The entire extracellular segment forms an elongated and relatively rigid rod made up of three immunoglobulin-like domains and a C-terminal lectin-like domain which interacts with the receptors. This domain resides on a rigid extracellular arm, which is most likely anchored to the amino-terminal transmembrane domain through a flexible hinge that includes two noncontiguous conserved glycine residues. They allow mechanical movement between the extracellular rod and the bacterial OM (Wentzel *et al.*, 2001; Luo *et al.*, 2000). Intimins form ring shaped structures with a diameter of 7nm and a channel which can accommodate peptide chains, but not fully folded passenger domains (Adams *et al.*, 2004).

The AT-3 (Int/Inv) family is just one of many distinct secretion mechanisms used by pathogenic gram-negative bacteria for the efficient surface display of binding domains which specifically interact with their complementary receptors on host cell surfaces (Saier, 2007; Wentzel *et al.*, 2001). While not much is known about the secretion mechanisms of Int/Inv, the passenger domains may be secreted by an autotransporter-like mechanism (Gal-Mor *et al.*, 2008).

F. Two-partner secretion (TPS) systems

Each TPS system (TC # 1.B.20) is composed of a substrate protein and a transport protein that are usually encoded by two neighboring genes (Jacob-Dubuisson *et al.*, 2001). Although TPS homologues have not been

identified in archaea, they have been found in bacteria and the animal, plant, and fungal kingdoms of eukaryotes (Yen *et al.*, 2002a). Most protein substrates of bacterial TPS systems are large proteins with adhesive activities that are related to bacterial virulence (Buscher *et al.*, 2006; Thanassi *et al.*, 2005). They can additionally serve as toxins, hemeaglutinins, and heme and hemoglobins receptors (Aoki *et al.*, 2005; Choi *et al.*, 2007). The transport protein consists of a β-domain with 19 predicted amphipathic β-strands that presumably form a β-barrel channel in the outer membrane (Jacob-Dubuisson *et al.*, 1999; Könninger *et al.*, 1999). Both proteins are secreted to the periplasm by the Sec system, and the passenger domain (TpsA) is further exported across the outer membrane by the transport constituent (TpsB) of the binary system (Newman and Stathopoulos, 2004). The mechanistic details of protein secretion by the outer membrane transporter are still unclear. The substrate protein contains a conserved N-terminal domain of approximately 115-amino acyl residues that is specifically recognized by its cognate transport protein (Jacob-Dubuisson *et al.*, 1997).

The first member of the TPS family to be characterized was the ShlB (HlyB) protein of *Serratia marcescens*, which exports the ShlA hemolysin from the periplasm of the Gram-negative bacterial envelope into the external medium (Poole *et al.*, 1988). ShlA reaches the periplasm by export from the cytoplasm via the GSP or Sec pathway. ShlB and some, but not all TPS homologues, include domains with both an outer membrane export channel and a ''hemolysin activator protein'' which activates ShlA by derivatization with phosphatidyl ethanolamine (Hertle *et al.*, 1997). Several ShlB homologues have been functionally characterized (Hirono *et al*, 1997; Jacob-Dubuisson *et al.*, 1997, 2000; Palmer and Munson, 1995). The channel activities of some of these homologues have been demonstrated (Jacob-Dubuisson *et al.*, 1999), and topological features of these putative β-barrel porins have been studied (Guédin *et al.*, 2000). Specificity for particular protein substrates has been demonstrated (Jacob-Dubuisson *et al.*, 1997). One such protein, FhaC of *B. pertussis*, exhibits a surface exposed N-terminus and an odd number of β-strands with large surface loops and small periplasmic loops (Guédin *et al.*, 2000; Kànninger *et al.*, 1999; Méli *et al.*, 2006).

Substrates of TPS family secretins include Ca^{+}-independent cytolysins, an iron acquisition protein and several adhesins. The hallmarks of TPS systems are the presence of (1) an N-proximal module where specific secretion signals in the substrate (TpsA) protein are found, and (2) a β-barrel channel (TpsB) homologue (Jacob-Dubuisson *et al.*, 2000). Usually, the genes encoding these two proteins occur within an operon. While transport via the GSP occurs in the unfolded state, the substrate protein may fold in the periplasm and on the periplasmic surface of the outer membrane before it is exported via the TPS porin (Jacob-Dubuisson *et al.*, 2000).

FhaC of *B. pertussis*, the TpsB protein that transports the TpsA partner, FHA, exhibits a surface-exposed N-terminus and 19 putative β-strands with large surface loops and small periplasmic turns as is typical of these proteins (Hodak *et al.*, 2006; Méli *et al.*, 2006). Surface exposed residues and pore formation in artificial membranes have been characterized with both FhaC and the ShlB outer membrane porins. Méli *et al.* (2006) reported that FhaC exhibits ion channel properties, and FhaC mutants altered for FHA transport exhibited altered ion channel activities. The N-terminal 200 residues probably form a functionally distinct domain that modulates the pore properties and may participate in FHA recognition. The C-terminal two-thirds of TpsB forms the transmembrane channel-forming β-barrel domain. A C-proximal motif (the family signature sequence) appears to be essential for pore formation (Méli *et al.*, 2006).

FhaC recognizes only nonnative conformations of the TPS domain, and *in vivo*, periplasmic FHA is not folded. Interaction determinants forming the secretion signal have been identified (Hodak *et al.*, 2006). They are found far into the TPS domain and include both conserved and variable residues, which most likely explain the specificity of the TpsA–TpsB interaction. The N-terminal domain of FhaC is involved in the FHA–FhaC interaction, in agreement with its proposed function and periplasmic localization.

Proteins showing large regions of sequence similarity to established members of the TPS family have been identified in Gram-positive bacteria, yeast, plants, and animals. They clearly share homologous domains. In Gram-negative bacteria, the two-partner secretion (TPS) pathway is dedicated to the secretion of large, mostly virulence-related proteins. The secreted TpsA proteins carry a characteristic 250-residue-long N-terminal ''TPS domain'' essential for secretion, while their TpsB transporters specifically recognize their respective TpsA partners and mediate their translocation across the outer membrane.

In Gram-negative bacteria, most surface-associated proteins are present as integral outer-membrane proteins. Exceptions include the *Haemophilus influenzae* HMW1 and HMW2 adhesins and a subset of other proteins secreted by the TPS system. HMW1 forms hair-like fibres on the bacterial surface and is usually present as pairs that appear to be joined together at one end. HMW1 is anchored to the multimeric HMW1B outer membrane translocator, resulting in a direct correlation between the level of surface-associated HMW1 and the quantity of HMW1B in the outer membrane. Anchoring of HMW1 requires the C-terminal 20 amino acids of the protein and is dependent on disulphide bond formation between two conserved cysteine residues in this region (Buscher *et al.*, 2006). The immediate C-terminus of HMW1 is inaccessible to surface labeling, suggesting that it remains buried in HMW1B. These observations may have broad relevance to many proteins secreted by the TPS

system, especially given the conservation of C-terminal cysteine residues among surface-associated proteins in this family.

G. OMP insertion porins (OmpIP)

Gram-negative bacterial OMPs are assembled from the periplasm into the outer membrane in a process that is poorly understood. Large (~800 aas) OMPs, complexed with several others, appear to play crucial roles. Omp85 of *Neisseria* species (TC #1.B.33.1.1) and the D15 antigen of *Haemophilus* species (1.B.33.1.2) were the first to be characterized. These proteins are distantly related to the TpsB proteins discussed in the preceding section. These large constituents are also distantly related to the chloroplast import-associated β-barrel channel protein, IAP75 (TC #1.B.33.2.1), a constituent of the chloroplast envelope protein translocase (CEPT or Tic-Toc) family (TC #3.A.9). IAP75 has been shown to be a β-barrel porin in the outer membrane of plant chloroplasts (Ertel *et al.*, 2005; Gentle *et al.*, 2005).

Another homologue is the yeast mitochondrial sorting and assembly machinery (SAM) constituent, SAM50 (Kozjak *et al.*, 2003). The SAM complex in yeast mitochondria consists of at least three proteins (Kozjak *et al.*, 2003; Milenkovic *et al.*, 2004; Wiedemann *et al.*, 2003) and is required for the assembly of outer membrane β-barrel proteins in mitochondria.

The functionally characterized homologue in *Neisseria meningitidis*, Omp85, is essential for bacterial viability (Gentle *et al.*, 2004). It has a two-domain structure with an N-terminal periplasmic domain rich in POTRA repeat sequences and a C-terminal domain that forms an integral outer membrane β-barrel (Gentle *et al.*, 2005). Unassembled forms of various OMPs accumulate when Omp85 is depleted (Voulhoux *et al.*, 2003). Moreover, immunofluorescence microscopy showed decreased surface exposure of OMPs, particularly at the cell division planes. Homologues of Omp85 are present in all Gram-negative bacteria examined (Voulhoux *et al.*, 2003).

Generally in Gram-negative bacteria, LPS or its precursors and phospholipids (PLs) destined for the outer membrane are made in the inner membrane. Genevrois *et al.* (2003) have reported that the Omp85 structural gene in *Neisseria* is cotranscribed with genes involved in lipid biosynthesis. Depletion of Omp85 results in accumulation of LPS and PL in the inner membrane and loss from the outer membrane. The effects on lipids were reported to precede the effects on OMP (PorA and Opa) insertion, suggesting that the latter effects were secondary to the effects on LPS and PL translocation (Genevrois *et al.*, 2003). However, Doerrler and Raetz (2005) came to the opposite conclusion when studying the effects of mutations in the homologous *yaeT* gene of *E. coli*. YaeT is the *E. coli* Omp85 orthologue. These investigators and others concluded that

YaeT functions as a principal constituent of a complex that catalyzes protein insertion into the outer membrane (Gentle *et al.*, 2004). A different protein, OstA or Imp (784 aas in *E. coli*; TC #1.B.42) may mediate LPS export (Bos *et al.*, 2004; Hu and Saier, 2006).

Normally OMPs are translocated into the periplasm via the Sec translocase (TC #3.A.5). They are believed to fold in the periplasm before being inserted into the outer membrane. Folding is stimulated by the small periplasmic chaperone protein SurA (P21202), and by a peptidyl prolyl *cis-trans* isomerase (PPIase) called Rotamase C or parvulin (P0A9L5). Two other periplasmic/OMPs, Skp (OmpH; HlpA; P11457) and another PPIase, FkpA (P45523) also function in this capacity (Missiakas *et al.*, 1996). Still other proteins may be involved. It is even possible that an energy source will prove to be required.

In *E. coli*, a multiprotein complex has been shown to be required for outer membrane biogenesis (Wu *et al.*, 2005). This complex includes YaeT, a lipoprotein, YfgL, and atleast two other proteins, YfiO and NlpB. It is believed that the activities of these proteins provide the primary function for OMP assembly. The specific biochemical roles of the individual protein constituents have recently been elucidated (Kim *et al.*, 2007; Malinverni *et al.*, 2006; Sklar *et al.*, 2007). YaeT of *E. coli* has one or more polypeptide transport-associated (POTRA) domains (Robert *et al.*, 2006). The crystal structure of a periplasmic fragment of YaeT (Kim *et al.*, 2007) revealed the POTRA domain fold and suggested a model for how POTRA domains can bind different peptide sequences, as required for a machine that handles numerous β-barrel protein precursors (Bos *et al.*, 2007). Analysis of POTRA domain deletions showed which are essential and provided a view of the spatial organization of this assembly machine (Kim *et al.*, 2007). The pore-forming regions of YaeT proteins are restricted to the C-termini (Stegmeier and Andersen, 2006). Based on phylogenetic analyses, the pore-forming domains display different evolutionary relationships than the N-terminal domains. The N-terminal domains are involved in gating of the pore, recognize the substrate proteins, participate in complex formation, and take part in homo-oligomerization. The differences in the phylogenies of the two domains are explained by different functional constraints acting on the two regions. The pore-forming domain is divided into two functional regions where the distal C terminus forms a dimeric pore. Based on functional and phylogenetic analyses, an evolutionary scenario that explains the origin of the contemporary translocon has been proposed (Bredemeier *et al.*, 2007).

YaeT of *E. coli* is essential for outer membrane biogenesis. However, a homologue to YaeT is present in *E. coli* denoted YtfM (Stegmeier *et al.*, 2007). Like YaeT, YtfM is predicted to consist of an amino-terminal periplasmic domain and a membrane-located carboxy-terminal domain. Nevertheless, YtfM is nonessential, and lack of the protein has no effect on

outer membrane composition and integrity. The only observable phenotype was reduced growth (Stegmeier *et al.*, 2007). Its molecular function has yet to be defined.

V. PROTEIN TRANSLOCASES OF EUKARYOTIC ORGANELLES

In discussing the bacterial protein secretory complexes and integral membrane insertases, referred to in preceding sections, brief reference was made to homologues in eukaryotic organelles. These bacterial proteins included the YidC inner membrane insertase and the Omp85/YaeT outer membrane component among others (Chan and Lithgow, 2008). In chloroplasts, the Tat and Sec systems are also functional (Dalbey and Kuhn, 2000; Müller and Klösgen, 2005; Robinson and Bolhuis, 2004; Steiner and Löffelhardt, 2005). Moreover, many protein constituents of the chloroplast-specific translocases have homologues in cyanobacteria. It seems clear that the chloroplast-bacterial connection is much tighter than the mitochondrial-bacterial connection, reflecting their relative dates of divergence.

The protein translocating systems that have evolved in eukaryotic organelles are usually more complex than those in prokaryotes with several novel accessory proteins providing peripheral functions. Moreover, additional systems have apparently evolved to provide novel functions (Dolezal *et al.*, 2006). Examination of the complexities and interrelated features of these organellar systems provides a more comprehensive view of complex molecular organization than would be possible if these systems were examined only in the more streamlined prokaryotes (Kutik *et al.*, 2007).

A. MPT complexes (TIM-TOM; TC #3.A.8; Fig. 6.7)

The MPT, which brings nuclearly encoded preproteins into mitochondria, is very complex with numerous identified protein constituents that comprise at least five translocons, two in the outer membrane: Tom (Tom = outer membrane translocase; Tom40/70/22/20) and Sam (Sam50/Sam35/Mas37), and three in the inner membrane, Tim23 (Tim23/17/44/50/Hsp70/Pam16/Pam18/Mge1; PAM = presequence-associated import motor); Tim22 (Tim22/54/18), and Oxa1p (TC #2.A.9; Neupert and Herrmann, 2007; Wiedemann *et al.*, 2004; see Figs. 6.7 and 6.8). These systems function in conjunction with several chaperone proteins (Rehling *et al.*, 2004).

The Tom consists of import receptors and the proteins of the Tom channel complex. Integral outer membrane receptor proteins are called Tom70, Tom22, and Tom20. Of these receptor proteins, only Tom22 is

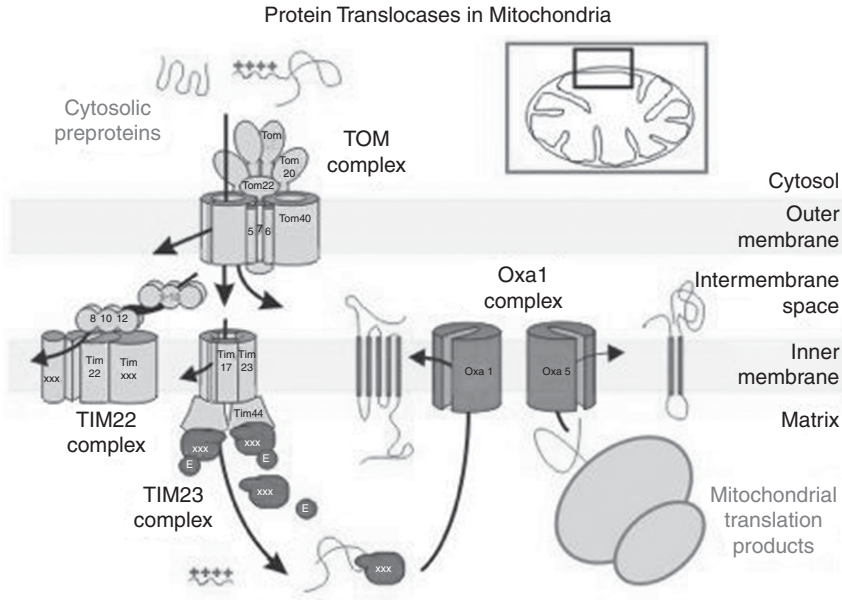

FIGURE 6.7 Protein translocases in mitochondria. Nuclear-encoded proteins in the cytoplasm dock to the translocator in the outer membrane (TOM) complex receptor before being translocated through that membrane. Intermembrane chaperones facilitate transfer to the translocators in the inner membrane, the TIM22 and TIM23 complexes, each specific for a different subset of substrate proteins. These two complexes transport proteins across or allow integration into the inner mitochondrial membrane. Transfer to the matrix is facilitated by chaperone proteins. The Oxa1 complex integrates a subset of the latter proteins as well as some mitochondrially-encoded proteins (mitochondrial translation products) into the inner membrane. The sorting and assembly machinery (SAM), for integration of outer membrane β-barrel proteins, is not shown (see Fig. 6.8).

essential for protein import. The receptor complex delivers the substrate proteins to the outer membrane channel consisting of five hydrophobic proteins, Tom40, Tom38, Tom7, Tom6, and Tom5. Tom40 is the core oligomeric subunit of this channel. It forms a β-stranded, cation-selective, high conductance pore that specifically binds to, and transports mitochondrial-targeting peptides. The inner pore diameter has been estimated to be about 22 Å. The small Tom proteins may function in regulatory or chaperone capacities and are not essential (Rehling *et al.*, 2003, 2004). The membrane-embedded C-terminal segment of TOM40 constitutes the pore. It also serves as a receptor, recognizing the preprotein substrate (Neupert and Herrmann, 2007; Suzuki *et al.*, 2004).

The TOM complex can apparently function independently of the two primary TIM complexes. It transports proteins from the cytoplasm to the intermembrane space (Herrmann, 2003). Conserved, intraprotein,

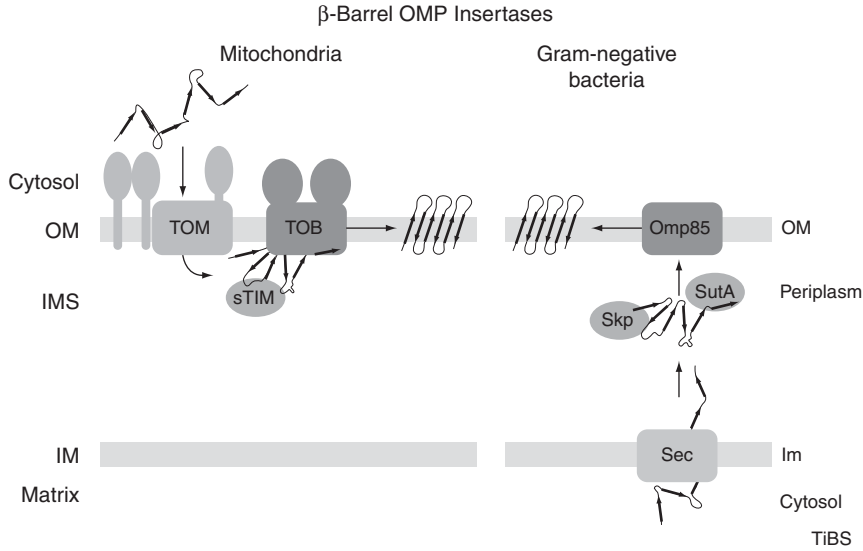

FIGURE 6.8 Comparison between β-barrel insertion of outer membrane proteins (OMPs) by the mitochondrial SAM (TOB; left) and the Gram-negative bacterial OmpIP system (right). The primary channel-forming proteins in both systems (Tob55 in yeast mitochondria; Omp85 in *Neisseria* species) are demonstrably homologous. IM, inner membrane; IMS, intermembrane spore; OM, outer membrane. Skp and SurA are periplasmic chaperone proteins. See text for further clarification.

hydrophilic targeting sequences, different from matrix targeting sequences, are involved. Targeting translocation across the outer membrane may be independent of ATP and the pmf, but subsequent transport steps require ATP.

About 30% of mitochondrial proteins lack matrix targeting sequences. They can be present in the outer membrane, the intermembrane space, and the inner membrane. These proteins may be imported initially via the TOM complex, but their transport across or into the inner membrane is achieved by two independent systems, each with a different subset of targeted proteins. The Tom complex distinguishes between β-barrel proteins and other types of preproteins, thus playing an active role in the transfer of preproteins to subsequent translocases for insertion into the correct mitochondrial subcompartment (Sherman *et al.*, 2006).

Mia40p and Erv1p are probably chaperone-like redox components of a translocation pathway for the import of cysteine-rich proteins into the mitochondrial intermembrane space. Oxidized Mia40p contains three intramolecular disulfide bonds. One disulfide bond connects the first two cysteine residues in a CPC-motif. The second and the third bonds belong to the twin Cx_9C-motif and bridge the cysteine residues of two

Cx_9C-segments. In contrast to the stabilizing disulfide bonds of the twin Cx_9C-motifs, the first disulfide bond is easily accessible to reducing agents. Partially reduced Mia40C, generated by opening of this bond, as well as fully reduced Mia40C, is oxidized by Erv1p *in vitro*, and mixed disulfides of Mia40C and Erv1p can be formed (Grumbt *et al.*, 2007). Thus, these proteins probably facilitate disulfide bond formation in substrate proteins.

The two inner membrane channel-forming complexes are both multicomponent. One, the Tim23 complex, consists of at least two integral membrane Tim proteins, Tim23 and Tim17, as well as peripheral membrane motor proteins, Tim44, Pam16, and Pam18. In this complex, other motor components (Tim14 and Mge1) are required (Mokranjac *et al.*, 2003a,b; Rehling *et al.*, 2004; Truscott *et al.*, 2003). Tim23, possibly together with Tim17, exhibits channel activity. Two ATP-dependent matrix chaperone proteins (mHsp70 and mGrpE) have been suggested to function together with Tim44 to drive active uptake. Tim44 (with or without the chaperone proteins) may function as an ATP-driven import motor that pulls the precursor polypeptide chain through the Tim23 channel into the matrix. The Tim23 complex imports presequence-containing matrix proteins as well as presequence-containing inner membrane monotonic (1TMS) proteins. The latter can laterally diffuse out of the channel into the lipid bilayer (Herrmann, 2003).

The second inner membrane channel-forming complex consists of at least three integral membrane proteins, Tim22, Tim54, and Tim18. The Tim22 complex has been purified and shown to insert inner membrane proteins by a twin-pore translocase that uses the pmf as the exclusive energy source and a half-of-sites mechanism (Rehling *et al.*, 2003, 2004). The polytopic substrate proteins of the Tim22 complex (including Tim22 and Tim23) lack typical presequences and instead have internal targeting signals. Tim22 is a dynamic, ligand-gated, high conductance, slightly cation-selective channel, solely active in the presence of its cargo protein (Peixoto *et al.*, 2007).

The Tom and Tim proteins have homologues in yeast, other fungi, animals, plants, and various unicellular eukaryotes. Some constituents are homologous between these eukaryotes, but others are nonhomologous (Lister *et al.*, 2007). Moreover, the Tim23, Tim17, and Tim22 proteins within a single organism are homologous to each other.

Some of the inner membrane proteins are translocated into the matrix via the Tim23 complex and then, following cleavage of its first signal sequence, they are targeted to the Oxa1 complex (TC #2.A.9). Moreover, most membrane proteins that are translated within the mitochondrial matrix are directly inserted into the inner membrane by the Oxa1 complex. Another group of inner membrane proteins is directly targeted to the inner membrane in a pathway requiring intermembrane protein

complexes as well as the integral inner membrane Tim22 complex. While the proteins that use the Tim22 complex are generally of eukaryotic origin (e.g., MC family members (TC #2.A.29)), those that use the Oxa1 complex are usually of bacterial origin (Chacinska *et al.*, 2002; Herrmann, 2003).

Most MCs (2.A.29) generally lack N-terminal targeting sequences, and those that have them do not require them for proper insertion. Instead, they become partially organized into partially assembled forms within the translocon. They present to the translocon noncontiguous targeting signals scattered throughout the protein sequence (de Marcos-Lousa *et al.*, 2006). A detailed multistep process has been proposed by de Marcos-Lousa *et al.* (2006).

While the Tim23 import system depends on a single water-soluble intermembrane protein, Tim50 (*Geissler et al.*, 2002; Yamamoto *et al.*, 2002), the Tim22 import system depends on two distinct water-soluble protein complexes that are present in the intermembrane space. One contains the essential Tim9 and Tim10 proteins and mediates transport of cytosolically synthesized integral inner membrane proteins including the metabolite carrier proteins from the outer to the inner membrane. The other complex includes the Tim8 and Tim13 proteins and mediates import of a different subset of integral inner membrane proteins. These two intermembrane complexes deliver substrate proteins to the Tim22 complex. Thus, multiple pathways are proposed for transfer of proteins across the intermembrane space, for transfer across the inner membrane, and for insertion into the inner membrane (Fig. 6.7).

B. The CEPT complex (Tic-Toc; TC #3.A.9; Fig. 6.9)

The vast majority of chloroplast proteins are encoded within the nucleus of the plant or algal cell. These proteins are made as preproteins on cytoplasmic ribosomes and are then targeted to the appropriate organellar subcompartment. Many of the proteins that comprise the translocation apparatus in the envelope and thylakoid membranes of the chloroplast have been identified. A single assembly is thought to catalyze translocation across the two-membrane envelope, and the protein components of the envelope that have been identified and sequenced appear to be unique (Schuneman, 2007). By contrast, translocation across the thylakoid membrane may occur via three or four pathways. One of these pathways involves ATP hydrolysis and proteins of the Type II GSP (IISP; TC #3.A.5; Schleitti and Soll, 2005). The SecY-dependent thylakoid membrane translocase is therefore not part of the translocase included under TC #3.A.9. A second thylakoid membrane translocase is GTP-dependent and includes proteins homologous to eukaryotic and bacterial SRP (TC #3.A.5; Steiner and Löffelhardt, 2005). This system probably uses the SecY system. A third transthylakoid membrane translocation pathway is pmf-dependent but

ATP- and GTP-independent. This is the Tat or VSP (TC #2.A.64) system (Robinson and Bolhuis, 2004).

Preproteins may be recognized at the outer envelope and translocated in a single step across the outer and inner membranes through contact zones where the two membranes are maintained in close apposition. The targeting signal for envelope translocation is in the N-terminal bipartite ''transit sequence.'' The N- and C-terminal portions of this sequence contain chloroplastic and intraorganellar targeting information, respectively. Translocation to the stroma occurs in at least two steps and requires the hydrolysis of both ATP and GTP. Subsequently, the transit sequence is removed in the stromal compartment.

In addition to chaperone proteins, four proteins of the outer chloroplast membrane translocase (TOC) and seven proteins in and associated with the inner chloroplast membrane translocase (TIC) of the chloroplast envelope import apparatus have been identified. The main Toc proteins (Toc(IAP)34/36), Toc(IAP)75 and Toc(IAP)86/159 or 160) are believed to form a recognition and translocation complex in the outer membrane (Fig. 6.9; Becker *et al*., 2004; Tu *et al*., 2004; Wallas *et al*., 2003). IAP34/36 and IAP86/160 are related to each other in sequence and possess cytoplasmically exposed GTP-binding sites. They are receptor GTPases. IAP34/36 and IAP86/160 each represents a single gene product. The ratios of Toc75:Toc34:Toc159 are 3:3:1 (Kikuchi *et al*., 2006). The protein translocons of plastid envelopes in roots and leaves include constituents of both the Tics and the Tocs that are tissue specific (Vojta *et al*., 2004). While the Tic proteins appear to be relatively stable, several Toc subunits seem to have a high rate of turnover (Vojta *et al*., 2004).

A. thaliana has two homologues of Toc159 (Toc120 and Toc132) that may serve as receptors for alternative Toc159-independent import pathways. Toc75 (also called OEP75) forms an outer membrane porin consisting largely of β-structure that may serve as the OMP-conducting channel. It is distantly related to Gram-negative bacterial OMPs of the YaeT family (TC #1.B.33) and the TPS family (TC #1.B.20). Toc75 has one sequenced homologue in the blue green bacterium, *Synechocystis* (22% sequence identity). The *Synechocystis* protein is located in the outer membrane of the cyanobacterium and forms a voltage-gated, high conductance channel with high affinity for polyamines and peptides in reconstituted liposomes. Toc34/36 and Toc86/160 have sequenced homologues in many if not all plants. They also have distant homologues in bacteria and slime molds. Toc75 of the pea chloroplast and alr2269 of the cyanobacterium, *Nostoc* PCC7120, which are homologous throughout their lengths, consist of two domains. The N-terminal domains function in recognition and complex assembly while the C-terminal domains provide the β-barrel pore. The pore is modulated by the N-terminal domain (Ertel *et al*., 2005).

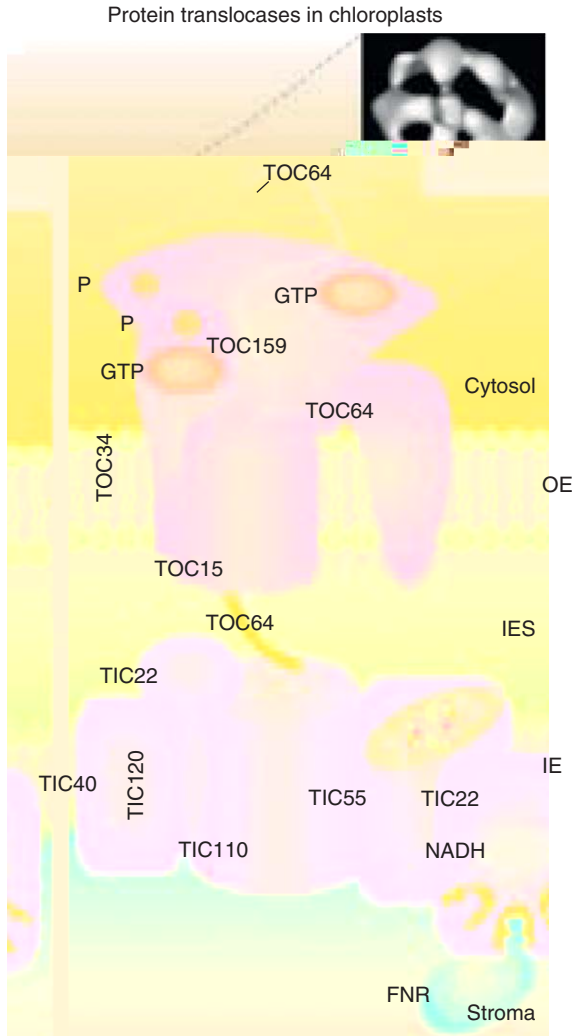

FIGURE 6.9 Schematic view of the translocases in the outer chloroplast membrane (TOC; top) and the inner chloroplast membrane (TIC; bottom). Proteins from the cytoplasm dock to the TOC receptor and are translocated through the TOC75 channel. They may be further translocated via the TIC system across in inner membrane. See text for details.

The Tic proteins include Tic110, Tic44, Tic22, and Tic20. Tic110 (IEP110; IAP100) is a 996 aa protein with one or two probable N-terminal, hydrophilic TMS(s). They form oligomeric channels in the inner membrane. Tic110 is also called PIRAC (protein-import-related anion channel). It is a 50-pS anion channel that becomes inactivated during protein

transport via a mechanism that requires both a functional transit peptide in the transported protein and stromal ATP. It is believed to be the protein import channel in the inner chloroplast membrane (van den Wijngaard and Vredenberg, 1999; van den Wijngaard *et al.*, 1999).

Other Tic components, Tic22, Tic20, and Tic55 may function in preprotein recognition/conduction via the Tic110 channel. Tic22 and Tic20 have homologues in various plants and in *Synechocystis* sp. (286 aas, D64003, and 160 aas, D90906, respectively). The driving force for inner membrane translocation may involve ATP hydrolysis by stromal chaperones, Hsp70, ClpC, and Cpn60. Several other Tic subunits are believed to function in regulation (Schünemann, 2007).

The Tic and Toc complexes in the inner and outer membranes, respectively, may form independently, but they probably form a single supercomplex in the presence of a substrate preprotein by forming contact sites between the two membranes. Some evidence suggests that each complex can function in protein transport independently of the other when contact sites are disrupted. However, even then, cooperative interactions probably coordinate their activities under all conditions (Schleiff and Soll, 2005; Schnemann, 2007).

VI. COMPARISONS AND OVERVIEW

Table 6.3 compares the properties of the four primary SP systems involved in inner membrane secretion in Gram-negative bacteria. The Tat system is tabulated separately for clarity. Each of these systems apparently evolved independently of each other, even though they exhibit overlapping properties and occasionally share one or two homologous protein constituents. Type I systems (ABC plus MFP and OMF proteins) consist of 3–4 protein constituents. Type II systems as defined here include the Sec or Tat system for inner membrane transport plus the MTB or a protein-specific translocase for transport across the outer membrane. Sec systems consist of 7–10 constituents. They transport proteins across or insert proteins into the cytoplasmic Gram-negative or Gram-positive bacterial cell membrane (Cao and Saier, 2003). The *E. coli* Tat system consists of four dissimilar protein constituents, but three of these proteins are homologous and of similar sizes with the same topology. Tat systems in other bacteria can be much simpler with only two nonhomologous constituents (Yen *et al.*, 2002b). Nevertheless, multiple subunits of TatA/B homologues generate the oligomeric pore structure (Mangels *et al.*, 2005; Müller, 2005; Sargent *et al.*, 2001). The MTB includes 10–12 essential constituents for transport across the outer membrane while the substrate-specific outer membrane translocases each usually consists of a single protein. Both type III and type IV systems consist minimally

TABLE 6.3 Comparison of the four principal protein export systems in Gram-negative bacteria[a]

	Characteristics	ISP	IISP	IIISP	IVSP	Tat
1	TC#	3.A.1; 8.A.1; 1.B.17	3.A.5; 3.A.15; 1.B.22[b]	3.A.6; 1.B.22[b]	3.A.7	2.A.64
2	# of constituents	3–4	7–10 (Sec; IM) 10–12 (MTB; OM)	10–12	10–12	2–4
3	Shared constituents	None	One each like type III or IV	One like type II (secretin)	One like type II (ATPase)	None
4	Spans the envelope (one-step transport)	+	–	+	+	–
5	ATPase(s)	ABC-type	SecA (for Sec); GspE[c] (for MTB)	YscQ	VirB4[c]; VirB11[c]	None
6	ATPase homologues of dissimilar function	–	+	+	+	–
7	IM pore	ABC-type	SecYEG	YscRSTU?	VirB4, B6	TatA/B/E
8	Transperiplasmic	OMF (+MFP)	None	YseJ?	VirB8, B9, B10	None
9	OM pore	OMF homotrimer	GspD (secretin)	YscC (secretin)	VirB8, B9, B10 (heterotrimer ?)	None

(*continued*)

TABLE 6.3 (*continued*)

	Characteristics	ISP	IISP	IIISP	IVSP	Tat
10	Distribution	Bacteria	Sec: Ubiquitous MTB: Gram-negative bacteria	Bacteria	Bacteria	Bacteria, archaea, and some eukaryotic organelles
11	Frequent lateral transfer	±	Sec: −; MTB: ±	+ (Path); −(Fla)	+	?
12	Shuffling of constituents	+	–	–	–	?
13	Protein substrate conformation	Unfolded	Unfolded (Sec), folded (MTB)	Partially folded subunits	Folded subunits	Fully assembled enzyme complexes

[a] IM, inner membrane; OM, outer membrane; G–, Gram-negative; G+, Gram-positive. The Tat system (TC #2.A.64), considered as part of the type IISP, is tabulated last for clarity.
[b] Secretins form the outer membrane channels for both MTB (IISP) and Path (IIISP) systems.
[c] GspE components of IISP systems are homologous to VirB4 and VirB11 of IVSP systems (Peabody *et al.*, 2003).

of about 10–12 constituents. Although these two systems share certain functional characteristics, none of the protein constituents is recognizably similar in sequence between the two systems. It is thus clear that all of these systems are distinct, complex, multicomponent systems that probably evolved independently of each other.

The basis for assuming that these systems arose independently in part results from the fact that homology cannot be demonstrated for constituents of one system with those of any other except for the secretins of the MTB, which are distantly related to the secretins of IIISP systems, and the ATPases of the MTB, homologues of which are found in IVSP systems. These ATPases may be involved in assembly of the complex and/or protein export (Table 6.2). While type I, III and IV systems span the two membranes of the Gram-negative bacterial envelope and translocate protein substrates across both membranes in a single energy-coupled step, type II systems, as defined here, actually consist of two distinct, independently-functioning complexes, one (Sec or Tat) for transport across the inner membrane and one (MTB) for transport across the outer membrane. The MTB system is transenvelope presumably to allow energy coupling to transport. There are also several types of protein-specific outer membrane export systems that probably function by energy independent mechanisms. Like Sec, Tat systems transport proteins only across the cytoplasmic membrane (Table 6.2), but unlike Sec, they transport fully folded and assembled enzyme complexes using the pmf instead of ATP as the primary energy source.

Well-characterized protein translocating ABC-type (type I) systems are found primarily in Gram-negative bacteria, although ABC peptide and small protein export systems are also found in Gram-positive bacteria and eukaryotes. While Sec systems are ubiquitous, being found in every living cell in which they have been sought, MTB systems seem to be restricted to Gram-negative bacteria. Homologues of Tat systems are widespread, but they are not found in all living organisms, being absent in some bacteria, some archaea, and in yeast and animals of the eukaryotic domain. However, they are represented in each of the three primary domains of life (Table 6.2). While type III pathogenesis-related systems are present only in Gram-negative bacteria, flagellar type III systems are present in Gram-positive bacteria as well, and similarly, the type IV virulence/conjugation-related systems occur in Gram-positive bacteria as well as Gram-negative bacteria. These last mentioned systems are particularly diverse, both with respect to sequence divergence of homologous constituents and with respect to the numbers and natures of apparently nonhomologous constituents.

Table 6.3 also reveals that while type III (Path) and type IV (Vir) systems have undergone frequent lateral transfer, shuffling of constituents during evolution has only been shown to occur in type I systems, not

in types II, III, and IV systems. In type I systems, OMFs have shuffled relative to the ABC and MFP constituents, but MFPs have not shuffled relative to their cognate ABC transporters (Dinh *et al.*, 1994; Paulsen *et al.*, 1997b). Specific protein–protein interactions, rendering essential the co-evolution of all or most protein constituents of a complex may account for these startling findings.

Finally, the protein species transported by these four primary types of systems are distinguishable in that Sec, ABC, and type III systems transport unfolded or partially folded protein substrates; the MTB and type IV systems transport fully or partially folded protein subunits, and Tat systems transport fully folded and assembled enzyme complexes (Table 6.3). It is therefore clear that the predominant well-characterized protein export systems in a Gram-negative bacterium like *E. coli* or *P. aeruginosa* use different protein constituents, energy sources, and mechanisms to transport their protein substrates which are transported in different states of assembly, depending on the system (Ma *et al*, 2003). The poorly characterized Oxa1/YidC-type systems are believed to be energized by the pmf as are the Tat systems (Luirink *et al.*, 2001; Yen *et al.*, 2001; Yi and Dalbey, 2005). However, the Oxa1-mediated mode of transport and the conformation of the translocated substrates of this system have not been extensively investigated (Luirink *et al.*, 2005).

It is hoped that this treatise allows the reader to conceptualize and discriminate between the various types of protein secretory systems found in nature. It is worth briefly marveling at the remarkable functional diversity that the evolutionary process has yielded, even within a single Gram-negative bacterium. Further, studies of protein translocators in less well studied organisms (e.g., Archaea, Gram-positive bacteria, and lower eukaryotes), as well as certain eukaryotic organelles (e.g., peroxysomes, amyloplasts, and endosomes) will undoubtedly expand our appreciation of structural and functional diversity. What we now see is but the tip of the iceberg.

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CHAPTER 7

Metabolic Behavior of Bacterial Biological Control Agents in Soil and Plant Rhizospheres

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Contents

I. INTRODUCTION

Rhizobacteria are soil-borne bacteria which live on or around the roots of plants in an area referred to as the rhizosphere, a soil zone which extends a few millimeters beyond the roots. As organic materials exude from plant roots, they create a nutrient-rich environment for soil microbes to inhabit and provide energy for their metabolic activity. Thus, the

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rhizosphere provides an important ecological niche for rhizobacteria and is a dynamic setting for complex interactions among and between plant and microbes (Bais *et al.*, 2006). While the interaction between plant hosts and microbes that colonize the rhizosphere can be detrimental, as in the case of soil-borne pathogens, many bacteria are known to be beneficial to their plant hosts. It is these interactions that provide interest and promise for future directions in agriculture. For example, natural antagonists to pathogens can be useful as biological agents for the suppression or prevention of disease in plants. Other examples include, the potential use of rhizobacteria as biofertilizers, phytostimulants, or in bioremediation (Compant *et al.*, 2005; Lugtenberg *et al.*, 2002; van Veen *et al.*, 1997).

The use of biological agents to combat plant disease in lieu of traditional chemical methods has great appeal as we strive to protect our environment. While small scale experiments with biological control agents often show great potential, variability in disease control is often observed in larger-scale tests (Compant *et al.*, 2005; Handelsman and Stabb, 1996). Without the prospect of better consistency in disease-controlling capabilities, the use of biological controls will not become cost-effective and thus will likely not gain widespread use.

A major factor contributing to inconsistencies in biocontrol performances is an ''ecological variability'' (Weller, 1988) that results from a dynamic and heterogeneous environment influencing the metabolic behavior of bacterial populations in soil. Without a better understanding of how ecological factors influence mechanisms of biocontrol, strategies to circumvent biocontrol inconsistencies will not be forthcoming. Considerable effort has been made in defining specific traits expressed by biocontrol agents that contribute to disease suppression. For example, colonization of plant parts is thought be an important trait to almost all biocontrol interactions (Compant *et al.*, 2005; Haas and Défago, 2005; Lugtenberg *et al.*, 2001; Weller, 1988). Other traits include those associated with various mechanisms of biocontrol, which generally fall into four categories: (1) competition for nutrients; (2) production of antibiotics or other inhibitory molecules; (3) predation and parasitism; and (4) induction of plant defense pathways (Compant *et al.*, 2005; Handelsman and Stabb, 1996). As a result of intensive characterization involving a broad range of experimental systems, significant advances have been made that improve our understanding of biocontrol mechanisms at the genetic and molecular levels. However, as represented by only a few cases (Haas and Défago, 2005; Kiely *et al.*, 2006), much less is know about environmental conditions and signals that induce biocontrol activity in rhizobacteria.

In association with our lack of knowledge regarding environmental stimuli that induce expression of biocontrol activity, there has been relatively little effort directed at determining the importance of the metabolic energy status of biocontrol agents, or the impact of environmental

conditions on the metabolic energy status within the context of biocontrol interactions. The metabolically active portion of bacterial populations are considered to be most important in beneficial interactions with plant hosts (Heijnen *et al.*, 1995; Ramos *et al.*, 2000; Unge *et al.*, 1999; van Veen *et al.*, 1997), as metabolic energy is ultimately responsible for expression of traits required for such activities (Crowley *et al.*, 1996; Dowling and O'Gara, 1994; Sørenson *et al.*, 2001). Similar to the variable performances inconsistencies observed with biocontrols, metabolic energy status of bacteria is known to vary greatly under different conditions (Atkinson, 1977; Brennerova and Crowley, 1994; Duncan *et al.*, 1994; Molin and Givskov, 1999). Understanding the impact of environmental conditions on the metabolic behavior, both on the expression of genes important for biological control and on the metabolic energy status of cells, is critical for achieving successful, replicable biological control (Haas and Défago, 2005; Ramos *et al.*, 2000; Sørenson *et al.*, 2001).

II. TECHNIQUES FOR STUDYING THE METABOLIC BEHAVIOR OF BACTERIAL BIOLOGICAL CONTROL AGENTS

Methods for studying gene expression in bacterial biological control agents in association with plants have been reviewed extensively (Haas and Keel, 2003; Kiely *et al.*, 2006; Rediers *et al.*, 2005) and will not be discussed here. A number of methods based on bacterial growth activity have been used to determine the metabolic energy status of cells that are in soil or in association with plants (Molin and Givskov, 1999). These methods include estimation of growth rates based on colony counts from plating (Rovira and Davey, 1974); analysis of cell size and shape (Bloem *et al.*, 1995; Givskov *et al.*, 1994; Møller *et al.*, 1995); estimation of DNA and protein synthesis rates based on incorporation of tritiated thymidine and leucine (Christensen *et al.*, 1992; Sengeløv *et al.*, 2000; Söderberg and Bååth, 1998); and determination of rRNA content of cells (Poulsen *et al.*, 1993). Other methods include determination of respiration rates (Winding *et al.*, 1994), oxygen consumption (Baker *et al.*, 2004), and the adenylate energy charge of cells (Brookes *et al.*, 1983; Contin *et al.*, 2001; De Nobli *et al.*, 2001). Molecular methods are available that determine and differentiate the metabolic energy status of specific, introduced bacterial populations from that of indigenous microflora. These include methods based on fluorescent antibodies and rRNA probes (Amann *et al.*, 1995; Assmus *et al.*, 1995; Christenson and Poulsen, 1994; Hahn *et al.*, 1992, 1997; Ramos *et al.*, 2000). Reporter systems are particularly well suited for assessment of the metabolic energy status of specific microbial strains, as demonstrated by bioluminescence- and green fluorescent protein (gfp)-based systems (Kiely *et al.*, 2006; Molin and Givskov, 1999; Ramos *et al.*, 2000; Unge *et al.*, 1999;

van Elsas and van Overbeek, 1993). In these cases, the reporter can not only indicate metabolic energy status, but also determine the spatial distribution of individual cells responding to different environmental conditions.

III. IMPACT OF SOIL EDAPHIC FACTORS AND INDIGENOUS MICROBES ON INTRODUCED MICROBES IN THE SOIL ENVIRONMENT

Edaphic features of soil are among the many factors known to affect the metabolic activity of its resident microorganisms. Soil consists of clay, silt, sand, and organic matter (humic material) that are clustered in aggregates of varying size and composition. These aggregates result in spatial voids of varying sizes that are filled by gases and aqueous solutions. Such variation in composition, further influenced by constant flux between liquid and gas phases, create microenvironments (Smiles, 1988; van Elsas and van Overbeek, 1993) that can individually have profound effects on localized microbial populations.

Numerous factors, the most obvious being nutrients, are known to influence both metabolism and persistence of soil microbes. Bulk soil lacking influence from plant roots has been described as ''grossly oligotrophic'', with most of the reduced carbon and other organic matter found within it to be in recalcitrant forms (Poindexter, 1981; van Elsas and van Overbeek, 1993). Although reduced carbon is the primary nutrient to have limiting effects on microbial activity in most soils, other components such as nitrogen, phosphorus, and iron have also been reported to be limiting in some soils (Aldén *et al.*, 2001; Haas and Défago, 2005). Availability of these and other essential inorganic nutrients have been shown to have significant impacts on survival and growth capabilities through interactions with other chemical, physical, and biological properties of the soil (reviewed in O'Hara, 2001).

Abiotic soil factors, such as soil texture, pH, temperature, moisture, high osmotic, or matric tension, are known to impose stresses on microorganisms that not only impact their metabolic behavior, but also their survival (van Elsas and van Overbeek, 1993; van Veen *et al.*, 1997). In a study by Bashan *et al.* (1995), the survival of *Azospirillum brasilense* was studied in 23 soil types representing different climatological conditions from tropical to arid zones. Of 15 physical and chemical soil parameters tested in this study, the percentages of clay, nitrogen, organic matter, and water-holding capacity were positively correlated with survival of the bacterium in soil. In contrast, the percentages of $CaCO_3$, fine sand, and rough sand were negatively correlated with survival. The percentages of silt, pH, conductivity, K, P, and C/N ratio were found to have no effect on survival of the bacterium in soil.

In addition to individual soil components, soil texture can also influence bacterial survival in different ways. For example, indigenous microorganisms can congregate in pores large enough to physically contain their population size, resulting in a composite of numerous, heterogeneous soil microbial communities that are each influenced by its own immediate environment (Foster, 1988; Hattori and Hattori, 1976). Pores of smaller sizes represent microenvironments that retain water longer during dry periods, preventing exposure of inhabiting microbes to desiccation stress (Foster, 1988). Pore size can also influence predation by protozoans. Small pores within soil can allow for occupancy by bacteria, thereby evading predation by excluding the larger protozoa (Postma and van Veen, 1990; vanVeen *et al.*, 1997).

Certain physical and chemical factors in soil not only influence microorganisms directly, but also influence the interactions that occur between organisms at different levels, ultimately affecting desirable processes such as biological control (Duffy and Défago, 1997; Duffy *et al.*, 1997; Ownley *et al.*, 2003). In a study by Ownley *et al.* (2003), 16 of 28 soil factors were found to be positively correlated biological control of take-all disease on wheat by *Pseudomonas fluorescens* strain 2–79, including key soil factors such as pH, ammonium-nitrogen ratio, and zinc concentration. In contrast, the same study identified other soil factors, such as cation-exhange capacity, iron, and percent silt, which were negatively correlated with disease suppression. This example not only reflects the multiplicity of factors, but also the complexity by which factors influence microbial activity.

A number of abiotic environmental signals are known to influence production of secondary metabolism in bacterial biocontrol agents (Haas and Défago, 2005). In certain *Pseudomonas* spp., excess iron concentrations repress biosynthesis of the iron siderophores pyoverdine and pyochelin, while low oxygen tensions and low iron availability are required for hydrogen cyanide production. In *P. fluorescens*, biosynthesis of the antibiotics 2,4-diacetylphloroglucinol and pyoluteorin is stimulated by zinc, while production of salicylic acid is stimulated by molybdenum and magnesium. Stresses induced by salt, or by low concentrations of inorganic nutrients such as ferric iron, phosphate, sulfate, and ammonium, are known to reduce biosynthesis of the antibiotic phenazine-1-carboxamide in *Pseudomonas chlororaphis* (van Rij *et al.*, 2004). These studies clearly indicate that variations between any of a number of soil factors can influence metabolic activity of microorganisms, and thus contribute to the variability that is often observed with biological control.

Biological components within soils can also have tremendous impact on biocontrol agents, such as by way of predation as mentioned previously, or by competition for nutrients and space. However, indigenous soil microorganisms can also impact the metabolic behavior of biocontrol

agents by affecting regulatory networks that control gene expression. One very good example of regulatory systems affected in different ways by indigenous soil populations is quorum-sensing, the cell density dependent regulatory mechanism that functions in a number of plant-associated soil bacteria (Pierson and Pierson, 2007). Quorum sensing is typically activated through the production of autoinducing compounds such as *N*-acyl homoserine lactones (AHL). Bacteria that use this regulatory system respond to the presence of the signal once a sufficient concentration is achieved, which is usually associated with a certain cell density. The biosynthesis of the antibiotic phenazine-1-carboxamide is dependent on *N*-acyl-homoserine lactone in *P. chlororaphis* and thus, the biocontrol activity of this bacterium is influenced in a cell density dependent manner (Molina *et al.*, 2003).

Bacteria that utilize quorum-sensing regulatory systems can be influenced by other bacterial species, or even plants, that produce similar signaling molecules (Pierson *et al.*, 1998; Teplitski *et al.*, 2000). Such heterologous cross-talk can result in gene regulation occurring prior to populations achieving cell densities originally designed to control gene expression. Alternatively, other microbial species can have an opposite effect on quorum-sensing systems, such as by producing inhibitory compounds or degrading quorum-sensing signals, thereby preventing regulation of gene expression from occurring when critical cell densities are reached (Molina *et al.*, 2003; Morello *et al.*, 2004; Uroz *et al.*, 2003).

IV. PLANT INFLUENCES ON MICROBIAL METABOLISM IN THE SOIL ENVIRONMENT

A number of factors released from roots and seeds, including ions, free oxygen, enzymes, and a diverse array of reduced carbon compounds, are known to influence metabolic activity of microorganisms (Bais *et al.*, 2006; Lynch and Whipps, 1990). In addition to factors released by roots, metabolic processes of roots affect the surrounding microenvironments in a number of ways, including the solubilization of mineral nutrients, as well as the uptake of water, ions, inorganic compounds, oxygen, and reduced carbon compounds (Bais *et al.*, 2006; Jones and Darrah, 1993; Jones *et al.*, 2004; Patterson, 2003), which in turn influence the activity of resident microbes (Jones *et al.*, 2004; Patterson, 2003). The rhizosphere effect represents another clear example where plant roots influence associated microbes, in which not only substantially higher populations of microbes are in association with plants than in bulk soil, but where specific microbial communities develop in association with certain plant species (Garbeva *et al.*, 2004; Kent and Triplett, 2002).

From the microbial perspective, the success of a bacterial colony is reflected by its ability to survive, colonize, and proliferate in its environment. A major factor in this success, or competence, relates to the presence of plant root exudates in the vicinity of the colony. These exudates provide a major source of nutrition and are the driving force behind their metabolic activity (Kraffczyk *et al.*, 1984; Lynch and Whipps, 1990). The importance of exudates on metabolic behavior is demonstrated by the effect different host plants and species have on their bacterial residents. Significant variation exists in these exudates, both in amount and composition, among plants of different species. Even among the same plant species, variations occur based upon the environmental conditions present. Such variations result in different nutrient availability and undoubtedly influence microbial metabolic activities such as growth, cellular function and gene expression.

Exudates are comprised of low molecular weight compounds such as amino acids, organic acids, carbohydrates, and other secondary metabolites, as well as high molecular weight mucilage and protein. Sugars, amino acids, and organic acids represent the most dominant of soluble reduced carbon compounds, and thus are the most influential on microbial growth (Farrar *et al.*, 2003; Lynch and Whipps, 1990; Meharg, 1994; Whipps, 1990). Availability of these compounds, and therefore the level of influence they have on microbial metabolism, varies in a spatial manner along the root (Bringhurst *et al.*, 2001; Jaeger *et al.*, 1999; McDougall and Rovira, 1970). Most reduced carbon compounds are released near root tips, making this region among the most active for microbial metabolism. The amount of compounds released in root exudates is known to differ between plant species and plant age, and is also influence by environmental conditions (Curl and Truelove, 1986; Grayston *et al.*, 1996; Walker *et al.*, 2003). Thus, nutrient availability in exudates typically varies by a number of influencing factors.

The significance of nutrient availability on successful colonization has been illustrated in studies investigating bacterial populations as they relate to carrying capacities of cucumber and pea seeds. These seeds are particularly suited to such studies due to the extreme difference in quantity, and therefore supply, of nutrients within the exudates. By way of example, a pea seed will exude nearly 3000 μg of glucose equivalents per seed within the first 24 hours after imbibition, whereas the cucumber seed will exude a mere 1 μg. Consequently, pea seeds support substantially greater growth and population sizes of colonizing microorganisms than cucumber seeds primarily due to higher levels of nutrients found in exudates (Roberts *et al.*, 1999, 2000).

Further evidence that the difference in carrying capacity between pea and cucumber is directly related to nutrient availability was provided in studies using mutant strains of *Enterobacter cloacae* with impaired catabolic

capability (Lohrke *et al.*, 2002; Roberts *et al.*, 2000). The *pfkA* gene encodes for phosphofructokinase, a key step in glycolysis that is necessary for utilization of most hexose sugars except fructose. Colonization experiments showed the *pfkA* mutant strain of *E. cloacae* was significantly reduced in population levels in the cucumber spermosphere as compared with the wildtype strain. However, when exogenous fructose is supplied to the spermosphere to compensate for the nutritional deficit incurred by the mutation, the colonization ability was restored to levels comparable to the wildtype strain (Roberts *et al.*, 1999, 2000).

Along with cell population levels, the metabolic energy status of colonizing bacteria has been found to correlate directly with the exudate content. Using a bioluminescence-based reporter system, *E. cloacae* produces significantly more relative luminescence units (RLU) per cell on pea seed than on cucumber seed (Roberts *et al.*, unpublished). These results indicate pea seed exudates not only support higher populations of cells, but the average bacterial cell colonizing a pea seed is more active metabolically than that colonizing a cucumber seed.

Interactions between plant and microbe goes beyond mere nutritional requirements, with root exudates also serving as chemical attractants to bacteria, as well as effective antimicrobials (Brigham *et al.*, 1999; Lugtenberg *et al.*, 2001, 2002). Successful colonization becomes a matter of fitting the niche provided by the particular plant host and is dependent upon multiple factors. Accordingly, bacterial traits can play important roles in bacterial competence for colonization. For example, possessing flagella-driven chemotaxis, which allow the bacteria to be mobile within the rhizosphere environment, can provide a significant advantage over competing organisms.

The significance of bacterial chemotaxis to root colonization, as well as its relationship with nutrient uptake, was highlighted in a study by de Weert *et al.* (2002). In this study, chemotaxis was found to be an important competitive colonization trait in *P. fluorescens* strain WCS365, as a *cheA* mutant deficient in flagella-driven chemotaxis was strongly reduced in root-colonizing ability. Chemotactic responses were also detected toward root exudate components, supporting the role of motility in colonization as a mechanism by which bacteria can seek out and reach nutrients exuded by roots. This and other studies (de Weger *et al.*, 1987; Simons *et al.*, 1996) support the role of motility in successful colonization, it should be pointed out that prior investigations have produced results which suggest no relationship between motility and root colonization (Howie *et al.*, 1987; Scher *et al.*, 1988). However, these conflicting findings could be explained by differences in experimental conditions as there is much support, as well as logic, behind the theory of motility enabling greater success in nutrient uptake in a competitive environment.

Perhaps a less obvious, but quite intriguing finding is a connection between motility and biocontrol. In a study investigating colonization traits and biocontrol activity, Chin-A-Woeng *et al.* (2000) demonstrated that motility was required for *P. chlororaphis* strain PCL1391 to suppress tomato foot and root rot caused by the fungal pathogen *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Prior research had identified the antibiotic phenazine-1-carboxamide as the primary mechanism of biocontrol for this disease (Chin-A-Woeng *et al.*, 1998). However, mutants impaired in known colonization traits that included motility, but were still capable of producing the antibiotic at wildtype levels were shown to be deficient in disease suppression. The authors presumed that this lack of biocontrol related to the inability of the mutant strain to deliver the antifungal metabolite in a spatially relevant manner so as to be effective against the pathogen. This study further supports the belief that successful management of biocontrol agents will require an in-depth understanding of these microbes. As demonstrated here, characteristics which initially seem to be distinct and of no consequence to each other may in fact play a significant role in determining the biocontrol capability.

Nutrient availability is not strictly a matter of what a plant root exudes, but is also dependent upon competition for those resources from other microorganisms living in the rhizosphere. Interestingly, this competition, creating a nutrient-limiting environment, can result in rhizobacterial metabolic behavior directly related to biocontrol. This has been clearly demonstrated by research involving a number of biocontrol systems.

Competition for essential resources is a factor in survival of all organisms and has been recognized for several years to play an important role in biocontrol. If a pathogenic organism is out-competed by an antagonistic organism for a limited resource, it stands to reason disease suppression will occur. van Dijk and Nelson (2000) presented compelling evidence for nutrient competition with a new twist. In interactions between *Pythium ultimum* and the biocontrol agent *E. cloacae*, biocontrol does not occur by competition directly between the two microorganism, but rather indirectly that leads to inhibition of pathogen spore germination that is necessary for plant infection.

Fatty acids are naturally present in the exudates of germinating seeds and act as signals for *Pythium* spp. spore germination. This concurrent germination strategy provides the pathogen an opportunity to infect the seeds while they are in a vulnerable state. To investigate the role of fatty-acid metabolism in this process, van Dijk and Nelson (2000) focused on linoleic acid, a fatty acid known to be utilized as a nutrient resource by the biocontrol bacterium *E. cloacae*, as well as a signal to break fungistasis in the oomycete pathogen *P. ultimum*. Two different mutants of *E. cloacae* were constructed that were deficient in linoleic metabolism. Both mutants

failed or were reduced in the ability to inactivate *P. ultimum* sporangium germination activity induced by either linoleic acid or by cottonseed exudate, which is rich in linoleic acid. Furthermore, both mutants failed to protect cotton seeds from infection in cottonseed bioassays assessing the ability of these strains to suppress *Pythium* seed rot. The results of this study succeed in demonstrating a mechanism, whereby *E. cloacae* can limit the availability of fatty acids to *P. ultimum*, thereby establishing competition in favor of *E. cloacae*. Since these findings also present persuasive evidence that this fatty-acid competition is a significant factor in the disease suppression exhibited by *E. cloacae* upon seed rot, they substantiate nutrient competition as an effective mode of biocontrol and provide insight into the specific manner in which these two microbes interact.

Based upon these results, one might conclude that the key to effective biocontrol using *E. cloacae* against disease caused by *P. ultimum* is a straightforward matter of competitive advantage for nutrients. However, studies have demonstrated that catabolic degradation of fatty acids by *E. cloacae* is subject to catabolite repression. Consequently, variation in nutrient availability between spermospheres of different plant species can have a profound effect on fungal antagonistic activity and biological control. Studies by Kageyama and Nelson (2003) not only demonstrated differential efficacy of *E. cloacae* against *Pythium* damping off in various plant species, but also provided new insight into the fatty-acid mechanism of biocontrol exhibited by *E. cloacae*. The data obtained in this study relates the inability of *E. cloacae* to afford biological control to certain plant species to its inability to inactivate the stimulatory activity of compounds (e.g., fatty acids) involved in *P. ultimum* spore germination found in the seed exudates of those plants.

The results of this study suggest that exudate composition, as opposed to overall exudate quantity, plays a pivotal role in whether fatty-acid competition between *E. cloacae* and *P. ultimum* occurs and points to the importance of *E. cloacae* metabolism in determining its biological control capability. As the mechanism of antagonism by *E. cloacae* against this pathogen has been demonstrated to be its ability to inactivate stimulatory activity of seed exudates via fatty-acid competition, this variable metabolic behavior of *E. cloacae* provides a basis for why differential biocontrol efficacy is observed in different plant species and further underscores the importance of nutrient availability on biological control activity.

As demonstrated by the study conducted by Mark *et al.* (2005), differences in exudate content go well beyond effects of competition, having global effects on gene expression. Transcriptomic analysis of *Pseudomonas aeruginosa* showed substantial differences in gene expression in response to exudates from two distinct varieties of sugarbeet (*Beta vulgaris* L.). Not

surprisingly, a significant proportion of genes with altered expression were involved with various aspects of metabolism.

From the practical biocontrol standpoint, the relevance of nutrient availability is well illustrated with antibiotic production by the biocontrol agent *P. fluorescens*. *P. fluorescens* is known to be effective against the fungal pathogen *P. ultimum*, the causal agent of damping off. Secondary metabolism in *P. fluorescens* is globally regulated by the sigma factor σ^s in response to various stresses imposed during growth, including nutrient exhaustion or starvation (Koch *et al.*, 2001; Sarniguet *et al.*, 1995). A mutant strain of *P. fluorescens* disrupted in the *rpoS* gene, which encodes σ^s, is altered in its ability to produce three antibiotics: pyrrolnitrin, pyoluteorin and 2,4-diacetylphloroglucinol (DAPG). A mutation in the *rpoS* gene results in a mutant strain that no longer produces pyrrolnitrin, but overproduces pyoluteorin and DAPG compared with the wildtype strain. In studies with cucumber, this differential regulation of antibiotic production by σ^s results in the mutant strain having greater biocontrol efficacy against *P. ultimum* when compared to the wildtype strain, presumably due to increased production of the antibiotics pyoluteorin and DAPG. Not only does this differential regulation illustrate the importance of nutrient availability on bacterial metabolism, it clearly demonstrates the potential for metabolic behavior to impact biocontrol activity.

In other studies with *P. fluorescens*, Duffy and Défago (1999) investigated the effects of various minerals and carbon sources on the *in vitro* biosynthesis of DAPG, pyoluteorin and pyrrolnitrin. From a nutritional standpoint, this study provides clear evidence that antibiotic production is significantly impacted by carbon sources typically found in the rhizosphere. In culture-based studies, the authors found that the sugars glucose, fructose, and mannitol stimulated production of DAPG and pyrrolnitrin. Production of pyoluteorin was repressed by the same sugars, but induced in the presence of glycerol. However, they provide strong support for the supposition that the nutritional environment plays a major role in metabolic behavior of these bacteria and, consequently, biocontrol activity. This study, together with findings by Sarniguet *et al.* (1995) regarding the sigma factor σ^s, argues for an integral role that nutrient availability plays in bacterial metabolism and biological control.

A classic example of competition for nutrients as a biocontrol mechanism is the production of siderophores for sequestering iron. Under iron-limiting conditions, certain bacteria secrete iron-chelating siderophores that have a high affinity for ferric iron, providing a competitive edge over other microorganisms (Loper and Buyer, 1991). Once siderophores have complexed with iron they can be recaptured, thus making this essential micronutrient available to the bacteria. Many rhizobacteria not only have the capacity to produce siderophores, they also have the ability to utilize exogenous siderophores produced by other rhizosphere inhabitants,

thereby increasing their ability to obtain iron (Jurkevitch *et al.*, 1992; Loper and Henkels, 1999).

As with antibiotic production, siderophore production has been shown to be differentially affected by nutrient levels. Concurrent with their studies on antibiotic production by *P. fluorescens*, Duffy and Défago (1999) investigated the effects of various minerals and carbon sources on the *in vitro* biosynthesis of the siderophore pyochelin. In general, they found that glucose alone or in combination with certain minerals resulted in increased production of pyochelin. This included addition of glucose to a rich nutrient medium, suggesting metabolic flow and nutrient balance influence production of siderophores and other secondary metabolites.

V. CONCLUSION

The complexity of biological control is epitomized by the wide range of effects observed on rhizosphere inhabitants. The past 20-plus years of research has advanced our knowledge of biocontrol at several levels, from establishing roles for specific genes and identifying environmental factors that affect the biocontrol activity of microbial agents. However, much remains to be elucidated in order to successfully develop it into a widespread, commercially viable alternative to chemical methods. Among the many challenges include the following: understanding the metabolic behavior of rhizobacteria as it relates to various environmental conditions, the relevance of cellular metabolic energy status as it relates to expression of biocontrol traits, and the various roles that nutrient availability contributes to biocontrol activity. The development of new and more accurate molecular methods, along with increasing availability of whole genome scale tools to study microbial activity in environmental settings, should proved helpful and exciting in future efforts to meet these challenges.

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CHAPTER 8

Copper Homeostasis in Bacteria

Deenah Osman and **Jennifer S. Cavet**[1]

Contents

I. INTRODUCTION

A. The properties of copper

Copper is widely distributed in the environment; present mainly in minerals as sulphides, oxides, or carbonates, with the major ores being chalcopyrite ($CuFeS_2$), chalcocite (Cu_2S), cuprite (Cu_2O), and malachite ($Cu_2CO_3(OH)_2$). Copper was the first metal to be worked by human societies and is thought to have been mined for around 5000 years. The discovery that copper could be hardened with tin, to form the alloy

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bronze, established the Bronze Age. Following its early use to craft tools and weapons, copper continues to have many different applications, with the greatest percentage of copper currently being used for electrical equipment such as wiring and motors.

Copper primarily exists in one of two oxidation states, the oxidized Cu(II) cupric form and the reduced Cu(I) cuprous form. Cu(I) and Cu(II) are the most effective monovalent and divalent ions, respectively, for binding to organic molecules and possess almost equal tendencies to form complexes with many organic ligands (Fraústo da Silva and Williams, 1993). Cu(I) is a closed shell $3d^{10}$ transition metal ion and hence diamagnetic. As a soft Lewis acid, it tends to bind to soft bases such as thiols, hydrides, alkyl groups, cyanide, and phosphines. Cu(II) has a $3d^9$ configuration and is paramagnetic. As an intermediate Lewis acid it forms complexes with additional ligands to Cu(I), including sulphate and nitrate (Crichton and Pierre, 2001). Cu(I) is susceptible to disproportionation to give Cu(II) and Cu(O), while Cu(O) is readily oxidized to Cu(I) and subsequently to Cu(II) by atmospheric oxygen. The high redox potential of the Cu(I)/Cu(II) couple makes it particularly useful and adaptable for enzymes, and most copper enzymes work in the 0.2–0.8 V range enabling them to directly oxidize substrates such as superoxide, ascorbate, catechol, and phenolates (Crichton and Pierre, 2001).

B. Copper requiring proteins

The use of copper in biological systems has only occurred since the release of oxygen into the atmosphere some 2.7 billion years ago, causing the oxidation of water-insoluble Cu(I) to soluble Cu(II) (Fraústo da Silva and Williams, 1993). Consequently, copper-requiring proteins are now widely distributed amongst aerobic organisms. Copper is usually bound to proteins via cysteine, methionine, or histidine amino acid side chains (Xue *et al.*, 2008). Cu(I) typically favors tetrahedral geometry involving sulphur donors while Cu(II) favors square planar coordination involving nitrogen donors. Due to the fact that copper can readily undergo redox cycling between Cu(I) and Cu(II), it can act as an electron donor/acceptor in the electron transport chain (e.g., in cytochrome oxidase) and in redox-active enzymes, which use molecular oxygen as a substrate (Fraústo da Silva and Williams, 1993). Enzymes that perform these roles can support both oxidation states of copper.

All bacterial copper-requiring enzymes appear to be extracytoplasmic and are found mainly in the periplasm or embedded in the cytoplasmic membrane. Hence, with the exception of cyanobacteria that possess copper-requiring proteins within internal membrane-bound compartments, there is currently no documented metabolic requirement for copper to cross the cytoplasmic membrane and enter the cytosol (Tottey

et al., 2005). Copper-dependent proteins in *Escherichia coli* include the periplasmic copper, zinc superoxide dismutase (SodC) and amine oxidase (MaoA), and the cytoplasmic membrane located NADH dehydrogenase-2 (Ndh-2) and bo_3-type (quinol) cytochrome oxidase (Gort *et al.*, 1999; Osborne *et al.*, 1999; Parsons *et al.*, 1995; Rapisarda *et al.*, 2002). Gram-positive bacteria, devoid of a periplasm, also possess copper-requiring proteins outside the cytosol. *Bacillus subtilis* has copper-requiring caa_3-type and aa_3-type (quinol) cytochrome oxidases at the cytoplasmic membrane (van der Oost *et al.*, 1991), and a copper laccase, CotA, is present in *B. subtilis* spore coats (Hullo *et al.*, 2001). Methane oxidizing bacteria switch from using an iron-requiring soluble methane monooxygenase (sMMO) to a copper-requiring particulate enzyme (pMMO) when copper is available (Kim *et al.*, 2004). pMMO is located within internalized membranes which are thought to be derived from the periplasm (Tottey *et al.*, 2005).

Many cyanobacteria contain internal membrane-bound compartments called thylakoids which, in contrast to those found in methane-oxidizing bacteria, are discrete from the periplasm. Thylakoids are the site of both photosynthetic and respiratory electron transport which involves the copper protein plastocyanin and a caa_3-type cytochrome oxidase, respectively (Tottey *et al.*, 2005). Plastocyanin is imported via the Sec pathway for unfolded proteins, while cytochrome caa_3 oxidase is membrane bound with the di-copper A site orientated inside the thylakoid lumen. Hence, cyanobacteria are currently the only bacteria with a known requirement for copper to enter the cytosol for delivery to copper-requiring proteins (Tottey *et al.*, 2005). However, it remains possible that in other bacteria, such as *E. coli* and *B. subtilis*, copper is also routed via the cytosol to supply copper sites located outside the cytosol.

C. Principles of copper homeostasis

The ability of copper to alternate between its cuprous Cu(I) and cupric Cu(II) oxidation states makes it an ideal biological cofactor. However, when unbound within a cell, redox cycling means copper is extremely toxic, largely due to its ability to catalyze Fenton chemistry, Eq. (8.1), causing the production of highly reactive hydroxyl radicals that damage biomolecules such as DNA, proteins, and lipids. The reduced form of the metal for this reaction can be generated by reaction with superoxide (or other cellular components such as low-molecular weight thiols), Eq. (8.2), with the sum of these reactions, Eq. (8.3), being referred to as the Haber–Weiss reaction (Liochev and Fridovich, 2002).

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH^{\cdot} \qquad (8.1)$$

$$Cu^{2+} + O_2^- \rightarrow Cu^+ + O_2 \quad (8.2)$$

$$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH^{\cdot} \quad (8.3)$$

Copper can also mediate toxicity by binding to adventitious sites in proteins, nucleic acids, polysaccharides and lipids, causing the displacement of native metal ions, as well as alterations to their structure and/or function. In addition, copper appears to be much more toxic under anaerobic conditions, possibly due to the reduction of Cu(II) to Cu(I), which can diffuse through the cytoplasmic membrane causing increased copper accumulation (Beswick *et al.*, 1976; Outten *et al.*, 2001).

Bacteria must therefore achieve precise copper homeostasis to avoid copper-mediated toxicity whilst maintaining a supply of copper for copper-requiring proteins. To achieve this, copper-sensing transcriptional regulators must distinguish copper from other metal ions and in response to levels above or below a threshold value, trigger an appropriate physiological response such as copper-import, -export, or -detoxification. Furthermore, cells may restrict access to copper by channeling copper away from adventitious sites, for example using copper-chaperones to deliver copper to its targets (Tottey *et al.*, 2005). The proteins involved in copper trafficking and resistance may also exhibit metal specificity. The way in which bacteria meet the conflicting demands imposed by copper varies depending upon environmental conditions, their adopted ecological niche and phylogeny. Well studied bacterial copper homeostatic systems include those in *E. coli, Enterococcus hirae, B. subtilis*, and *Synechocystis* PCC 6803 (Fig. 8.1). This review will attempt to summarize the various mechanisms of copper trafficking and resistance used by bacteria with an emphasis on *E. coli*, while the different approaches used by other bacteria will also be highlighted.

II. MECHANISMS OF COPPER TRAFFICKING AND RESISTANCE

Bacteria use copper homeostasis proteins to maintain a strict cellular copper quota, which in *E. coli* is estimated to be about 10^4 atoms per cell (c. 10 μM) (Finney and O'Halloran, 2003; Outten *et al.*, 2001). Furthermore, the extraordinarily high (zeptomolar, 10^{-21} molar) sensitivity of the *E. coli* copper sensor CueR to free Cu(I), measured at less than one atom per cell, indicates that there is essentially no free cytosolic copper (Changela *et al.*, 2003) and most copper ions in the cell must be compartmentalized or protein bound.

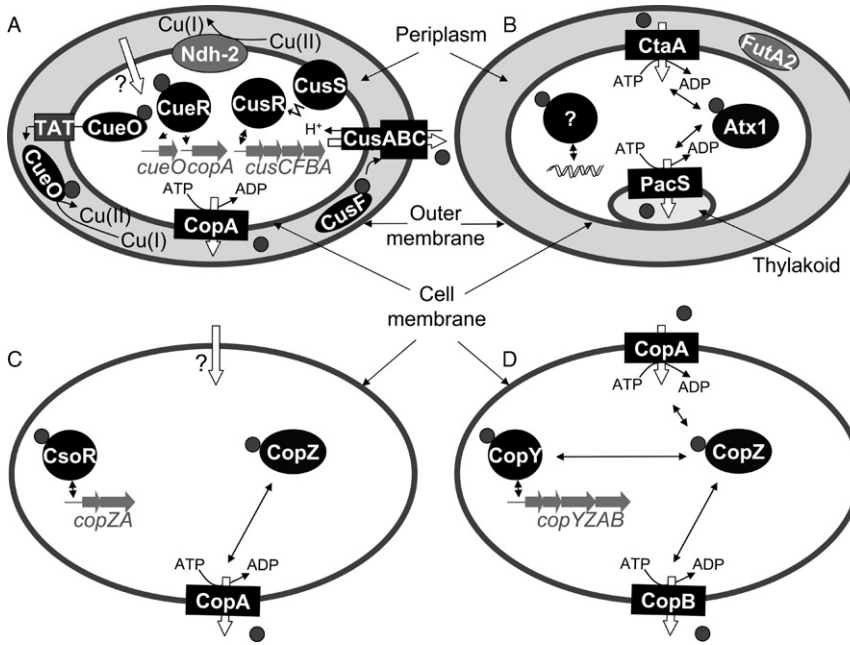

FIGURE 8.1 Principal copper homeostatic and trafficking mechanisms. (A) In *E. coli*, the cytoplasmic copper sensor CueR triggers production of the P_{1B}-type ATPase CopA and the multicopper oxidase CueO. CopA transports Cu(I) from the cytosol into the periplasm where CueO is proposed to convert Cu(I) to less toxic Cu(II). CueO is exported via the TAT pathway for prefolded proteins and may also contribute to copper-export. The CusR/S two component sensory system detects surplus copper in the periplasm and triggers transport across the outer membrane by the CusABC and F proteins. Ndh-2 acts as a Cu(II)-reductase. (B) In the cyanobacterium *Synechocystis* PCC 6803, copper is trafficked to thylakoids through the actions of two P_{1B}-type ATPases, CtaA and PacS, and the copper-chaperone Atx1. Atx1 is presumed to acquire copper from the importer CtaA at the cytoplasmic membrane and donate it to PacS for export into the thylakoid. (C) In *B. subtilis*, the cytosolic copper sensor CsoR triggers copper-export by the P_{1B}-type ATPase CopA. The copper-chaperone CopZ is thought to channel copper to CopA. (D) In *E. hirae*, according to the model the P_{1B}-type ATPase CopA imports copper, which is routed to the cytosolic copper sensor CopY and to the copper-exporting P_{1B}-type ATPase CopB by the CopZ copper-chaperone.

The metal-binding motifs within the regulatory and homeostatic proteins have atypical coordination chemistry, relative to the enzymes that incorporate copper as a cofactor, which relates to their particular functions (Finney and O'Halloran, 2003). For example, copper-chaperones (e.g., CopZ and Atx1, Fig. 8.1) must bind Cu(I) tightly enough to prevent adventitious reactions or release of the ions, whilst allowing for easy transfer of Cu(I) to target sites. They achieve this by binding Cu(I) using

two cysteines (in a G*XX*C*XX*C motif) and Cu(I) transfer is facilitated via the formation of low-coordination-number anionic intermediates (Banci *et al.*, 2004; Cobine *et al.*, 2002; Finney and O'Halloran, 2003). In contrast, extracellular or periplasmic copper homeostatic proteins operate in a more oxidizing environment than the cytosol where the use of cysteines is less appropriate; for example, due to disulphide bond formation, and utilize clusters of methionines occasionally with histidine (Arnesano *et al.*, 2003; Davis and O'Halloran, 2008; Wernimont *et al.*, 2003) or even tryptophan (Xue *et al.*, 2008).

A. P_{1B}-type ATPases

The most widely distributed copper-trafficking proteins in both prokaryotes and eukaryotes are the copper-transporting P_{1B}-type ATPases, with representatives associated with both copper-uptake and -export (Fig. 8.1). The P_{1B}-type ATPases are a subgroup of the P-type family of ATPases (Axelsen and Palmgren, 1998), so called because an invariant aspartate residue in the conserved motif DKTGT becomes transiently phosphorylated during the reaction cycle (Pedersen and Carafoli, 1987). P_{1B}-type ATPases are known that transport a range of metal ions including cadmium (Tsai *et al.*, 1992), zinc and lead (Rensing *et al.*, 1997, 1998), cobalt (Rutherford *et al.*, 1999), and silver (Gupta *et al.*, 1999). Phylogenetic studies have led to a further sub-division into two groups, one containing copper and silver transporters and the other containing transporters of the divalent metal cations (Rensing *et al.*, 1999). These proteins comprise eight transmembrane helices, that presumably form the ionic pathway across the membrane, and couple metal transport to the hydrolysis of ATP at a large cytoplasmic loop (c. 400 amino acids) located between transmembrane helices 6 and 7. Other features common to these proteins include a proline residue flanked by probable metal-binding residues (CP*X* motif) within the sixth transmembrane helix and a soluble amino-terminus typically comprising one or more metal-binding domains (one or two in bacteria). These amino-terminal domains possess typical βαββαβ ferredoxin-like folding (Banci *et al.*, 2002a) and bear the motif G*XX*C*XX*C associated with metal-binding and the formation of copper-bridged hetero-dimeric species during copper transfer with copper-chaperones (Arnesano *et al.*, 2001; Banci *et al.*, 2003; Gitschier *et al.*, 1998; Pufahl *et al.*, 1997). The amino-terminal metal-binding domains are not necessary for metal ion transport but may interact with the intracellular ATP-binding loop of the transporter to modulate activity (Bal *et al.*, 2001; Mitra and Sharma, 2001; Tsivkovskii *et al.*, 2001; Voskoboinik *et al.*, 2001).

There is a high degree of specificity with respect to the metal ions transported by the different P_{1B}-type ATPases. The metal-binding sites within the membrane-transport sites and the amino-terminal domains

both appear to contribute to metal-specificity (Banci *et al.*, 2002b, 2006b; Borrelly *et al.*, 2004; Finney and O'Halloran, 2003; Hou *et al.*, 2001; Mandal *et al.*, 2004). Furthermore, studies with a zinc-transporter, AztA, have revealed that possession of two amino-terminal metal-binding domains, as opposed to just one, may provide a further level of metal-discrimination (Liu *et al.*, 2007). In this case, export of cadmium is prevented due to the thiophilic nature of cadmium causing it to recruit cysteines from both metal-binding domains and forming nonproductive bridged complexes, while the ''correct'' metal, zinc, binds to a pair of cysteines in one or other domain, but not both, and thereby migrates along an affinity gradient toward the membrane for transport. Restricted contact of the amino-terminal domains of the P_{1B}-type ATPases with copper-chaperones (refer to Section II.E) may also contribute toward metal-specificity (Borrelly *et al.*, 2004). Although bacterial copper-transporting P_{1B}-type ATPases are known that have roles in import and export to/from the bacterial cytosol, this cannot be predicted based upon sequence analysis.

B. Copper acquisition

No specific copper import system has been identified in *E. coli*, possibly reflecting no cytosolic requirement for copper, and the mechanism of copper entry into *E. coli* is largely unknown. Copper has been suggested to cross the outer membrane through porins, since porin-deficient mutants have been obtained when selecting for copper-resistance (Lutkenhaus, 1977). However, no increase in metal-tolerance has since been reported for isogenic OmpF and/or OmpC mutants (Li *et al.*, 1997). Within the periplasm, the respiratory chain Ndh-2 has specific cupric-reductase activity (Rapisarda *et al.*, 1999), which possibly contributes to Cu(I) uptake by passive diffusion or by yet unidentified copper-importers and/or nonspecific uptake systems for other metals such as sodium or potassium. In contrast to *E. coli*, specific copper-importers have been described in other bacteria including *Synechocystis* PCC 6803, *E. hirae*, and *Listeria monocytogenes* DRDC8 (Francis and Thomas, 1997b; Tottey *et al.*, 2001; Wunderli-Ye and Solioz, 2001), despite the latter two having no known cytosolic copper-requirement.

1. P_{1B}-type ATPases: *E. hirae* CopA and *Synechocystis* PCC 6803 CtaA

Current evidence that *E. hirae* CopA is involved in copper-import remains indirect. *E. hirae* mutants lacking *copA* show no change in copper-resistance but, unlike wild-type cells, fail to grow after two to three generations in metal-limited media (treated with the chelator 8-hydroxyquinoline). In addition, the *copA* mutants are more resistant to silver than wild-type cells, which is presumed to result from reduced fortuitous silver uptake by

CopA and subsequent adventitious binding (Odermatt *et al.*, 1993). Purified CopA can catalyze ATP hydrolysis and form an acylphosphate intermediate, which is inhibited by vanadate (typical of P-type ATPases). Inhibition has also been detected in the presence of Cu(I) chelators, but not chelators of divalent cations, further supporting a role in Cu(I)-transport (Wunderli-Ye and Solioz, 2001). CopA possesses a single amino-terminal metal-binding domain, which interacts with the CopZ copper-chaperone (Multhaup *et al.*, 2001). CopZ is suggested to channel copper supplied by CopA to both the CopY repressor and CopB for export (Fig. 8.1).

Deletion of the *ctaA* gene in the cyanobacterium *Synechocystis* PCC 6803 caused no change in copper-tolerance but reduced the amount of copper accumulated per cell (Tottey *et al.*, 2001), while deletion of the related gene from *Synechococcus* PCC 7942 caused (slightly) elevated copper-resistance, which was attributed to reduced copper-import at the cytoplasmic membrane (Phung *et al.*, 1994). Consistent with a role for CtaA in copper-import, *Synechocystis* PCC 6803 mutants lacking *ctaA* have low cytochrome caa_3 oxidase activity in purified membranes and are impaired in photosynthetic electron transport via plastocyanin (Tottey *et al.*, 2001). In the model (Fig. 8.1), the copper-chaperone Atx1 interacts with the single amino-terminal metal-binding domain of CtaA, where it is presumed to acquire copper, and donates it to PacS for transport into the thylakoid (Banci *et al.*, 2006a; Tottey *et al.*, 2001, 2002). Mutants deficient in *atx1* and *pacS* also show reduced cytochrome caa_3 oxidase activity and plastocyanin mediated electron transport (Tottey *et al.*, 2001, 2002).

2. FutA2

A periplasmic iron-binding protein, FutA2, has also been shown to contribute to copper-import in *Synechocystis* PCC 6803. Mutants lacking *futA2* have low cytochrome caa_3 oxidase activity and accumulate cytochrome c_6 for electron transport whilst copper-accumulation in plastocyanin is impaired (Waldron *et al.*, 2007). Furthermore, the total amount of copper in the major soluble protein and small-molecule complexes from whole cell extracts is less in *futA2* mutants, compared to wild-type cells, while additional copper accumulates in periplasmic soluble protein and small-molecule complexes. It is proposed that FutA2 contributes towards the inward supply of copper from the periplasm by sequestering Fe(III) and thereby limiting Fe(III) associations with vital binding sites for other metals, such as the copper-binding sites of CtaA (Waldron *et al.*, 2007).

3. Methanobactin

Some methane-oxidizing bacteria, such as *Methylosinus trichosporium* OB3b and *Methylococcus casulatus* (Bath), are known to secrete a small siderophore-like compound called methanobactin ($C_{45}N_{12}O_{14}H_{62}Cu$, 1216 Da) which is suggested to have a role in copper-uptake and/or

copper-detoxification (DiSpirito *et al.*, 1998; Kim *et al.*, 2004, 2005; Tellez *et al.*, 1998). Methanobactin binds both Cu(I) and Cu(II) and may itself reduce Cu(II) to Cu(I) (Choi *et al.*, 2006; Hakemian *et al.*, 2005). It has been postulated that the active site of pMMO (refer to Section 1.B) includes methanobactin or alternatively methanobactin scavenges copper from the environment and supplies pMMO in the periplasm and/or periplasmic invaginations (Kim *et al.*, 2004, 2005).

C. Copper detoxification

The use of copper-transporting P_{1B}-type ATPases for the rapid export of available copper ions from the cytosol is a common theme amongst bacterial copper-resistance mechanisms and there are numerous examples of these proteins conferring copper-tolerance throughout the literature. In *Synechocysis* PCC 6803, the export of copper by PacS not only provides copper-resistance but also has a role in supplying copper to proteins within the thylakoid (Tottey *et al.*, 2001, 2005). Similarly eukaryotic cells use copper-specific P_{1B}-type ATPases to remove copper from the cytosol into the *trans*-Golgi, where it is incorporated into proteins (Yuan *et al.*, 1995). Some bacteria, including *Synechocystis* PCC 6803, *E. hirae*, and *B. subtilis* (not *E. coli*), in common with eukaryotic cells, also possess copper-chaperones (e.g., CopZ and Atx1, Fig. 8.1), which may contribute to copper-trafficking by channeling copper to these proteins for export. If copper concentrations overwhelm these proteins, some bacteria invoke additional mechanisms that confer additional copper-resistance.

The responses of *E. coli* to elevated copper levels have been studied in detail and primarily involve the chromosomally-encoded *cue* and *cus* systems. The *cus* system only confers copper-tolerance under extreme copper stress while the *cue* system is the primary copper homeostasis system (Outten *et al.*, 2001). As extracellular copper levels increase, CueR mediates production of a copper-exporting P_{1B}-type ATPase CopA at the cytoplasmic membrane and a periplasmic multicopper oxidase CueO, these three proteins constituting the *cue* system. Although these proteins apparently clear the cytosol of excess copper, this is retained within the periplasm and thus may serve as a compartmentalized store (Outten *et al.*, 2001). If copper levels continue to increase, expression of the *cusCBFA* operon is induced by the CusR/S two-component sensory system, which detects surplus Cu(I) in the periplasm, mediating export of copper across the outer membrane. The *cus* system is particularly important in providing copper-tolerance under anaerobic conditions (Outten *et al.*, 2001). Some *E. coli* strains also harbor the plasmid-borne *pco* system which involves seven genes, *pcoABCDRSE*, providing yet further copper-resistance. The mechanism of copper detoxification provided by this system is largely unknown but includes the multi-copper oxidase

PcoA and its putative partner PcoC, both of which are exported to the periplasm (Lee *et al.*, 2002), PcoD is thought to transport copper across the cytoplasmic membrane, PcoB is a predicted outer membrane protein and PcoE is a periplasmic protein that binds copper (Rensing and Grass, 2003). PcoRS form a two-component regulator required for copper-inducible expression of the *pco* genes. A number of other proteins have also been linked to copper-resistance in *E. coli* (Kershaw *et al.*, 2005; Yamamoto and Ishihama, 2005), including the products of the *cutABCDEF* genes, which were identified based on the preliminary characterization of copper-sensitive mutants (Rouch *et al.*, 1989). However, few of these genes have been directly linked to copper metabolism, transport, or regulation (Crooke and Cole, 1995; Fong *et al.*, 1995; Gupta *et al.*, 1997) and hence are not described here.

1. The *E. coli cue* system

a. CueR Expression of both *cueO* and *copA* is substantially induced in response to elevated copper levels by the action of the CueR transcriptional regulator which has zeptamolar sensitivity to free Cu(I), less than one atom per cell (Changela *et al.*, 2003; Outten *et al.*, 2000; Stoyanov *et al.*, 2001). CueR belongs to the MerR-family of metal-responsive transcriptional regulators (reviewed by Brown *et al.*, 2003), other members of which include zinc-sensing ZntR (Brocklehurst *et al.*, 1999; Outten and O'Halloran, 2001), mercury-sensing MerR, cadmium-sensing CadR (Brocklehurst *et al.*, 2003), lead-sensing PbrR (Borremans *et al.*, 2001), and cobalt-sensing CoaR (Rutherford *et al.*, 1999). These proteins bind to the promoters of their target genes in the presence and absence of their effector metals (Stoyanov *et al.*, 2001). In the presence of elevated metal ions, they allosterically activate transcription initiation by RNA polymerase by realigning abnormally spaced consensus RNA polymerase recognition sequences, while in the absence of metal ions these proteins may cause slight (*c.* twofold) repression (Ansari *et al.*, 1992; Outten *et al.*, 1999; Parkhill *et al.*, 1993).

The operator-promoter regions upstream of *copA* and *cueO* reveal unusual spacing (19-bp, rather than 17-bp) between −10 and −35 RNA polymerase consensus binding sequences, characteristic of genes regulated by MerR-family transcriptional activators (Stoyanov *et al.*, 2001). Mutants lacking *cueR* show reduced copper-tolerance and lack copper-induced expression of *copA* and *cueO* (Outten *et al.*, 2000; Stoyanov *et al.*, 2001). In addition to Cu(I), CueR is responsive to both Ag(I) and Au(I) ions *in vivo* and *in vitro* (Changela *et al.*, 2003; Stoyanov and Brown, 2003; Stoyanov *et al.*, 2001), although the biological relevance of this is not clear and induction may simply result from Ag(I) and Au(I) mimicking Cu(I) in the CueR metal-binding site. The crystal structure of a CueR homodimer reveals a buried metal-receptor site at the dimer interface that contributes to selectivity toward the monovalent metals by restricting binding to a linear two-coordinate geometry involving two cysteines, Cys^{112} and

Cys^{120} (Changela *et al.*, 2003). Disruption of these cysteines causes loss of copper, silver, and gold sensing *in vivo* and *in vitro* (Chen *et al.*, 2003; Stoyanov and Brown, 2003).

In addition to copper-responsive regulation by CueR, *copA* has been suggested to be under the control of other transcriptional regulators including CpxR that responds to cell envelope stress (Outten *et al.*, 2000).

b. Multi-copper oxidase CueO Multi-copper oxidases catalyze the oxidation of a variety of substrates, such as polyphenols, aromatic polyamines, L-ascorbate and metal ions, and transfer the liberated electrons to a copper cluster causing the concomitant four-electron reduction of dioxygen to water (Sakurai and Kataoka, 2007). These proteins require dioxygen for activity and hence are inactive under anaerobic conditions. Prominent members of this protein family are the fungal laccases, which are implicated in a variety of processes including lignin degradation and biosynthesis, pigmentation, sporulation, and pathogenesis (Sakurai and Kataoka, 2007). As a multi-copper oxidase, *E. coli* CueO is proposed to contribute to copper-detoxification by converting Cu(I) to less toxic Cu(II) in the periplasm (Outten *et al.*, 2001), although its precise role remains to be established.

CueO is known to bind four copper ions and is exported to the periplasm via the TAT pathway (for prefolded proteins) where it exhibits copper-inducible oxidase activity (Grass and Rensing, 2001; Outten *et al.*, 2001; Roberts *et al.*, 2002; Singh *et al.*, 2004). The molecular chaperone DnaK is essential for TAT targeting of CueO, and is required after protein-folding (Graubner *et al.*, 2007). However, it is not known whether CueO acquires copper in the periplasm or is exported pre-metallated and thereby contributes to copper-export from the cytosol. Mutants lacking *cueO* show a slight reduction in copper-tolerance under aerobic conditions, but not in the absence of dioxygen, and CueO can protect periplasmic enzymes from copper-induced damage (Grass and Rensing, 2001; Outten *et al.*, 2001). In addition to Cu(I), CueO can oxidize a variety of other compounds in the presence of copper, including the catechol iron siderophore enterobactin (Grass *et al.*, 2004; Kim *et al.*, 2001). By oxidizing enterobactin, CueO may protect *E. coli* from copper-toxicity by preventing enterobactin mediated reduction of Cu(II) to Cu(I). Substrate specificity of CueO appears to result from a methionine-rich helix which obstructs the substrate-binding site in the absence of elevated Cu(I) (Kataoka *et al.*, 2007; Li *et al.*, 2007).

In addition to CueO, some *E. coli* strains possess a second multi-copper oxidase PcoA as part of the plasmid-encoded *pco* copper-resistance system (Brown *et al.*, 1992). CueO-related proteins have also been associated with copper-resistance in a number of other bacteria and include CopA from *Pseudomonas syringae* pathovar *tomato* associated with the plasmid encoded *cop* copper-resistance operon (Cha and Cooksey, 1993), CutO from the photosynthetic purple bacterium *Rhodobacter capsulatus*

(Wiethaus *et al.*, 2006), and CuiD from *Salmonella typhimurium* which provides resistance to hydrogen peroxide in addition to copper (Lim *et al.*, 2002). Unlike *E. coli* CueO, the multi-copper oxidases from *R. capsulatus* and *S. typhimurium* are reported to somehow provide copper-tolerance under both aerobic and anaerobic conditions (Espariz *et al.*, 2007; Wiethaus *et al.*, 2006).

c. P_{1B}-type ATPase CopA *E. coli* CopA has a role in copper-export under both aerobic and anaerobic conditions (Outten *et al.*, 2001). Mutants lacking *copA* are hypersensitive to copper in the presence or absence of oxygen (Outten *et al.*, 2001; Rensing *et al.*, 2000), while tolerance to other metals (silver, cadmium, zinc, and lead) is unaffected, at least under aerobic conditions (Rensing *et al.*, 2000). Furthermore, these mutants show decreased copper efflux and accumulate copper (Petersen and Møller, 2000), while everted-vesicles from cells expressing *copA* show ATP-coupled accumulation of copper in the presence of the reductant dithiothreitol (Rensing *et al.*, 2000). CopA can catalyze ATP hydrolysis to form an acylphosphate intermediate (inhibited by vanadate) in the presence of Cu(I) or Ag(I), but not Zn(II), Cu(II) or Co(II) (Fan and Rosen, 2002), and the use of a luminescent biosensor under the control of the *copA* promoter also indicates a role for CopA in regulating silver levels, in addition to copper, but not gold (Stoyanov *et al.*, 2003).

CopA possesses two amino-terminal metal-binding domains, neither of which are required for formation of the acylphosphate intermediate or copper-resistance and transport (Fan and Rosen, 2002; Fan *et al.*, 2001). It is tempting to speculate that the possession of two such domains contributes to the metal-selectivity of this transporter, for example by forming nonproductive complexes (Liu *et al.*, 2007) with highly thiophilic metals such as gold. Interestingly, *S. typhimurium* possesses two P_{1B}-type ATPases, CopA and GolT (Fig. 8.2), both of which contribute to copper-resistance (Espariz *et al.*, 2007). However, CopA (the *E. coli* CopA orthologue) appears to be the principal copper-exporter and provides only slight resistance to gold, whereas GolT with a single amino-terminal metal-binding domain is primarily associated with gold-resistance (Checa *et al.*, 2007; Espariz *et al.*, 2007) (refer to Section II.D.1). The Cu(I)-exporting P_{1B}-type ATPases CopA from *B. subtilis* possesses two amino-terminal metal-binding domains (Banci *et al.*, 2002a) whereas a single domain is found in PacS from *Synechocystis* PCC 6803 (Tottey *et al.*, 2001). CopB from *E. hirae* lacks an amino-terminal G*X*X*C*X*X*C metal-binding motif although a histidine rich region is present which likely constitutes a metal-binding site (Solioz and Stoyanov, 2003).

2. The *E. coli cus* system

While the *cue* system confers copper-tolerance under moderate to high copper levels, the *cus* system is only required under conditions of extreme copper stress and is particularly important in anaerobic environments

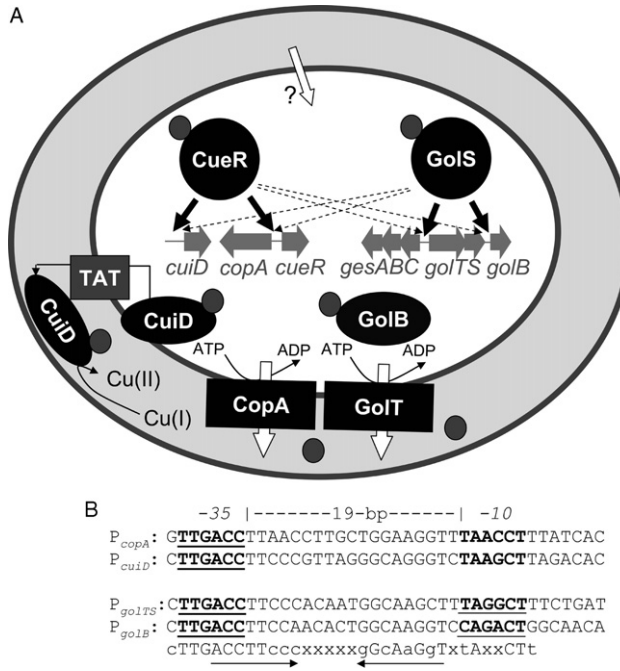

FIGURE 8.2 *S. typhimurium* possesses two MerR-type copper-responsive regulators CueR and GolS. (A) In response to elevated copper levels, CueR triggers expression of a copper-exporting P_{1B}-type ATPase CopA and a multicopper oxidase CuiD associated with copper-resistance, while GolS triggers expression of a second P_{1B}-type ATPase GolT and a CopZ/Atx1-like protein GolB. GolS, GolT, and GolB have primarily been associated with gold-sensing and -resistance (Checa *et al.*, 2007; Espariz *et al.*, 2007). (B) An alignment of the operator-promoter regions of the target genes of CueR (P_{copA} and P_{cuiD}) and GolS (P_{golTS} and P_{golB}) showing a consensus motif (below) containing a region of dyad symmetry (arrows) and RNA polymerase consensus −10 and −35 binding sequences (bold and underlined). The −10 and −35 sequences are separated by a long (19-bp) spacer region typical of promoters under the control of MerR-family transcriptional activators. It remains to be tested whether or not there is an overlap in the target promoters of the two sensors.

(Outten *et al.*, 2001). The *cusCFBA* structural genes are induced in response to highly elevated copper levels by the CusRS two-component sensory system which typically monitors stress at the cell envelope and is thought to respond to Cu(I). No expression of *cusCFBA* is detected in mutants lacking *cusR* (Outten *et al.*, 2001). In the presence of oxygen, Outten and co-workers (2001) have shown that while half maximal expression from the *cueO* and *copA* promoters occurs at c. 10 μM copper, half maximal expression from the *cusCFBA* promoter occurs at much

higher levels of copper (200 μM), suggesting that the *cus* system is only activated once the *cue* system is overwhelmed. In contrast, under anaerobic conditions half maximal expression from the *cusCFBA* promoter occurs at lower copper levels (70 μM) than observed aerobically yet the level of copper required for half maximal induction of *cueO* is increased (60 μM) implying a greater role for *cus* under anaerobic conditions and possibly relating to a shift in the periplasmic copper ratio to the Cu(I) state. Furthermore, mutants lacking *cusR* or *cusA* have normal copper-tolerance under aerobic conditions (Franke *et al.*, 2001; Outten *et al.*, 2001) but copper-sensitivity and increased copper accumulation is detected for the *cusR* mutant under anaerobic conditions, consistent with disruption of anaerobic copper-export (Outten *et al.*, 2001). However, in the presence of oxygen, *cueR/cusR* double mutants do show a substantial reduction in copper-tolerance, compared to either single mutant or wild-type cells, and deletion of any of the four *cusCFBA* structural genes in conjunction with *cueO* also causes loss of copper-tolerance (Franke *et al.*, 2003; Grass and Rensing, 2001). Hence, the *cus* system can play a role in aerobic conditions when the *cue* system cannot be induced or is impaired. A role for the *cus* system in providing silver-resistance has also been shown (Franke *et al.*, 2001).

The products of the *cusC*, *cusB*, and *cusA* genes are thought to form a multiunit transport complex that spans both membranes and the periplasmic space (Outten *et al.*, 2001). CusC is an outermembrane protein with homology to the TolC stress-response protein (Koronakis *et al.*, 2000), CusB belongs to the membrane fusion protein family which are anchored into the cytoplasmic membrane with a long periplasmic-spanning domain (Zgurskaya and Nikaido, 1999), and CusA is a member of the RND family of proton antiporters associated with the export of a range of substrates including metal ions (Saier *et al.*, 1994). Clusters of methionines within the deduced periplasmic domains of CusA and CusB have been shown to be required for copper-resistance and, in the case of CusB, Cu(I)-binding (Bagai *et al.*, 2007; Franke *et al.*, 2003). The CusCBA complex is proposed to transport Cu(I) from the cytosol across the cell envelope into the extracellular milieu.

CusF is a small (10 kDa) periplasmic protein which binds a single Cu(I) (or Ag(I)) ion and interacts with both CusC and CusB (Franke *et al.*, 2003). The recent elucidation of the structure of the Cu(I) bound form of CusF has revealed a new metal-binding site in which Cu(I) is tetragonally displaced from a Met_2His ligand plane toward a tryptophan which involves cation-π interactions (Xue *et al.*, 2008). The tryptophan in CusF appears to block access to its bound Cu(I), while the weaker cation-π interactions versus a coordinate covalent bond have implications for metal transfer, for example with the methionine clusters of CusB and/or CusA. This novel site in CusF has therefore been proposed to meet the

demands of controlling metal exchange whilst preventing adventitious redox reactions (Xue *et al.*, 2008).

D. Sensors of elevated copper levels

Copper-responsive transcriptional regulators detect surplus copper-ions and modulate transcription of genes with roles in copper-uptake, -channeling, -efflux, or -detoxification. In *E. coli*, CueR is the intracellular Cu(I) sensor that activates expression of CopA and CueO, while the two component CusRS system senses periplasmic Cu(I) and triggers expression of CusCFBA. In addition, some strains possess a second two component Cu(I) sensor, PcoRS, as part of the plasmid-borne *pco* resistance determinant. Bacteria that lack CueR homologues may use other, structurally distinct, intracellular copper sensors that functionally substitute for CueR, such as CopY in *E. hirae* and CsoR in *Mycobacterium tuberculosis* and *B. subtilis* (Fig. 8.1).

1. MerR-family copper-sensors

CueR belongs to the MerR-family of transcriptional activators (refer to Section II.C.1a). Other bacteria that possess copper-tolerance systems under the control of CueR-like proteins include *Pseudomonas putida* (Adaikkalam and Swarup, 2002), *Rhizobium leguminosarum* (Reeve *et al.*, 2002), and *S. typhimurium* (Checa *et al.*, 2007; Espariz *et al.*, 2007; Kim *et al.*, 2002). In the case of *S. typhimurium*, two CueR-like proteins are present (Fig. 8.2), one designated SctR (Kim *et al.*, 2002) or CueR (Espariz *et al.*, 2007) and the second designated GolS (Checa *et al.*, 2007). The former is an orthologue of *E.coli* CueR (91% identity) and has been shown to mediate copper-responsive expression of the multi-copper oxidase CuiD and P_{1B}-type ATPase CopA, conferring copper-resistance (Espariz *et al.*, 2007; Kim *et al.*, 2002; Lim *et al.*, 2002). GolS on the other hand, regulates expression of a second P_{1B}-type ATPase GolT, a CopZ/Atx1-like copper-chaperone GolB, and a CBA family drug-efflux system GesABC, and is primarily associated with gold-resistance (Checa *et al.*, 2007; Pontel *et al.*, 2007). Elevated expression of GolT, GolS, and GolB occurs in response to copper and gold, while expression of GesABC is only induced by gold (Pontel *et al.*, 2007). However, a role for GolS in gold-resistance is somewhat unexpected from a biological point of view and the physical chemistry of Au(I) ions would be consistent with them mimicking Cu(I) in the metal-binding sites of these proteins as has been suggested for *E. coli* CueR (Changela *et al.*, 2003; Stoyanov and Brown, 2003). Hence, it remains possible that both *S. typhimurium* CueR and GolS have evolved to sense Cu(I) under different surplus copper conditions and by causing differential expression of the *cue* and *gol* systems may allow subtle adaptation to excess of these metal ions in the cytosol.

2. CopY-family copper sensors

The *copYZAB* operon in *E. hirae* is regulated by the copper-responsive repressor CopY (Odermatt and Solioz, 1995). The current model (Portmann *et al.*, 2004) indicates that at low copper concentrations CopY is present as a Zn(II) containing homodimer and binds to the *copYZAB* operator-promoter region repressing transcription (Cobine *et al.*, 1999). In the presence of copper, the copper-chaperone donates Cu(I) to CopY, displacing Zn(II), alleviating DNA-binding and allowing transcription of *copYZAB* to proceed. The carboxyl-terminal region of CopY possesses a C*X*C*XXXX*C*X*C metal-binding motif. Each Zn(II) ion is coordinated to all four cysteines in the repressing form of CopY, and is replaced by two Cu(I) ions in the induced, non DNA-binding form (Cobine *et al.*, 2002).

CopY-like regulatory proteins are not widely distributed amongst bacteria but have been associated with the copper-resistance determinants of *Enterococcus faecium* (Hasman *et al.*, 2006), *Lactococcus lactis* IL1403 (Magnani *et al.*, 2008), *Streptococcus mutans* (Vats and Lee, 2001), and *Streptococcus gordonii* (Mitrakul *et al.*, 2004). The amino-terminal half of CopY has structural similarity to the BlaI and MecI repressors that belong to the ''winged helix'' family of proteins and mediate resistance to β-lactam antibiotics (Portmann *et al.*, 2006). Consistent with this, CopY, BlaI, and MecI bind to similar sequences within the promoters of their target genes with the consensus binding motif TACA*XX*TGTA (Portmann *et al.*, 2006).

3. CsoR-family copper sensors

The recently described intracellular copper sensor CsoR from *M. tuberculosis* is the founding member of what appears to be a large family of bacterial Cu(I)-responsive repressors (Liu *et al.*, 2007). *M. tuberculosis csoR* is part of the *cso* operon which also contains a small open reading frame *Rv0968* of unknown function and *ctpV* encoding a P_{1B}-type ATPase with sequence features of a Cu(I)-transporter. Expression of the *cso* operon is induced by elevated copper levels and to a lesser extent silver, but not other metals (lead, zinc, cadmium, nickel, or cobalt), consistent with a role in copper-export (Liu *et al.*, 2007; Ward *et al.*, 2008). CsoR binds to a region of the *cso* operon containing dyad symmetry and DNA-binding is weakened upon Cu(I)-binding, thus alleviating repression in elevated metal (Liu *et al.*, 2007). The structure of Cu(I)-CsoR reveals a homodimer with two Cu(I) ions, each Cu(I) bound in a triganol coordination complex involving two cysteines and a histidine in a subunit bridging site (Liu *et al.*, 2007).

CsoR-related sequences are widely distributed in bacterial genomes and a second Cu(I)-sensing CsoR has recently been characterized from *B. subtilis* which mediates copper-responsive expression of *copZ* and *copA* (Smaldone and Helmann, 2007). The majority of identified CsoR-related

sequences appear to possess all three Cu(I)-binding ligands, consistent with Cu(I)-sensing (Liu *et al.*, 2007). However, some of the more distantly related sequences lack all three ligands raising the intriguing possibility that they are involved in sensing metals other than Cu(I) or other substrates such as organic molecules.

E. Copper-chaperones

In eukaryotes, highly conserved proteins are used for copper-import, copper-channeling, or for ejecting copper from the cell. These include copper-chaperones that direct copper to its three destinations: copper, zinc superoxide dismutase; mitochondrial cytochrome *c* oxidase; and the *trans*-Golgi apparatus for incorporation into proteins (O'Halloran and Culotta, 2000). Specific interactions and metal exchange between the copper-chaperones and their targets allow proteins to acquire their copper cofactor under physiologically low metal conditions *in vivo* (Carroll *et al.*, 2004). The trafficking of copper into the *trans*-Golgi involves the copper-chaperone, Atx1 in yeast or Atox1 (Hah1) in humans, interacting with, and donating copper to, a copper-specific P_{1B}-type ATPase for transport across the membrane (reviewed in O'Halloran and Culotta, 2000; Singleton and Le Brun, 2007). Similarly, the copper-chaperone CCS is the primary source of copper for yeast and mammalian copper, zinc superoxide dismutase (Sod1) (Culotta *et al.*, 1997; Rae *et al.*, 2001; Wong *et al.*, 2000). CCS physically interacts with Sod1 to insert a copper ion into the active site (Culotta *et al.*, 1997; Gamonet and Lauquin, 1998; Schmidt *et al.*, 2000; Torres *et al.*, 2001), but also has a role in catalyzing the oxidation of a conserved disulphide that is essential for Sod1 function (Furukawa *et al.*, 2004). Only a few mammalian cell types are able to support a low level of copper incorporation into Sod1 in the absence of their copper-chaperone (Culotta *et al.*, 1997; Wong *et al.*, 2000) consistent with the assertion that most cells lack freely available copper and a pool of free copper ions is not used in physiological activation of metallo-enzymes (Rae *et al.*, 2001). *E. coli* lacks sequences with similarity to known copper-chaperones. However, some bacteria possess Atx1 homologues, for example Atx1 from *Synechocystis* PCC 6803 (Tottey *et al.*, 2002), CopZ from *B. subtilis* (Banci *et al.*, 2001; Radford *et al.*, 2003), CopZ from *E. hirae* (Magnani and Solioz, 2005), and GolB from *S. typhimurium* (Checa *et al.*, 2007). Like their eukaryotic counterparts, the bacterial Atx1 and CopZ proteins are suggested to play a role in preventing adventitious Cu(I)-binding and contribute to cytosolic Cu(I)-export.

1. Bacterial copper-chaperones: CopZ/Atx1

The cyanobacterial Atx1 acts with two P_{1B}-type ATPases, CtaA and PacS at the cytoplasmic and thylakoid membranes, respectively, to traffic copper to the thylakoid for cytochrome caa_3 oxidase activity and

photosynthetic electron transport via plastocyanin (Tottey *et al.*, 2002). *B. subtilis* CopZ acts with copper-exporting CopA (Banci *et al.*, 2003; Radford *et al.*, 2003). *E. hirae* CopZ interacts and exchanges copper with the CopA transporter, which appears to import copper (Multhaup *et al.*, 2001), and may also interact with copper-exporting CopB (Solioz and Stoyanov, 2003). A role for *E. hirae* CopZ in routing copper to the cytoplasmic copper sensor CopY, to alleviate CopY-mediated repression of the *copYZAB* operon, has also been demonstrated (Cobine *et al.*, 1999) (Fig. 8.1). Differential protease-mediated degradation of apo-CopZ and Cu(I)-CopZ in *E. hirae* is also suggested to contribute to regulation (Magnani and Solioz, 2005).

The Atx1/CopZ proteins adopt a very similar structure to the amino-terminal metal-binding domains of the P_{1B}-type ATPases with typical βαββαβ ferredoxin-like folding and a G*XX*C*XX*C metal-binding motif present on a flexible solvent-exposed loop (Arnesano *et al.*, 2001; Banci *et al.*, 2003, 2004; Rosenzweig *et al.*, 1999). Cu(I)-bridging between the cysteines of donor and acceptor proteins mediates protein–protein interactions and transfer, with complementary charged surfaces also contributing to these interactions (Banci *et al.*, 2006b). The formation of tricoordinate Cu(I)-bridged intermediates is presumed to be a general feature in the Cu(I) transfer mechanism and has been reviewed extensively elsewhere (Huffman and O'Halloran, 2001; Singleton and Le Brun, 2007; Tottey *et al.*, 2005).

2. *B. subtilis* Sco1

Cytochrome caa_3 oxidase activity in yeast and mammals requires the action of Sco proteins, in addition to the mitochondrial copper-chaperone Cox17, to assemble the di-copper A site at the inner mitochondrial membrane (Banci *et al.*, 2007). *B. subtilis* possesses a single Sco homologue (*Bs*Sco or YpmQ), which is required for the activity of cytochrome caa_3 oxidase but not of the aa_3-type (quinol) oxidase, with only a copper B site, consistent with a similar role to its mitochondrial counterparts (Balatri *et al.*, 2003; Mattatall *et al.*, 2000). Genes encoding Sco proteins have also been identified in the genomes of a number of other bacteria, some of which lack a cytochrome oxidase with a di-copper A site, and a more general role for Sco proteins as copper-chaperones for other metalloenzymes has been suggested (Banci *et al.*, 2007).

III. COPPER AND BACTERIAL PATHOGENICITY

During infection of a host, bacterial pathogens must sense and respond rapidly to a wide variety of fluctuating conditions, such as changes in the availability of metal ions and exposure to reactive oxygen species. Indeed, copper homeostatic mechanisms are increasingly being linked to bacterial

pathogenicity, but the nature of their role in virulence is largely unknown and is anticipated to vary depending on the site of infection and the adaptive strategies used by different bacteria. Changes in copper levels have been detected in the livers and spleens of animals infected with a range of pathogens, including viruses, bacteria, and trypanosomes (Crocker *et al.*, 1992; Ilbäck *et al.*, 2003; Matousek de Abel de la Cruz *et al.*, 1993; Tufft *et al.*, 1988). These changes have been reported to be up, down, or even biphasic during the course of infection. Within the gastrointestinal tract and gall bladder, the concentration of copper may be high due, at least in part, to the action of the Wilson P_{1B}-type ATPase (ATP7B) which transports copper from the liver into bile for excretion (Lutsenko *et al.*, 2007). Furthermore, the toxicity of copper is likely to be increased due to the acidic anaerobic conditions. Thus, within the gastrointestinal tract, bacterial pathogens likely have a requirement for copper-resistance.

Pathogen killing within professional phagocytes involves the one-electron reduction of oxygen by the respiratory burst (NADPH) oxidase generating superoxide (Fig. 8.3). This is highlighted by the rare syndrome of chronic granulomatous disease (X-CGD) in which loss of respiratory burst oxidase function results in an inability of patients to combat microbial infections. For some bacterial pathogens, such as *S. typhimurium*, protection from the products of the respiratory burst oxidase is dependent upon the function of extracytoplasmic copper and zinc superoxide dismutases (SodC proteins) (Battistoni, 2003). These enzymes catalyze the dismutation of superoxide into oxygen and hydrogen peroxide by the alternate reduction and oxidation of a copper ion that constitutes the active site. Highly virulent *S. typhimurium* strains possess multiple *sodC* genes (Battistoni, 2003). These genes are dispensable for growth under normal laboratory conditions but are crucial for bacterial–host interactions (Ammendola *et al.*, 2005; Battistoni *et al.*, 2004; De Groote *et al.*, 1997; Fang *et al.*, 1999; Farrant *et al.*, 1997; Sansone *et al.*, 2002; Uzzau *et al.*, 2002). The ability of these proteins to acquire copper within the periplasm is therefore crucial for virulence. However, within the phagosome it is likely that bacterial pathogens must also avoid copper-mediated toxicity. Indeed, the toxic potential of copper is now thought to constitute a crucial component of the respiratory burst killing mechanism (Fig. 8.3) and an increase in copper levels is detected in phagosomes isolated from macrophages infected with *S. typhimurium* (unpublished). Consistent with these findings, the ability of phagocytes to kill ingested bacteria is reduced in copper deficiency (Percival, 1998), and copper deficient animals are highly vulnerable to bacterial infection while protection is restored by copper supplementation (Suttle and Jones, 1989). The contribution of bacterial copper trafficking proteins, such as *S. typhimurium* CopA, GolT, and GolB, in avoiding copper mediated toxicity in the phagosome and supplying copper to the periplasmic SodC proteins remains to be established.

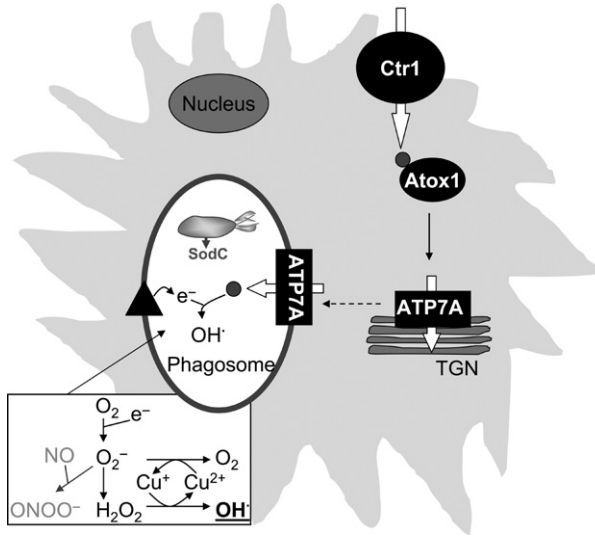

FIGURE 8.3 The toxic potential of copper may be exploited by phagocytes as a crucial component of the oxidative burst. Copper enters the cell through the high-affinity copper transporter Ctr1 and binds to cytosolic copper-chaperones. Atox1 shuttles Cu(I) to the Cu(I) transporting P_{1B}-type ATPase ATP7A at the *trans*-Golgi network (TGN) where it supplies Cu(I) for maturing secreted copper proteins. In elevated copper levels, ATP7A may be relocated to both perinuclear vesicles and the phagosome (Leary and Winge, 2007). Within the phagosome, Cu(I) may contribute to the bacteriocidal action of the respiratory burst oxidase (triangle) by catalyzing Fenton chemistry causing the production of highly toxic hydroxyl radicals ($OH^{\cdot}$). The antimicrobial activity of the respiratory burst may also be increased when combined with the activity of nitric oxide (NO) synthase (also present in phagocytes) forming highly toxic peroxynitrite ($ONOO^{-}$). By catalysing the dismutation of superoxide (O_2^{-}) into oxygen and hydrogen peroxide (H_2O_2), copper-requiring bacterial SodC proteins have a role in providing resistance to reactive oxygen and nitrogen species, although in the presence of Cu(I) this may contribute to Fenton chemistry.

Consistent with the requirement for different bacterial pathogens to adapt to changing copper levels during infection, genes encoding copper-exporting P_{1B}-type ATPases have been identified amongst the major genes expressed during infection of lungs and/or macrophages by the intracellular pathogens *M. tuberculosis* and *S. typhimurium* (Graham and Clark-Curtiss, 1999; Heithoff *et al.*, 1997; Talaat *et al.*, 2004), and by *in vivo* expression technology (IVET) using *Staphylococcus aureus* in the murine renal abscess model (Lowe *et al.*, 1998). Furthermore, disruption of the gene for a copper-exporting P_{1B}-type ATPase has been shown to reduce the pathogenicity of *Pseudomonas aeruginosa* in mice (Schwan *et al.*, 2005) while mutants of *L. monocytogenes* DRDC8 lacking a P_{1B}-type ATPase,

CtpA, thought to import copper, were cleared much more rapidly from the livers of infected mice than the wild type strain (Francis and Thomas, 1997a). Hence, copper homeostasis and adaptation to different copper stresses is clearly important for bacterial pathogens surviving and growing in different host tissues.

IV. COPPER AS A BIOCIDE

The cytotoxic properties of copper have been exploited by man since ancient civilizations. Its use as a biocide is recorded as far back as c. 2400 BC in an ancient Egyptian medical text (Smith Papyrus) which describes copper as a sterilizing agent for drinking water and wounds. More recent exploitation of copper cytotoxicity includes the protection of ship's hulls by copper sheathing or by antifouling paints containing copper (subsequently replaced by tin compounds), its use in algicides and wood preservatives, and incorporation of copper into fabrics used for clothing and bedding to prevent microbial growth. Copper is also commonly used as a disinfectant for veterinary purposes and in the food industry. Copper is added to animal feed to inhibit bacterial populations in the intestinal tract and for disinfecting the claws of cattle (Aarestrup and Hasman, 2004), as well as promoting growth in both industrial and organic pig production (Edmonds *et al.*, 1985; Hasman *et al.*, 2006). In addition, the toxic effects of copper are used in agriculture to control bacterial and fungal diseases on a variety of crops (Aarestrup and Hasman, 2004).

The emergence of nanoscience and nanotechnology in the last decade has also provided the basis for examining the bacteriocidal effects of copper nanoparticles with promising results (Cioffi *et al.*, 2005; Ruparelia *et al.*, 2007; Yoon *et al.*, 2007). These nanoparticles can be immobilized and coated onto surfaces and used in a range of applications such as medical devices, water treatment, and food processing. Furthermore, there is considerable new interest in the use of copper alloys as a way of controlling drug-resistant bacterial infections such as methicillin-resistant *S. aureus* (MRSA). A recent study (Noyce *et al.*, 2006) demonstrated that strains of MRSA were completely killed within 90 min on pure copper surfaces whereas they can live for up to three days on stainless steel, thereby providing the rationale for replacing stainless steel door handles and taps with copper ones in hospitals (http://www2.uhb.nhs.uk).

Clearly, a concern is that the widespread use of copper as a biocide will select for copper-resistant strains of bacteria. Indeed, some bacterial pathogens are known to have acquired plasmid-borne copper-resistance mechanisms (Brown *et al.*, 1992), in addition to the chromosomal copper homeostatic mechanisms described above. Well documented examples are an *E. coli* strain found in the feces of copper-fed pigs and *P. syringae*

pathovar *tomato* isolated from copper exposed plants where copper-resistance was attributed to the plasmid encoded *pco* and *cop* operons, respectively, which encode related proteins involved in periplasmic copper handling (Brown *et al.*, 1995; Cooksey, 1994; Lee *et al.*, 2002; Rensing and Grass, 2003) (refer to Section II.C). Of particular concern is the finding that selection of copper-resistance may result in co-selection of antibiotic-resistance determinants (Hasman *et al.*, 2006). Hence, a high degree of caution needs to be applied to the widespread use of copper to control bacteria in the environment and hospitals.

V. CONCLUDING REMARKS

The copper homeostatic mechanisms described herein ensure that bacteria are able to satisfy the needs of copper-requiring proteins whilst avoiding any excess that would otherwise engage in adventitious interactions. The use of copper-transporting P_{1B}-type ATPases to export copper from the cytosol appears as a common theme amongst the different bacterial groups. All bacterial copper-dependent enzymes known to date are extracytoplasmic and for the vast majority it is not known how they acquire their copper. However, it is tempting to speculate that with analogy to eukaryotic cells, bacteria use P_{1B}-type ATPases to mediate transport of available copper ions from the cytosol into a more specialized compartment, i.e., the cell envelope (or thylakoid), for incorporation into proteins whilst protecting the cytosol from copper-toxicity. Many bacteria also possess Atx1/CopZ-like copper-chaperones which may further contribute to copper-trafficking and -resistance. If these housekeeping copper-homeostatic systems are overwhelmed, some bacteria can invoke additional systems providing further resistance to increased copper stress.

Copper homeostatic mechanisms are increasingly being linked to bacterial pathogenicity. However, the basis for this remains largely unknown and is anticipated to vary depending on the site of infection and the diverse survival tactics used by different bacteria. Understanding the environmental challenges that occur in an infected host and the mechanisms bacteria use to adapt to these challenges offers possibilities for the design of drugs that disrupt such mechanisms thereby allowing host clearance of the infecting organism. Furthermore, there is a new appreciation that copper may be used as a means of controlling bacterial growth, particularly with respect to controlling antibiotic-resistant strains of bacteria (such as MRSA) in clinical settings. An understanding of the mechanisms of copper homeostasis and resistance in bacterial pathogens is therefore needed to better inform future control strategies and offer the possibility of improving current copper-based treatments. Both Ag(I) and Cu(I) are chemically similar and in many cases appear to be treated interchangeably; many of the proteins involved

in copper-resistance also protect against silver-toxicity. Clearly this has implications regarding the wide and uncontrolled use of both copper and silver products in the environment and the potential for the development of more resistant bacteria.

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CHAPTER 9

Pathogen Surveillance Through Monitoring of Sewer Systems

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I. INTRODUCTION

It is now possible to monitor sewage systems for pathogen occurrence in a community. An epidemiological approach to monitoring sewer systems is especially relevant for an early warning of pathogens used as biological weapons. In many situations, bioterrorist contamination events will result in the pathogen shedding to wastewater before a community level epidemic begins. Detecting the organism early allows the governments to respond on time and eliminate a potential catastrophe.

A. Monitoring for human pathogens in sewage

Monitoring of human pathogens in sewage is possible because they may be excreted in a range of bodily fluids, skin, and hair during active infection (Feachem *et al.*, 1983). All of these materials will find their way into sewage systems during the process of waste elimination (toilet flushing) and cleaning (e.g., bathing, hand washing). In addition to release during active infection, pathogens can be washed into sewage systems from cleaning of indoor (floor washing, kitchen sink use) and outdoor (auto washing, driveway cleaning, storm water collection) facilities. Thus, sewer systems collect pathogens from over a wide area to a common carrier, where they are transported to a central facility for processing.

Wastewater presents a time dynamic collection point where many physical, chemical, and biological substances of our society are brought to a central location. Monitoring of centralized wastewater allows detection of intentional, natural, or accidental contamination events. Because of recent bioterrorism concerns in the U.S., routine monitoring is potentially useful since it can result in better preparedness of utilities and the public health response system (Meinhardt, 2005). The qualitative microbial risk assessment (QMRA) framework can be used as a tool to develop and interpret this type of wastewater monitoring system. Because the threat level drives the risk assessment analysis, a monitoring system should be coordinated with findings from modeling studies on the survival and dispersion of contaminants (Kim *et al.*, 2007; Romero *et al.*, 2008; Sinclair *et al.*, 2008), the contaminant point of introduction (Danneels and Finley, 2004), the health risk (Haas *et al.*, 1999), and the locations of early warning systems/sensors in wastewater and water treatment systems (Murray *et al.*, 2004).

A recent U.S. National Research Council study called for more resilient design/operation of wastewater and drinking water systems (USNRC, 2007) to improve response and recovery from adverse water quality events in collection systems, water distribution systems, and water/wastewater treatment systems. Monitoring programs for pathogens or surrogates could potentially aid in the accomplishment of these goals.

The aim of this review of published literature and reports is to assess the feasibility of monitoring sewage systems as an early warning system for the release of pathogens from an intentional, natural, or accidental biological contamination event. We address issues from a QMRA perspective and explore methods to detect and monitor pathogens in wastewater. The review presents our conclusions on: (1) the potential biological agents that might be released into a sewage system, (2) the likely background level of those agents in sewage, (3) laboratory methods and detection, and (4) the probability of detecting select biological agents in sewage.

II. POTENTIAL BIOLOGICAL AGENTS IN SEWAGE

A wide variety of pathogenic organisms pass through municipal wastewater treatment systems. One study found that a single toilet flush containing poliovirus was detectable at a nearby treatment plant for more than 4 days (Ranta *et al.*, 2001). The toilet flush study was designed to replicate the number of virus released from an infected individual. Pathogenic microorganisms can also grow in the host but not produce sickness in the infected host. It is estimated that 50% or less of those individuals infected with enteric viruses or bacteria actually become ill (Haas *et al.*, 1999). In the case of some respiratory pathogens, 90% or more of the persons infected will become ill (Belshe, 1991).

During the growth of the organism in the host, the organism will be found in various organs and bodily fluids. Organisms transmitted by the fecal–oral route are usually excreted in large numbers in the feces, since the initial or primary site of replication is in the intestinal tract. However, this does not preclude their replication in other parts of the body. For example, enteroviruses (e.g., poliovirus) will replicate in nerve tissue causing paralytic disease, while Hepatitis A virus will replicate in the liver causing damage there (Belshe, 1991). Respiratory infections are usually the result of replication of the organism in the nose, throat, or lungs. Infection of other organs of the body often leads to the presence of the organisms in the blood and then the urine after their elimination by the kidneys. This explains the occurrence of insect-borne encephalitis viruses and enteric viruses in the urine (Pichichero *et al.*, 1998). Any type of infection (Fig. 9.1) within a community is likely to lead to pathogen excretion in bodily fluids/substances and therefore, transported into the community sewage system.

This review considers biological agents prioritized by the Centers for Disease Control (CDC) as potential biological weapons that could be used by terrorists (Table 9.1 and 9.2). They are listed in three categories (i.e., A, B, and C) of decreasing concern. Category A agents require the most intensive public preparedness efforts due to the potential for mass

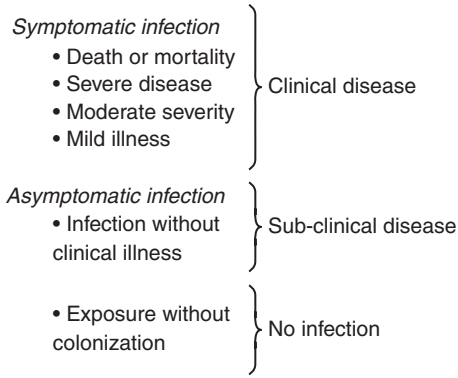

FIGURE 9.1 Outcomes of exposure to a microbial infection.

causalities, public fear, and civil disruption. Category B agents are also moderately easy to spread, but have lower mortality rates. Category C agents do not present a high public health threat, but could emerge as future threats (Rotz *et al.*, 2002). Many other pathogenic agents are present in sewage, but not on the CDC select agent list. Table 9.2 lists some common blood and respiratory agents and emerging pathogens, all of which could potentially be engineered for mass dissemination and detected through monitoring of wastewater. The methods described in this paper apply to many other pathogens and are not limited to those agents listed in Tables 9.1 and 9.2.

A. Human pathogens secreted in bodily fluids

A literature search was conducted to determine the occurrence of the agents in bodily fluids, feces, skin, and sewage. As indicated in Table 9.3 and a previous publication (Sinclair *et al.*, 2008), many select agents may occur in bodily excretions or secretions even though this may not be their primary site of replication. It would appear that all of the viral agents are excreted in the urine and most of the bacterial agents in the feces or saliva. Since none of the organisms cause enteric infections they have seldom been sought in sewage, however, *Bacillus anthracis* and *Yersina pestis* (plague) have been detected in sewage. The source of *B. anthracis* spores in the sewage was believed to be from an African import tannery operation (Perone and Gelosa, 1982) and presumably not from enteric infections, which would normally result in the presence of spores in the feces. Category B agents differ in that, and they include many enteric pathogens which are excreted in large numbers in the feces (Table 9.3). All of the other agents in this category appear to be excreted in the feces; many of the viral agents are excreted in the urine. No studies

TABLE 9.1 The center for disease control select agents (Rotz *et al.*, 2002)

Category A	Category B	Category C
Anthrax	Brucellosis	Nipah virus
Bacillus anthracis	*Brucella abortus*	Tick-borne HFV
Botulism	Water and Food-borne	Crimean-Congo HFV
Clostridium	agents	Tick-borne encephalitis
botulinum	Enteroviruses	viruses
Plague	Poliovirus and	Yellow fever
Yersinia pestis	Rotavirus	Multidrug resistant TB
Smallpox	Salmonellosis	Influenza
Variola major	*Salmonella*	Other Rickettsias
Tularemia	Caliciviruses	Rabies
Francisella	Hepatitis A virus	
tularensis	Protozoan parasites	
Hemorrhagic	*Cryptosporidium*	
fever virus[a]	*parvum*	
Arenaviridae	*Giardia lamblia*	
Bunyaviridae	Toxoplasma	
Filoviridae	Microsporidium	
Flaviviridae	Glanders	
Lassa fever	*Burkholderia mallei*	
Hantavirus	Psittacosis	
Dengue fever	*Chlamydia psittaci*	
Ebola	Q fever	
Marburg	*Coxiella burnetii*	
	Typhus fever	
	Rickettsia prowazekii	
	Viral Encephalitis	
	West Nile	
	La Crosse	
	Venezuelan equine	
	encephalitis	
	Japanese encephalitis	

[a] Hemorrhagic fever virus (HFV).

were found that report examining sewage for their presence. The Category C viral agents appear to be excreted in the saliva and urine (Tables 9.3 and 9.4). No references for the presence of these agents in sewage could be found. Some typical blood-borne agents such as Hepatitis B virus (Alter *et al.*, 1977) and Human Immuno-Deficiency virus (Levy, 1989) have been detected in sewage by molecular methods (Table 9.4). The coronavirus, which causes Severe Acute Respiratory Disease (SARS), is

TABLE 9.2 Nonenteric pathogens found in sewage and other emerging agents of concern

Nonenteric agents	Emerging agents
Severe Acute Respiratory Syndrome (SARS)	Parvoviruses
John Cunningham virus (JC Virus)	Picobirnaviruses
Human Immuno-deficiency Virus (HIV)	Enteroviruses types 78–100
Hepatitis B Virus (HBV)	Torque teno virus (TTV)

also excreted in the feces and other bodily fluids such as tears (Loon *et al.*, 2004; Wang *et al.*, 2005) (Table 9.4).

B. Duration of release and concentration in bodily fluids and skin

The duration and concentration of pathogens released by a host during the course of an infection varies, with greater numbers being released in more severe infections. After infection, the number of organisms released usually rises rapidly reaching a peak when the symptoms appear in symptomatic infections. This is usually followed with a long decline in the amount of agent released by the host as long as death does not occur. For example, poliovirus appears in the throat and feces 7–10 days before clinical illness (fever) is apparent and may be excreted for more than 30 days after infection (Fig. 9.2). Poliovirus will also be detectable in the blood and urine during the course of infection (Pichichero *et al.*, 1998). Hepatitis A virus appears in the stool of infected individuals 2–3 weeks before clinical illness (Belshe, 1991). Parainfluenza, a virus related to influenza, can be detected in nasal secretions in less than 24 h after infection and up to 2 weeks afterward (Belshe, 1991). In the case of SARS, the virus may still be present in the feces for 37 days after infection (Holmes, 2003). Variola major, the virus that causes smallpox, is released for up to 19 days after infection at concentrations of 10^2–10^5 per ml of urine (Table 9.5) (Sarkar *et al.*, 1973). In many infections, the greatest concentrations are released during the first few days after the initial infection. *Brucella abortus* is excreted in concentrations as high as 10^6 per ml of urine for up to 12 weeks (Table 9.6). Marburg virus and flaviviruses are excreted in the urine of animals for 10–12 days. In summary, all of the nonenteric agents of interest (Categories A, B, and C) are released in the host for at least days to weeks in concentrations likely to be detectable in sewage systems (Table 9.6) (Sinclair *et al.*, 2008).

Most of the existing data on the occurrence and concentration of pathogens was gathered using culture of viable or infective organisms. Molecular methods such as the polymerase chain reaction (PCR) or

TABLE 9.3 Select category B and C agents found in human bodily fluids and sewage

Agent	Category	Urine	Feces	Saliva	Sewage	Reference
C. psittaci	B	?	Yes[a]	?	?	Midura and Arnon (1976); Anderson (1996); Smith *et al.* (2005)
C. burnetii	B	Yes	Yes[b]	?	?	Tylewska-Wierzbanowska and Kruszewska (1993)
Viral encephalitis	B	Yes	Yes[c]	?	?	Mathur *et al.* (1995)
Nipah virus	C	Yes	?	Yes	?	Chua *et al.*, (2002)
Rabies	C	Yes	?	Yes	?	Wacharapluesadee and Hemachudha (2002)
Influenza	C	?	Yes[c]	Yes	?	Buchy *et al.* (2007)

[a] Nasal.
[b] Semen.
[c] Animals.

TABLE 9.4 Occurrence of other agents of interest in bodily fluids

Agent	Urine	Feces	Saliva	Sewage	Reference
Severe Acute Respiratory Syndrome	?	Yes	Yes	?	He *et al.* (2007); Petrich *et al.* (2006)
John Cunningham virus (poliovirus)	Yes	?	?	Yes	Coleman *et al.* (1980)
Human Immuno-deficiency Virus	Yes	Yes	Yes	Yes	Levy (1989); Yolken *et al.* (1991)
Hepatitis B virus	?	?	Yes	Yes	Alter *et al.* (1977); Bancroft *et al.* (1977); Arvanitidou *et al.* (1998)

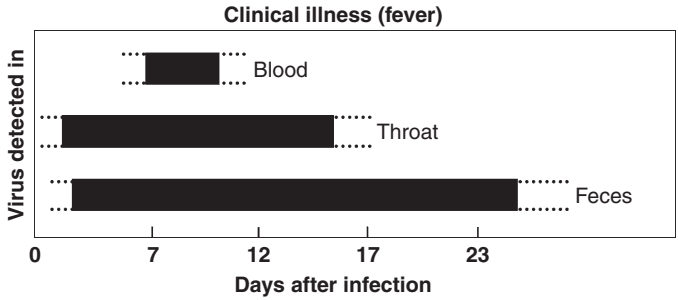

FIGURE 9.2 Occurrence of nonpolio enterovirus in bodily fluids and feces during the course of infection (interpreted from Pichichero *et.al.*, 1998).

immuno-chemical methods (enzyme-linked immunoassays or ELISA) can detect both infectious and noninfectious organisms. These molecular techniques can detect concentrations from 1 to 10,000 greater than culture methods because some of the organisms may be inactivated (dead) or may not be able to grow on the selected media (bacteria) or cell culture (used for viruses). In the case of enteric viruses, the ratio of viruses detected by infectivity assay may be 100–50,000 times less than that detected by a molecular method (Ward *et al.*, 1984). This is because cell culture methods have a low efficiency in virus quantification from clinical and environmental samples; however, they do provide a robust measure of viral activity not feasible with molecular methods.

Agents causing enteric and respiratory infections are released in large numbers in feces and respiratory secretions (Table 9.4). Many of the

TABLE 9.5 Concentration of select agents in sewage and duration of agent release after infection of individuals. see Sinclair *et al.*, (2008) for the following category A agents: Variola major, Hantavirus, Marburg virus, and Flavivirus

Agent	Category	Fluid	Concentration[a]	Duration	Reference
C. Botulinum	A	Feces	10^8	?	Paton *et al.* (1983)
C. Psittaci	B	Feces	10^2–10^3	28 days[b]	Takahashi *et al.* (1988)
B. Abortus	B	Urine	10^2–10^6	8–12 weeks	Bicknell and Bell (1979); Carmichael and Joubert (1988); Serikawa *et al.* (1981)
Japanese encephalitis	B	Urine	1–4	3 days	Mathur *et al.* (1995)
Enteroviruses	B	Feces	10^8–10^{12}	Weeks to Months	Maier *et al.* (2000)
Protozoa	B	Feces	10^6–10^7	Weeks to Months	Maier *et al.* (2000)
C. Burnetii	B	Feces	10^3–10^4	7 days	Tylewska-Wierzbanowska and Kruszewska (1993)
Influenza	C	Nasal	10^5–10^7	5 days to Weeks	Belshe (1991)

[a] per "milliliter" of volume or "gram" of solid.
[b] Animals.

TABLE 9.6 Titer of smallpox virus in urine (Sarkar *et al.*, 1973)

Days after infection	Titer(mL)
3	10^3–10^5
4	10^2–10^5
5	10^2–10^4
6	10^1–10^4
7	10^1–10^3
8	10^1–10^2
10	10^1–10^2
15	10^1–10^2
19	10^1
20	0

TABLE 9.7 Factors that affect concentration of the biological agent in sewage

Site of replication in the host
GI, upper respiratory, nose, skin, internal organs
Duration of release from the host
Concentration in the source
Incidence of disease in the population
Water use per capita
Season
Survival in the sewer system

enteric viruses such as the enteroviruses and adenoviruses may replicate both in the intestinal and respiratory tract. Using molecular methods the number of enteric viruses detected can approach peak concentrations of 10^{12} organisms per gram of stool while protozoa can approach 10^6–10^7 per gram. Cultivatable enteric bacterial pathogens such as *Salmonella* may also occur in concentrations as large as 10^{11} per gram (Feachem *et al.*, 1983). By infectivity assays, the concentration of respiratory viruses ranges from 10^5 to 10^7 per ml of respiratory secretion. Even blood-borne viruses such as HIV will be found in the feces of infected persons (Ansari *et al.*, 1992) and it appears that many viruses will occur in the urine during infection of the host (Table 9.6), although these excreted viruses may not be infectious. Little information is available on the concentration of pathogenic viruses or bacterial agents of interest in the urine. The total amount of virus released by a person is, of course, also related to the amount of feces, urine, respiratory secretion, and skin that is released by the person. On average, a person excretes between 100 to 400 g of feces and 700–2000 ml of urine per day (Table 9.7).

TABLE 9.8 Sources of biological agents in sewers (Feachem *et al.*, 1983)

Feces (100–400 g/person/day)
Urine (700–2000 ml/day)
Skin—from bath and hand washing
Saliva, respiratory secretions
Blood
Food
Wash water (kitchen, drains)
Storm water[a]

[a] Some sewer systems are combined with the storm-water collection system.

A person with an enteric viral infection may excrete as many as 10^{14} viral particles per day and over 10^{15} during the course of an infection (Table 9.8). Nonenteric bacterial agents of interest appeared to be released in concentrations from 10^{0} to 10^{8} by viability assays (Boone and Gerba 2007). Respiratory pathogens end up in the feces from the swallowing of secretions.

Ecological studies of bovine tuberculosis in badgers introduce the concept of ''super-excretors,'' which maintain the disease and pass infectious organisms in their stool or urine continuously. Super-excretors are individuals who excrete larger numbers than average of a pathogen during an infection. These super-excretors were almost exclusively animals with a progressive infection, which does not resolve and contributed to a higher mortality (Delahay *et al.*, 2000). The occurrence of a similar ''super-spreader'' was also noted in a clinical epidemiological report of SARS in humans (Holmes, 2003).

III. CONCENTRATION OF BIOLOGICAL AGENTS IN SEWAGE

The occurrence and concentration of pathogens in sewage is dependent upon a number of factors listed in Table 9.9. One of the most important considerations is the amount of pathogen released by a person daily from bodily fluid, feces, skin, and urine. Because one infected individual typically produces at least 100 g of feces per day, a pathogen present at 10^{8} per gram will introduce at least 10^{10} or more of the pathogen into the sewer system. Logically, pathogens excreted in urine and feces will be released several times during a 24-h period. Enteric and respiratory pathogens are almost always detected in sewage because of the long duration of release from the host during infection, the large concentrations released from the host, and the many infections that are asymptomatic.

Studies have shown that the types and concentration of enteric microorganisms in sewage is directly related to the incidence of disease in the

TABLE 9.9 Comparative occurrence of enteric agents (category B) in feces and sewage

Agent(s)	Feces (per gram)	Stool[a]	Sewage (100 ml)
Enteric viruses (infectivity assay)	10^8	10^{10}	10^2
Enteric viruses (PCR assay)	10^{10}–10^{12}	10^{12}–10^{14}	10^4–10^5
Giardia	10^6	10^8	10–10^2
Cryptosporidium	10^6	10^8	0.1–10^2

[a] 100 g stool (150 g average in the U.S.).

community (Riordan 1962; Sellwood *et al.*, 1981). The concentration of enteric pathogens in sewage ranges from 0.1 to 100,000 per ml of sewage (Table 9.8). While many biological agents of interest have been detected in sewage (Table 9.3), the studies are limited and vary by location.

IV. LABORATORY METHODS AND DETECTION

A. Detection of pathogens

Culture based methods can be used for the detection of pathogens in wastewater, but they may take days to weeks to perform. Alternative molecular methods, such as the PCR, have been successful in detecting bacterial, viral, and protozoan pathogens in sewage without the need for cultivation (Gilbride *et al.*, 2006). These new techniques detect live and dead organisms, have a high sensitivity for wastewater, and can reduce detection time to a few hours (He and Jiang, 2005; Holmes, 2003). Some promising new wastewater methods use nucleic acid microarrays or antibody/receptor technologies to detect multiple pathogens simultaneously (Boehm *et al.*, 2007). Combining these multiplexed methods with fiberoptic sensors and lab-on-a-chip technology can allow utilities to rapidly screen, identify, and quantify multiple pathogens in real time.

Because these technologies rely on PCR DNA techniques, the many interfering substances in raw sewage pose a problem. Without proper sample extraction, the sample analytes are exposed to many varying inhibitors, which can negatively impact the DNA isolation and amplification steps. These methods are also limited by their inability to differentiate between viable and nonviable or nonculturable organisms (Josephson *et al.*, 1993), a vital characteristic when assessing the microbial risk assessment for any given community.

Certain methods are in development to automate the sample collection, sample processing, and concentration to separate analytes from inhibitors and deliver a suitable clean sample to a real-time detection microarray technology. These methods use latex beads, carbohydrates,

anion exchange resins, or similar substances as part of sample collection and sample processing step (Straub and Chandler, 2003), but no fully automated method has been proposed for wastewater. A biosensor capable of identifying and quantifying a wide group of pathogens is necessary, but future development is needed in the areas of extraction from environmental samples, selection of a suitable target sequence of the pathogen (specificity), detection and differentiation of the signal from interfering sequences (sensitivity), and automation of all processes towards a functional real-time biosensor for wastewater (Gilbride *et al.*, 2006).

B. Survival of pathogens in sewer systems

A principal benefit of wastewater monitoring is that most pathogens of interest are expected to remain viable for at least several days in the sewerage environment (Table 9.10). Enteric and respiratory agents are particularly stable, while data is limited for viral encephalitis agents because transmission in water and other liquid media does not occur naturally. Using molecular methods, survival of the pathogens in the viable form is not necessary for their detection, thus increasing the length of time for which the pathogen may be detected. In the case of select

TABLE 9.10 The Helsinki poliovirus experiment (Ranta *et al.*, 2001)

Helsinki population = 740,000
Sewage flow 2×10^8 l/day
Contamination Event
5×10^{10} $TCID_{50}$ Poliovirus vaccine
Flushed down toilet in one liter volume
20 km from sewage treatment plant
Detection
Automatic sampler = 200 ml per 5×10^6 liters of sewage flow
Four samples pooled per day
Concentrated from 400 to 1 ml before assay
Result
Virus was detected for the next 4 days (cell culture)
Peak 24–48 h after flush
Virus detected after passage of 800 million liters of sewage pass through system
Conclusion
Monitoring of sewage could detect 1 infected person in 10,000
Assumes:
10^8 infectious virus excreted by child in 4 days

agents, knowing the presence of the organism in the sewer system may be all that is needed to trigger further investigation regardless of viability.

C. Lessons learned from poliovirus: Monitoring as an early warning system

The benefits of pathogen monitoring in sewage have been recognized for poliovirus for more than 40 years. The relationship between the occurrence of poliovirus in sewage and clinical incidence of disease in a community was first noted in the late 1960s (Nelson *et al.*, 1967). These early detection studies were designed as longitudinal epidemiological investigations to assess the success of polio vaccination campaigns (Riordan, 1962). The results of these studies demonstrated that a definite correlation exists among the isolation of enteroviruses in sewage, and the isolation of viruses in stools, and the number of recognized clinical cases within the community. Using cell culture assay techniques (which measure only infective viruses) and only grab samples (i.e., no steps to concentrate the sample) poliovirus could be detected when only 0.27–0.4% of the population was excreting the virus. It was also demonstrated that small outbreaks and epidemics of enterovirus and adenovirus disease within a community can be predicted by monitoring a community's sewage. Virulent or wildtype (nonvaccine strain) poliovirus type 1 was detected in sewage 9 days before the first clinical case became evident (Kuwert *et al.*, 1970). In an outbreak of Coxsackievirus B5, the virus was detected in the sewage 10 days before clinical cases were positive (Nelson *et al.*, 1967). These studies make it clear that grab samples collected on a regular (weekly or every few days) basis could be used to assess the introduction of a new infectious agent in the community. This approach was later adapted to monitor the success of poliovirus vaccine campaigns internationally (WHO, 2003).

To assess the sensitivity of poliovirus monitoring, one study (Ranta *et al.*, 2001) flushed a one-time bolus of 1 l containing 2×10^{10} infective poliovirus type 1 vaccine strain down a toilet 20 km (12 miles) from the sewage plant (Table 9.11). Samples were automatically collected and assayed for the next 4 days. Infectious poliovirus was still detected after 800 million liters had passed through the system. The authors concluded that their monitoring system could detect one infected person in 10,000 residents of the community, assuming that 10^8 infective viruses are excreted by a child over a 4-day period of time. The study showed that pathogens appear to be greatly retarded in sewage systems, where a one-time event resulted in a detection period over 4 days. The pathogen was also easily detected in 200-ml samples for every 5×10^6 l of sewage flow.

Surveillance of poliovirus in sewage has been used by several nations to assess the success of vaccination programs and to identify the potential

TABLE 9.11 Survival time of pathogens in the environment (water, feces, urine, sewage) (Belanov *et al.*, 1996; Belshe, 1991; Mitscherlich and Marth, 1984; Sinclair *et al.*, 2008)

Organism	Days of survival
B. anthracis	Weeks to years
C. botulinum	Weeks
Y. pestis	Days
Variola major (smallpox)	Weeks to months
F. tularensis	12–60 days
Marburg virus (surfaces)	4–5 days
Enteric pathogens	Days to months
B. mallei	28–35 days
Psittacosis (*C. psittaci*)	Days
Q fever (*C. burnetti*)	30–1000 days
Typhus fever (*Rickettsia typhi*)	Hours to days
Influenza (surfaces)	3 days

need for vaccination to prevent outbreaks (Deshpande *et al.*, 2003; Manor *et al.*, 1999; Tambini *et al.*, 1993). The World Health Organization has published guidelines for the environmental surveillance of poliovirus circulation (WHO, 2003). These guidelines assume that a single infected person will excrete 10^7 polioviruses per day and that one person infected in 100 could be detected using an infectivity assay without concentrating the sewage. However, if the tested sample is concentrated 100 fold then one infected person among 10,000 could be detected.

The Public Health Laboratories of Israel have been conducting an environmental surveillance of sewage on a monthly basis since 1989 (Manor *et al.*, 1999) to assess the spread of the wild type poliovirus strains capable of causing paralytic disease. This was done to determine the success and need for vaccination programs. Between 1989 and 1998, four ''silent'' separate episodes of wild-type poliovirus circulation were detected when no clinical cases were observed. The study described how surveillance of the sewage is much more effective than surveillance of clinical cases. The greater sensitivity of sewage surveillance was also validated in Mumbai, India where wild type poliovirus was detected 3 months before any clinical cases were observed (Deshpande *et al.*, 2003).

D. Differentiation of vaccine and virulent strains

In the poliovirus surveillance of sewage it is necessary to differentiate between vaccine strains and wild type strains of the virus. In the past this has been accomplished by using different cell lines or incubation

conditions to limit the growth of the vaccine strains. However, today this can be accomplished by the use of molecular methods and sequence analysis. Sequences amplified directly from processed sewage samples by PCR using primer pairs specific for the indigenous type 1 genotype could be used to assess its occurrence in the presence of vaccine strains (Tambini *et al.*, 1993). Vaccine strains have unique sequences from wild type strains of pathogens allowing easy differentiation. In addition, sequence analysis of sewage isolations has been shown useful in tracking the spread of wild type poliovirus from one country and community to another (Deshpande *et al.*, 2003; Manor *et al.*, 1999). This review of poliovirus is offered here as a case study and justification for the use of monitoring additional CDC select biological agents. With current molecular techniques and updated concentration methods, a much greater sensitivity and specificity can be achieved for poliovirus and many other CDC select agents.

V. CONCLUSIONS: THE PROBABILITY OF DETECTION

Studies with poliovirus demonstrated the feasibility of how monitoring sewage for virulent pathogens can be used to assess the success of vaccine programs. This review identified three important benefits of developing a wastewater monitoring system. Sewage surveillance system has been shown to be more sensitive than reporting of clinical cases of serious illness in a community. It was also demonstrated that pathogens can be greatly retarded in a sewage systems allowing a detection time over many days for a one-time release into a sewage system. Finally, it was shown that infectivity assays have the ability to detect one infected person in 10,000 individuals.

Sewage surveillance can detect the presence or increased amount of infections from enteric pathogens excreted in the feces or urine during infection. However, the success of such a surveillance system for nonenteric pathogens has not been demonstrated, although they have been found in sewage. The sensitivity of a sewage surveillance system will depend on several important factors including the amount and duration of the agent released into the sewers, the frequency of monitoring, and the sensitivity of the monitoring method.

Nonenteric pathogens are released from the host for a minimum of several days. This has already been demonstrated for HIV, hepatitis B, and *Y. pestis* (see Tables 9.2 and IV). Given this fact and the expected several day retardation in sewer systems, all or most of the nonenteric category agents will be present in the sewer system if there is an infection in the population served by the sewer system. Based upon the conclusions of the Helsinki experiment, which measured infectious poliovirus

(Table 9.11), one individual excreting 10^8 infectious virus per gram of feces for a period of 4 days could be identified in a population of 10,000. If we consider the concentration and amount of infectious agent in the fluid or feces released during infection, this same sensitivity should be achieved with the agents of smallpox, *Brucella*, botulism and perhaps influenza. Based on existing information in Table 9.5 at least one person in 100 could be detected for most of the agents for which information is available.

Because many of the agents take several days to detect by conventional culture methods the preferred detection system would be by a rapid, but highly specific method such as the quantitative real time PCR or other similar molecular detection system. Because PCR can detect both culturable and nonculturable organisms, it can be expected to be more sensitive than methods that have been used in the past for sewage surveillance. Use of PCR should increase sensitivity by as much as 50,000 over cultivation methods (Ward *et al.*, 1984). Also, when using PCR to detect viruses in sewage, a 10-fold loss in sensitivity is likely with current methods. This loss is due to interfering substances present in the sewage, but still leaves a method that may be 5000 times more sensitive than conventional culture methods. Increasing the volume of wastewater that is tested may also increase the sensitivity of current methods. Technology is available (Hurst and Crawford, 2002) which allows for the concentration of bacteria and viruses from up to 10 l of raw sewage. Thus, increasing the volume analyzed from 400 to 4000 ml could increase the sensitivity of detection another 10-fold.

Surveillance of pathogens in wastewater has several advantages over aerosol and other monitoring methods. Longer survival times in soil, water, and wastewater (Sinclair *et al.*, 2008) facilitate a retardation of pathogens in sewage which allows a longer sampling window than aerosols where organisms are much more susceptible to factors such as settling, desiccation, and relative humidity. Additionally, wastewater is collected in a central location, allowing monitoring to be defined or subdivided to specific areas. Lastly, wastewater systems can include many pathogens originating in aerosol, surface water, tap water, or fomites as storm-water and watersheds will often flow into sewerage systems.

Of course background levels and alert levels of the agents of interest would have to be established. Most agents of interest are likely to occur at one time or another in wastewater or at least have some normal range of background. Further research would be needed to determine what these levels might be and normal variation of concentrations of the agents in wastewater. The types and concentration would be expected to vary by location and the size of the population, area served and type of connections (e.g., the presence of a slaughterhouse may increase the likelihood of finding animal pathogens).

In summary, given the potential enhanced sensitivity of molecular methods and current abilities to test larger volumes of all of the CDC

select agents of interest (enteric and nonenteric), it is possible to detect if an infected individual enters a monitored population. Although the concentration and duration of release of all of the agents of interest are not known, it is still possible to detect at least one infection in populations of 1000 or more. This monitoring is especially useful when combined with other components of the QMRA framework such as modeling of sewage dispersion, back calculation of contaminant point of introduction, and calculations of the health risk.

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