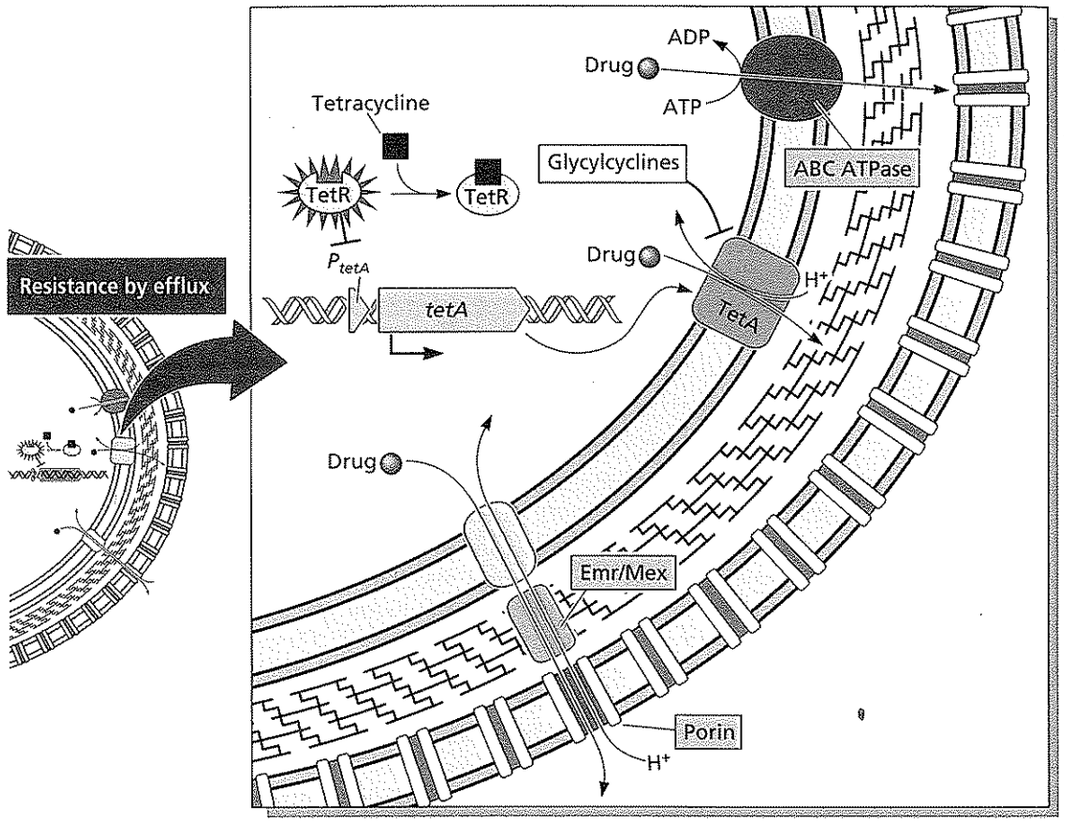


Resistance by action of H^+ and ATP-coupled efflux pumps in bacterial membranes.

chapter 9

Antibiotic Resistance by Efflux Pumps

The second major route by which drug resistance is manifested in bacteria is by the active export or efflux of antibiotics such that therapeutic concentrations are not attained in the bacterial cytoplasm. The chapter opening figure focuses on the portion of Fig. 2.2 that deals with resistance by active efflux of antibiotics.

The active efflux is mediated by transmembrane proteins, both in the cytoplasmic membranes and also in the outer membranes of gram-negative bacteria, with the transmembrane proteins acting as pumps that export the antibiotics, often against concentration gradients (**Table 9.1**). Active efflux can be clinically relevant for β-lactam antibiotics, macrolides, the pristinamycin peptides, fluoroquinolones, and most classically the tetracyclines. As we shall note below, some efflux pumps have relatively narrow specificity, e.g., the tetracycline pumps, while others have broad tolerance and confer multidrug resistance (Mdr) phenotypes. Bacteria have large numbers of efflux pumps, used physiologically for export of specific metabolites and to pump out foreign toxic substances. The integrated array of pumps with overlapping activities can lead to a remarkable capacity to pump out antibiotics either as a chromosomally encoded metabolic capacity, which makes *Pseudomonas aeruginosa* intrinsically antibiotic insensitive, or by acquisition of pump genes carried on plasmids and transposons.

Classes of membrane efflux pumps

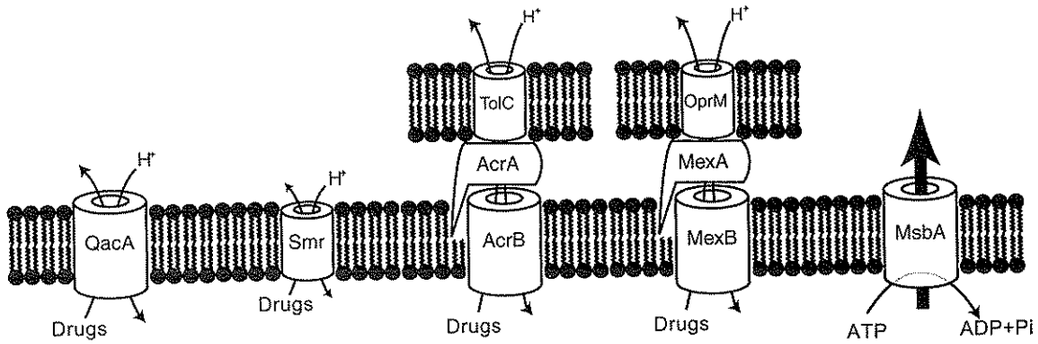
From bioinformatic analysis four protein families of efflux pumps that can function in antibiotic resistance have been described (**Fig. 9.1**). The first three couple drug efflux to counterflow of protons, or sometimes to Na^+ ions, while the fourth family uses the hydrolysis of ATP to provide the energy for active transport of the antibiotic or other foreign compound out of the cell (Paulsen et al., 1996). The pumps driven by proton motive force (ΔpH) are categorized in the major facilitator subfamily (MFS), the small multidrug regulator (SMR) family,

Table 9.1 Summary of reported drug resistance profiles for multidrug-resistance-inducing efflux pumps (modified from Putman et al. [2000])

	MFS						SMR	RND						ABC
	12-TMS cluster				14-TMS cluster									
Drug	Blt	Bmr	EmrD	NorA	EmrB	VceB	EmrE	AcrB	AcrF	MexB	MexD	MexF	MexY	LmrA
Aminoglycosides		–					–	–		–	–	–	+	+
β-Lactams														
Carbapenems							–			+		–		–
Cephalosporins							–			+				+
Penicillins								+	+	+	–			+
Chloramphenicol		+	–	+	–	+	–	+		+	+	+		+
Glycopeptides							–	–	+					–
Lincosamides														+
Macrolides														
14-Membered		–		–	–	+	+	+	+	+	+	–	+	+
15-Membered										+	+			+
16-Membered														
Novobiocin						–		+	+	+				
Quinolones														
Hydrophilic	+	+	–	+	–	–		+		+	+	+	+	+
Hydrophobic		+	–	+	+	+		+		+	+		+	+
Rifampin						+		+	+					
Sulfonamides										+				
Tetracyclines		–		–	–	–	+	+	+	+	+	+		+
Trimethoprim										+		+		–

From Putman et al. [2000], with permission.

Figure 9.1 Four protein subfamilies of proton-dependent efflux pumps and the ATPase family of efflux pump in antibiotic resistance. (From Paulsen et al. [1996], with permission.)

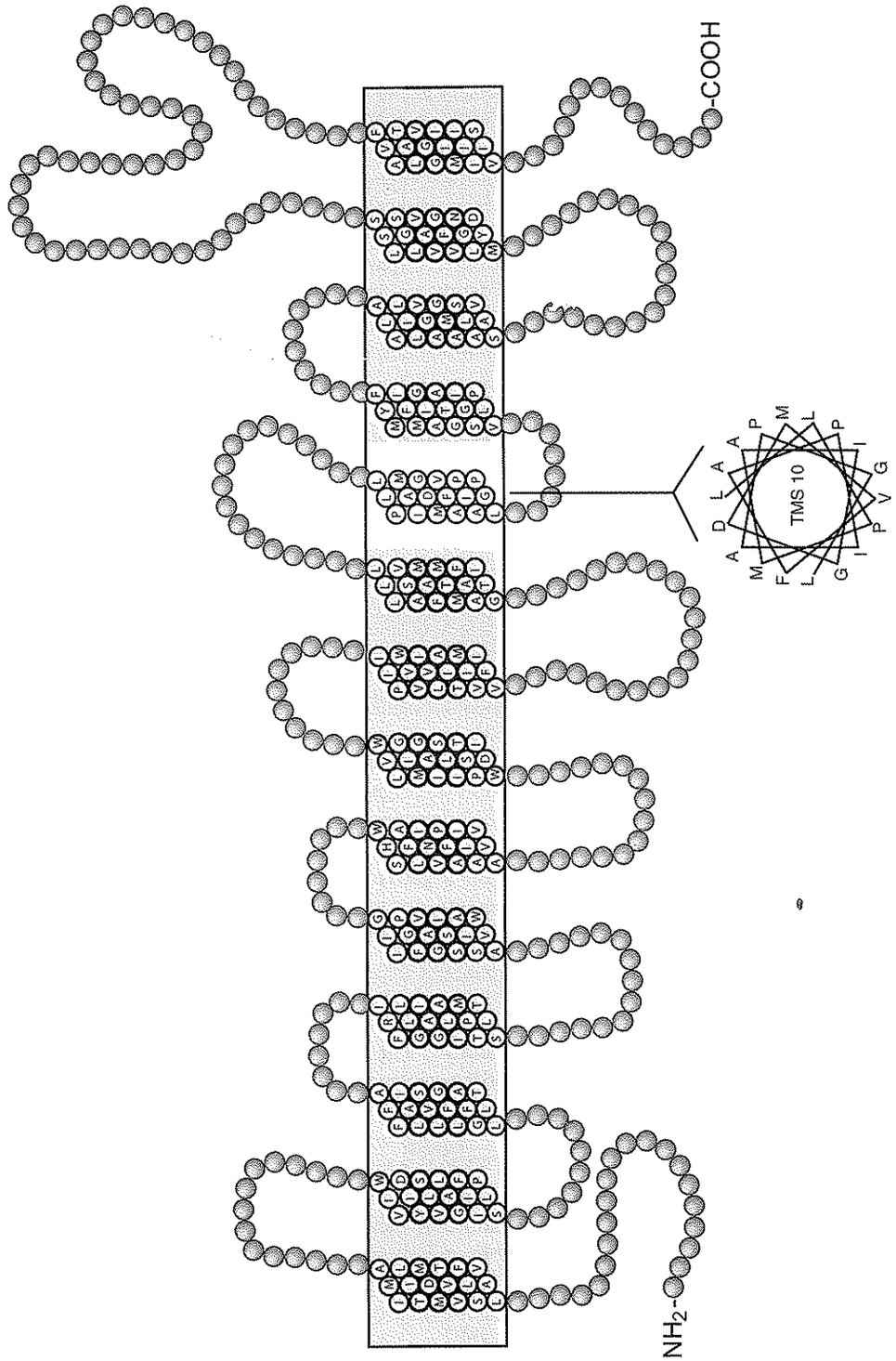


or the RND (resistance/nodulation/cell division) family, based on projected size and the need for partner proteins and subunits. The second major category of efflux pumps, those hydrolyzing ATP, is called the ATP-binding cassette (ABC) family. The schematic orientation of the four classes of efflux pumps in the bacterial cytoplasmic membrane is shown in Fig. 9.1, with counterflow of H^+ or hydrolysis of ATP as the obligate coupling mechanism for efflux. While ATP-driven pumps predominate in eukaryotes, the proton-driven antiporters predominate in bacterial genomes. For example, *Escherichia coli* is predicted to have 18 MFS, 3 to 4 SMR, and 5 to 6 RND members, for a total of 26 efflux pumps driven by counterflow of protons, compared to only 3 to 5 ABC-type ATPase pumps (Borges-Walmsley and Walmsley, 2001; Saier et al., 1998). Further annotation has raised the projected total from 31 to 37 putative drug transporter genes for *E. coli* (Nishino and Yamaguchi, 2001).

The MFS subfamily, represented by QacA (quaternary ammonium compounds [QAC], used as disinfectants and antiseptics, were among the first ligands detected to be substrates for this efflux pump) in Fig. 9.1, has more than 300 predicted members in prokaryotes and eukaryotes and includes the bacterial lactose permease LacY and the GLUT family of human glucose transporters. The use of some bacterial family members for drug efflux is probably a minor variation of a broad range of physiological functions. **Figure 9.2** shows that both N- and C-terminal ends of the MFS pumps are predicted to be on the cytoplasmic face, as exemplified in this prediction for the *Staphylococcus aureus* QacA protein. The N termini of MFS membrane proteins are thought to be involved in energization of transport while the C termini, more variable in sequence, may be involved in specific ligand recognition for export.

Some MFS pumps are predicted to have 14 transmembrane (TM) helices: QacA, EmrB in *E. coli*, TetK from *S. aureus*, TetL from *Bacillus stearothermophilus*, and TcmA from the producer organism *Streptomyces glaucescens* for tetracenomycin. Others have 12 TM domains predicted: Blt and Bmr from *Bacillus subtilis*, EmrD from *E. coli*, NorA from *S. aureus*, and the proteins TetA, TetG, and TetH from a series of gram-negative bacteria (Lomovskaya et al., 2001). Two-dimensional crystals of the TetA pump have been produced, leading to low-resolution structural models (Yin et al., 2000). One hypothesis about the origin of these two MFS subfamilies is evolution by gene duplication from a common six-transmembrane gene precursor. The QacA pump probably functions on its own in the gram-positive *S. aureus* strains, as do the tetracycline pumps TetK and TetL from gram-positive strains. The pumps in the gram-negative strains have partner proteins, in both the periplasm and the outer membrane, indicated in Fig. 9.1 for EmrB, EmrA, and an as yet unidentified outer membrane partner, perhaps the TolC protein.

The SMR family are small 12-kDa proteins, with four predicted transmembrane domains, and may function as oligomers. The RND family of pumps are predicted to have 12 TM domains, again perhaps evolving by gene duplication from a six-TM precursor. In *E. coli* this family includes the AcrB and AcrF pumps (ACR denotes acridine resistance); in *P. aeruginosa* it includes MexB (Fig. 9.1) and MexD; and in clinical isolates of *Neisseria gonorrhoeae* the pump is MtrD.



All 30 of the putative *E. coli* proton-driven drug transporter pumps (20 MFS, 3 SMR, and 7 RND family members) have been expressed in multicopy plasmids in an *E. coli* mutant lacking the AcrAB efflux pump (Nishino and Yamaguchi, 2001) and evaluated for increased resistance to 13 antibiotics. Six novel gene products joined 13 known pumps to yield 20 genes that conferred drug resistance of two-fold or more to at least one of the antibiotics. This might make a useful library set for screening new antibiotic candidates for susceptibility to this battery of pumps.

The fourth family is the ABC family of ATPases. It represents a minority of the antibiotic efflux pumps but is an extremely common form of transmembrane transport system. For example, it is estimated that 70 ABC-type ATPases are encoded in the *E. coli* genome, representing almost 5% of the genes. The typical organization of ABC proteins is to be part of multicomponent membrane transport systems, including periplasmic protein components. In *E. coli*, 44 ABC genes are predicted to be part of uptake systems from the periplasm while 13 are predicted to be dedicated for ligand export. The typical domain organization involves two hydrophobic domains, embedded as transmembrane pores, and two hydrophilic domains on the cytoplasmic face of the membrane that serve as ATP binding and hydrolysis sites. Various configurations of the domains are seen, from a four-domain single polypeptide to four separate subunits. The latter configuration occurs in the *E. coli* maltose permease system, $(MalK)_2 \cdot MalF \cdot MalG$, where MalK is the ABC subunit (**Fig. 9.3**). An X-ray structure of the nucleotide binding domain (NBD) of the MalK pump has been reported (Hung et al., 1998), giving a baseline picture of the catalytic ATPase domain but not revealing how the NBD interacts with the transmembrane domains (TMDs), how docking of the MalK-ATP complex to the MalF/MalG membrane subunit triggers ATP hydrolysis, or how the stored energy is utilized for protein conformational changes to transiently open a pore for maltose to be pumped in.

A significant breakthrough in understanding the architecture of an ABC-type transporter has been obtained by crystallization of the MsbA protein from *E. coli*, at a relatively low resolution (4.5 Å) (Chang and Roth, 2001), but sufficient to reveal orientation of NBDs to TMDs and allowing a model for transporter action. MsbA is homologous to human MDR-1 and mouse MDR3, multidrug resistance transporters that are thought to act physiologically as lipid and phospholipid "flippases," moving phospholipid molecules from the inner to the outer layer of the membrane bilayer. *E. coli* MsbA transports lipid A (see chapter 15) through the inner membrane to the outer membrane of the gram-negative envelope, where lipid A is a major structural component. It is proposed that MsbA and homologs act as "hydrophobic vacuum cleaners" to remove lipids and hydrophobic drugs from the inner membrane leaflet (Chang and Roth, 2001; Raviv et al., 1990). MsbA crystallizes as a homodimer, with the transmembrane domain helices (52 Å long) spanning the membrane at a tilt of 30 to 40° from the normal plane of the membrane, creating a chamber between the dimers large

Figure 9.2 Predicted orientation of MFS pumps in the bacterial cytoplasmic membrane. (From Paulsen et al. [1996], with permission.)

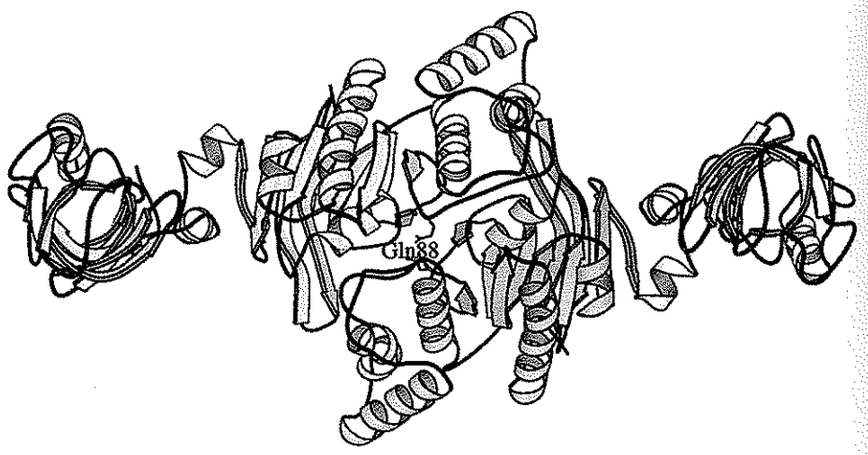


Figure 9.3 The *E. coli* maltose transport system: architecture of the MalK dimer. (From Diederichs et al. [2000], with permission.)

enough to bind the lipid A ligand (**Fig. 9.4**). A linking region between the TMD and the NBD, nestled in the aqueous region on the cytoplasmic side of the membrane, is thought to couple conformational changes from ATP binding and hydrolysis in the NBD to the TMD in the membrane. The models proposed by Chang and Roth (2001) allow for recruitment of lipid or drug from the lower (cytoplasmic) leaflet of the membrane bilayer into the chamber as an initial binding/sequestration step (**Fig. 9.4B**). ATP binding to the NBD is sensed by the TMDs that rotate to close the chamber and bring a cluster of charges from the TM helices into the closed chamber. This would destabilize the microenvironment for the hydrophobic ligand, allowing a vectored flipping to an energetically more favorable position in the upper part of the chamber, poised to enter the outer leaflet of the membrane (Chang and Roth, 2001). While the MsbA may not be a good prototype for transporters that move hydrophilic ligands, it is probably a paradigm for ABC transporters that move hydrophobic ones, including antibiotics. Chang and Roth (2001) noted that the chamber could accommodate and transport a wide variety of molecules, consistent with low selectivity for MDR-type transporters, and also that MsbA (and related transporters) is not acting as a pump but as "a molecular machine scanning the lower bilayer leaflet for substrates, accepting them laterally, and flipping them to the outer membrane leaflet."

A second bacterial transporter of the ABC type, in this case the *E. coli* BtuCD protein pair, transporting the hydrophilic ligand vitamin B_{12}, has also recently been solved by X-ray analysis at 3.2-Å resolution (Locher et al., 2002). The functional transporter is the $BtuC_2BtuD_2$ heterotetramer, where the BtuC subunit is membrane spanning, with 10 α-helices per subunit, and the BtuD is the ATPase cassette. Vitamin B_{12} on the periplasmic face is presented to the external face of $BtuC_2$ by the periplasmic binding protein BtuF, initiating a signal across the membrane that leads to ATP hydrolysis and a presumed power stroke that opens the channel between the BtuC subunits to allow passage of vitamin B_{12}. The orientation of the ATPase domains to the membrane-spanning protein por-

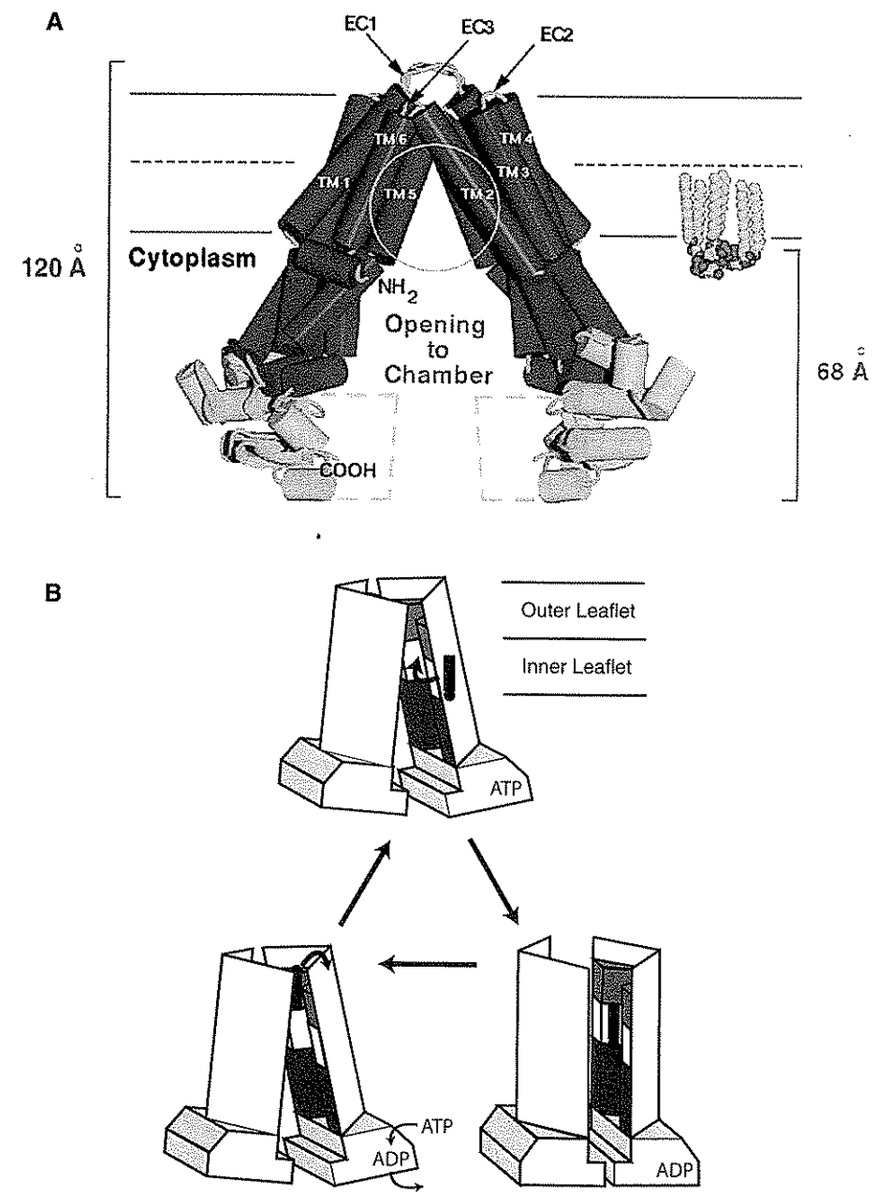


Figure 9.4 Schematic of an ABC transporter. (A) The MsbA dimer and its orientation towards membrane bilayer leaflets. (B) Schematic of lipid A transport by *E. coli* MsbA. (From Chang and Roth [2001], with permission.)

tion and the number and orientation of helices in BtuC are different from the MsbA transporter. The two variants of ABC transporters will create a new platform for design and analysis of alternate ligands and blockers of channel function.

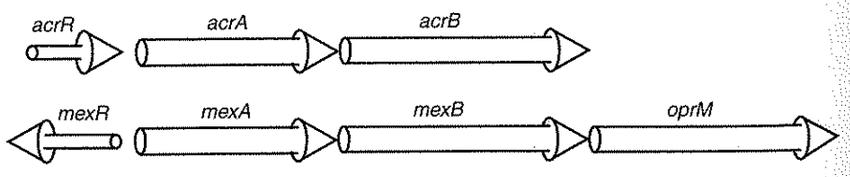
In terms of antibiotic pumps in the ABC family, the resistance to erythromycin in clinical isolates of *Streptococcus epidermidis* (Chu et al., 1996) is due to the *msrA* gene, which encodes such an ATPase subunit for pumping out erythromycins and pristinomycins. Also, the LmrA pump of *Lactococcus lactis* is a broad-spectrum MDR pump. When five candidate ABC-type open reading frames from *E. coli* were expressed and assayed as efflux pumps for the macrolide erythromycin, the *ybjYZ* gene pair was found to encode such a pump and so has been renamed *macAB* (Kobayashi et al., 2001; see also Nishino and Yamaguchi, 2001). MacA is thought to pass through the cytoplasmic membrane once and be mostly in the periplasm, while MacB is thought to be an intrinsic inner membrane protein with the cytoplasmic ATPase domain. As noted in Fig. 9.1 and expounded below, an outer membrane protein is needed to complete ligand efflux across the outer membrane and is provided by the TolC protein. Given X-ray structures for MsbA (Fig. 9.4) and TolC (see Fig. 9.6), we are approaching knowledge of the molecular architecture of multicomponent antibiotic efflux pumps.

Function of the MFS and RND pumps in physiological and antibiotic efflux

The physiologic roles of the various MFS and RND pumps are beginning to be deciphered to give some clue how they may be adapted or taken over for xenobiotic and antibiotic efflux. The Blt pump of *B. subtilis* appears to be used for spermidine efflux and is transcribed with a spermidine acetyltransferase (Paulsen et al., 1996). It is coopted for antibiotic export. The MFS family Ptr pump in *Streptomyces pristinaespiralis* appears to be an autoimmunity pump for this organism when it turns on production of pristinamycins I and II, since both induce ptr transcription. We have noted the OleB efflux pump for glucosyl-oleandomycin in the *Streptomyces antibioticus* producer in chapter 7 as part of the immunity mechanism to its own antibiotic.

In the RND superfamily of pumps, the MexA-MexB-OprM operon (Fig. 9.5) has been proposed to be involved in secretion of the nonribosomal peptide siderophore pyoverdin by *P. aeruginosa* in iron-deficient microenvironments.

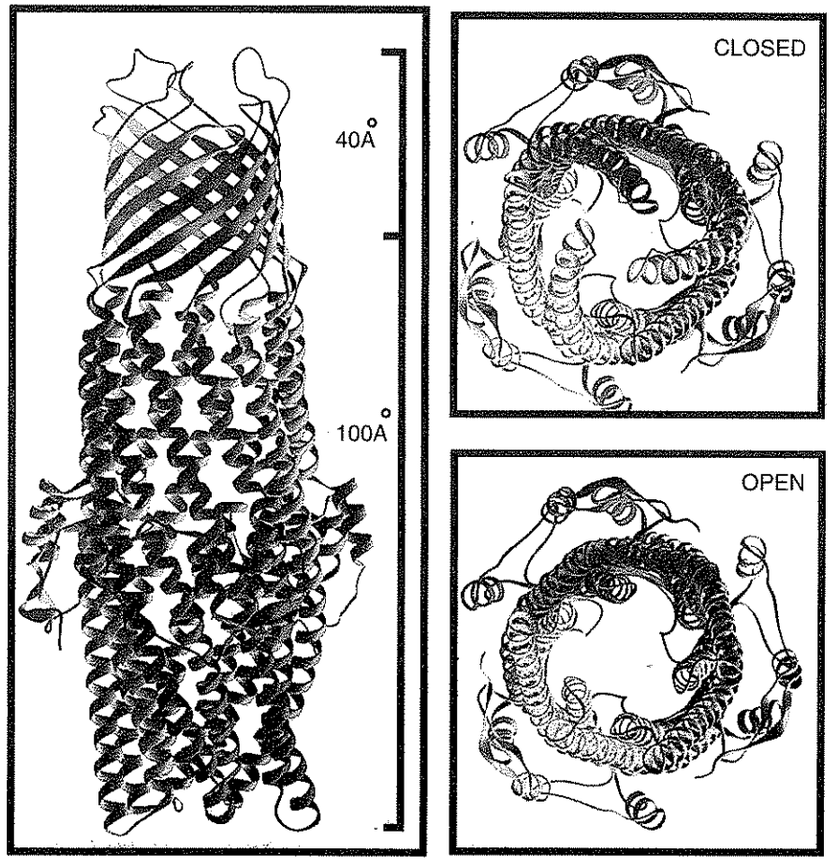
Figure 9.5 Operon organization of three-component Mex efflux pumps in *P. aeruginosa* and *E. coli acrA-acrB* operon.



The presence of two membrane barriers in gram-negative bacteria requires a pump component in both the inner and outer membranes and some connecting protein to bridge the periplasm or bring the two membranes transiently into contact (Fig. 9.1). This trio of proteins is conscripted for efflux of tetracyclines, ciprofloxacin, chloramphenicol, and β-lactams. In fact there are four such multicomponent RND family efflux pumps identified in *P. aeruginosa*, with overlapping ranges for pumping out antibiotics such that the total makes this pathogen intrinsically insensitive to most classes of antibiotics. The *E. coli* Acr system probably has a physiological role of pumping out bile acids and fatty acids to lower their toxicity (Paulsen et al., 1996). AcrB is the inner membrane protein component, while AcrA is thought to span the periplasm (Fig. 9.1) and interact with an outer membrane general porin/channel protein, suggested on genetic grounds to be the protein TolC (Fralick, 1996). TolC is also noted below as an outer membrane channel component of the hemolysin secretion system.

The TolC outer membrane protein of *E. coli* (**Fig. 9.6**) can interact with several different inner membrane translocases to form transient conduits across

Figure 9.6 (Left) The architecture of TolC; (Right) models for TolC closed and open states. (Modified from Koronakis et al. [2000], with permission.)



both membranes to produce a tunnel from cytoplasm to external environment that can be traversed by both small molecules and large proteins that get exported (see "Protein secretion machinery in gram-negative pathogens and connection to disease" below) (Koronakis et al., 2000). TolC is thus a prototype for the outer membrane partner proteins of gram-negative efflux pumps. The X-ray structure (Koronakis et al., 2000) of the functional TolC trimer reveals both a 12-stranded β-barrel domain (four per monomer) and an α-helical domain (Fig. 9.6, left panel) contiguous with the barrel domain. It is argued that the β-barrel domain, typical for outer membrane porin structures (Koebnik et al., 2000), delimits the outer membrane portion and the helical region protrudes into and through the periplasm to form interactions, via coil-coil interactions with coiled-coil domains of the inner membrane translocase pair, e.g., AcrAB. As isolated, the channel in the α-helical barrel of TolC is closed, as seen in the top view of Fig. 9.6 (right panels). It is postulated that during small-molecule and protein efflux through this channel, the channel interior is dilated by rotation of the coiled coils, an allosteric untwisting, induced by protein-protein interaction with the inner membrane components of the pump (Koronakis et al., 2000). The open form could produce a tunnel diameter in TolC (bottom panel of Fig. 9.6, right) of 30 Å. When the TolC dissociated again from its inner membrane partners such as AcrAB, it would revert to the closed state to avoid leakage of periplasmic components.

Regulation of efflux pumps

The best studied of the antibiotic efflux pumps are probably the tetracycline pumps, with TetA-L noted above as MFS family members in both gram-negative and gram-positive bacteria. When tetracycline enters such a bacterial cell, it is bound with high affinity to a protein, TetR, that functions as a repressor of the Tet pump gene, *tetA*, in *E. coli*. The Tet·TetR complex relieves the at rest, negative repression of the Tet pump gene expression. The structure of TetR, with and without the tetracycline ligand, bound to Tet operator DNA[9] reveals how binding of the Mg^{2+}-tetracycline complex causes the TetR to lose affinity for its DNA site (Orth et al., 2000) (**Fig. 9.7**). The K_d for binding of the Mg^{2+}-tetracycline complex to TetR is about 10^{-9} M, some thousand-fold tighter than the 10^{-6} M binding to the 30S ribosome, so the relief of *tetA* transcriptional repression kicks in at low levels of drug in the bacterial cell. When Mg^{2+}-tetracycline is bound to TetR, its affinity for DNA upstream of *tetA* drops an estimated nine orders of magnitude (Orth et al., 2000) via ligand-induced, pendulum-like motion of one of the helices in TetR that pries apart the DNA binding domains.

The 42-kDa TetA pump protein is then overproduced, inserts into the cytoplasmic membrane, and acts in antiport mode with entering protons to pump out tetracycline. The Tet efflux pumps may have evolved from such pumps in the tetracycline producers, such as the one encoded by the *otrB* gene in *Streptomyces rimosus* (McMurry et al., 1998). This regulatory circuit logic appears to be generalized for the operons in *P. aeruginosa* for the *mexA, mexB*, and *oprM* genes, controlled by a divergently transcribed repressor *mexR*; for the comparable *mtr* gene system in *N. gonorrhoeae* (**Fig. 9.8**); and for the *AcrF,E,S* operon in *E. coli*. The *bmr* pump gene in *B. subtilis* is similarly under transcriptional control by the *bmrR* gene, which encodes a repressor. Bmr functions as an efflux pump for lipophilic cations, such as trimethylphosphonium ions, as well as for fluoroquinolones, chloramphenicol, and doxorubicin. The X-ray structure of the BmrR repressor, free and in complex with triphenylphosphonium, has been solved (**Fig. 9.9**), indicating that the lipophilic ligand induces selective unfolding and repositioning of an α-helix. This exposes a drug binding pocket that interacts with the ligand by hydrophobic stacking and by electrostatic pairing with a buried glutamate carboxylate anion (see Fig. 4 and 5 in Zheleznova et al., 1999). The helix-to-coil transition and repacking of the BmrR binding domain is req-

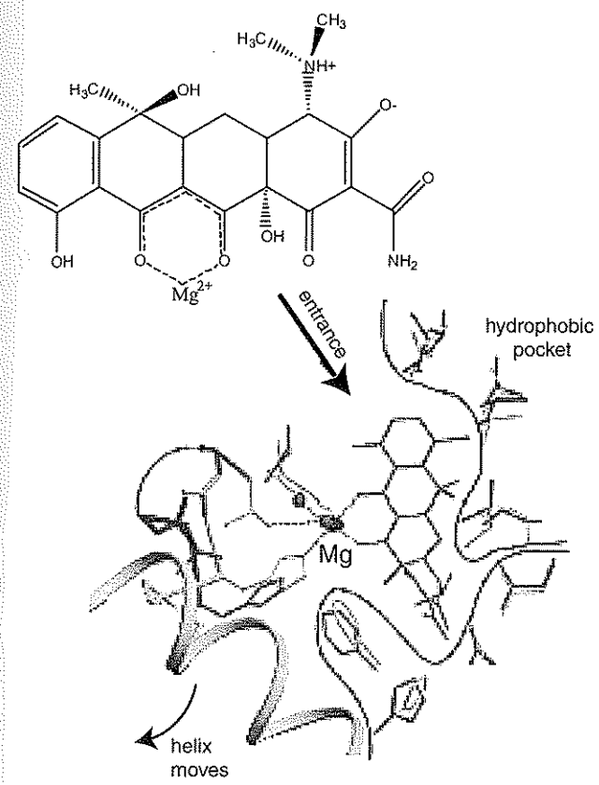


Figure 9.7 Structural basis of relief of repression of *tetA* transcription when Mg^{2+}-tetracycline binds to TetR. (From Orth et al. [2000], with permission.)

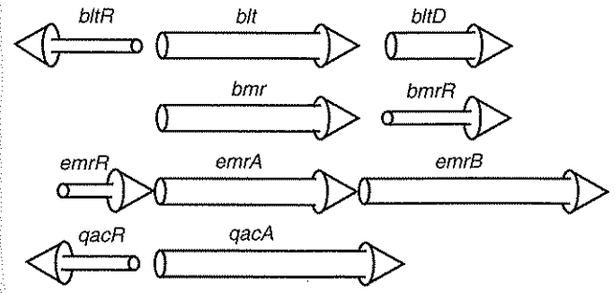


Figure 9.8 Regulatory circuit logic for efflux pump genes.

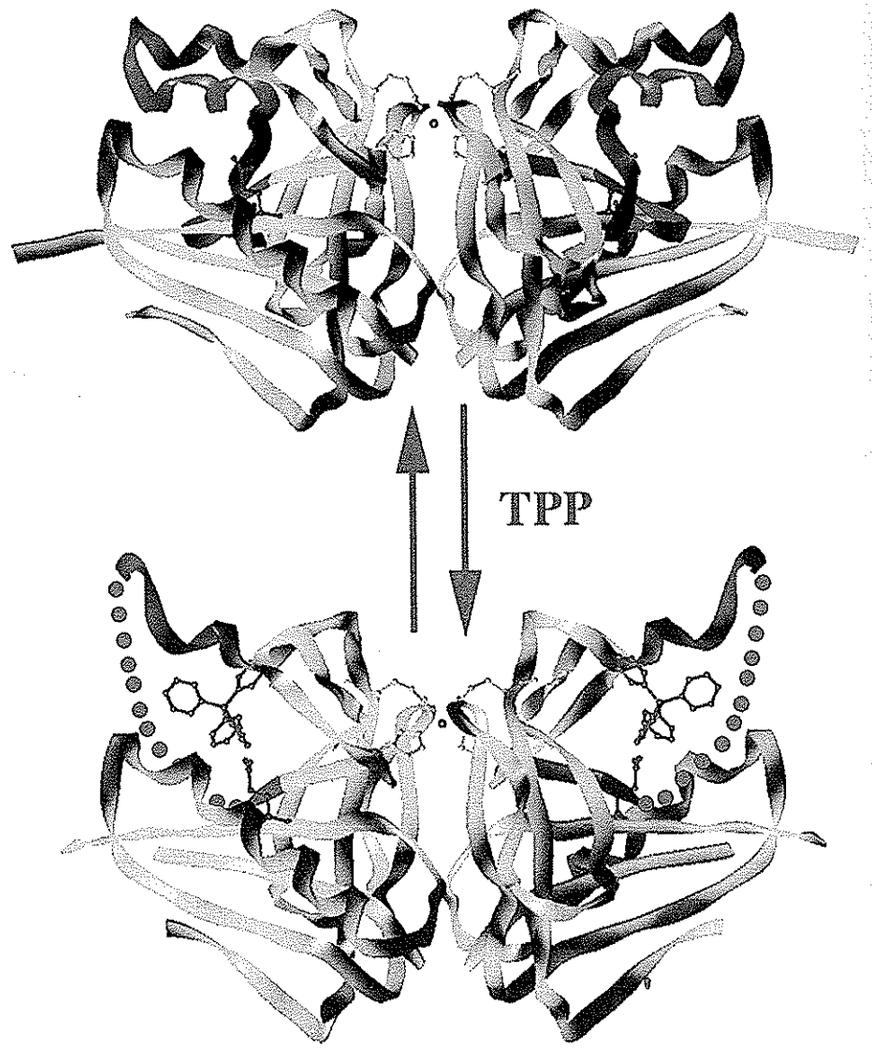


Figure 9.9 Binding of lipophilic cation trimethylphosphonium ion to the BmrR repressor. (From Zheleznova et al. [1999], with permission.)

uisite for high-affinity recognition. Brennan and colleagues have used this observation to propose that this logic may be used for drug binding and recognition by the membrane efflux pump proteins, such as Bmr (Zheleznova et al., 2000), EmrA and B, QacA, and others of the MFS family, many of which have a conserved glutamate in the first transmembrane domain, as do SMR family pumps. They suggest the hydrophobic portions of antibiotics partition into the cytoplasmic membrane at the inner leaflet. If the ligand binding domain of the efflux pump undergoes transient conformational changes via the above helix-to-coil transitions, with one conformation exposing the buried glutamate to bound ligand, the antibiotic will find this site by two-dimensional diffusional encounter.

How the drug-pump complex then senses the ΔpH across the membrane and unidirectionally expels bound antibiotic to the outside in a low-affinity state remains to be analyzed.

The regulation of porin and pump genes is of clinical relevance in carbapenem treatment of *P. aeruginosa* infections (Enne et al., 2001). Point mutations in the *mexR* gene can lead to MexR mutant forms of this repressor protein with lower affinity for promoter targets, allowing relief from repression. This is a common path for upregulation of the *mexA-mexB-oprM* operon noted above. This broad-spectrum, three-component efflux pump provides an exit portal for quinolones, tetracycline, chloramphenicol, and normal β-lactams. Of the two approved carbapenems, imipenem, lacking lipophilic side chains, is not exported. Meropenem, with its heterocyclic side chain, is pumped out and its MICs typically rise from 0.12 to 0.5 mg/liter to 2 to 4 mg/liter. On the other hand, imipenem use selects for *P. aeruginosa* mutants lacking the outer membrane porin OprD; the absence of that pore restricts the drug's entry, and imipenem MICs values rise from 1 to 2 mg/liter to 8 to 32 mg/liter (Enne et al., 2001). Meropenem also has some transit through OprD as its MICs in strains with in this mutant rise an order of magnitude to 2 to 4 mg/liter. Livermore (2000) notes that these observations may favor meropenem use since two mutations (*mexR* and *oprD*), at multiplicatively low frequency, are required to bring the meropenem MIC values out of the useful range. He notes the additional mechanism of the metallo β-lactamases acting as carbapenemases (chapter 8) as an additional resistance determinant.

Reduced outer membrane permeability of *E. coli* O157:H7

We note earlier in this chapter and elsewhere in this book that certain bacteria are better pathogens than others. The gram-negative bacterium *P. aeruginosa* does a good job of keeping intracellular antibiotic concentration low, both by turning on many variants of efflux pumps and also by restricting uptake by expression of outer membrane pore proteins, porins, that restrict inward diffusion of antibiotics and other antibacterial small molecules. Recent studies (Martinez et al., 2001) indicate that the outer permeability barrier of virulent, toxigenic strains of *E. coli* may also be more restrictive for antibiotic permeation. *E. coli* O157:H7, first detected in 1982 in fecal-contaminated undercooked beef, causes up to 75,000 cases of infection annually in the United States, outbreaks of hemorrhagic colitis that can progress to hemolytic uremic syndrome (Mead et al., 1999) (**Fig. 9.10**).

The O157:H7 strain enters through the stomach and colonizes epithelial cells in the intestine, proliferating and producing a toxin. One component of the toxin interacts with a membrane glycolipid, and the other then enters the cells and blocks protein biosynthesis (see Kaper and O'Brien, 1998; Karmali, 1989). *E. coli* O157:H7 exhibits resistance to streptomycin, tetracycline, and sulfa drugs and may do so through reduction of outer membrane permeability for uptake. Martinez et al. (2001) used a kinetic assay for detection of periplasmic alkaline phosphatase activity to calculate that, compared with nonvirulent control strains, *E. coli* O157:H7 has six-fold lower permeability to an anionic substrate small

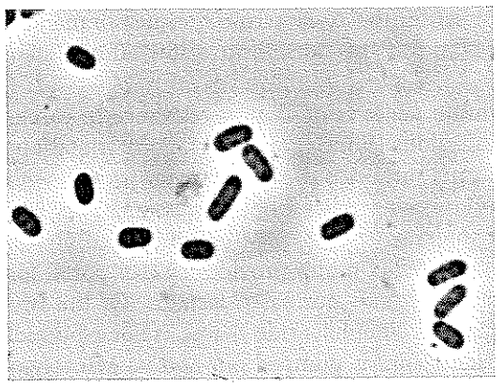


Figure 9.10 Culture of *E. coli* O157:H7. (Courtesy of D. E. Graham.)

molecule and a thousand-fold reduction to phage transformation. The recent sequencing of the *E. coli* O157:H7 genome (Perna et al., 2001) revealed small numbers of changes in the major porins OmpF and OmpC among the 1,387 genes out of 3,500 which are distinct and in strain-specific gene clusters in this pathogenic strain of *E. coli*. Doubtless there will be many contributing factors to the toxicity of the O157:H7 strain, with 15 gene islands of >15 kb encoding putative virulence factors (Perna et al., 2001), but the permeability assay may allow screening for antibiotic agents with more favorable uptake properties in these pathogens.

Protein secretion machinery in gram-negative pathogens and connection to disease

In addition to transporter- and protein pump-mediated export of small molecules, including lipophilic antibiotics, both gram-negative and gram-positive pathogenic bacteria secrete proteins by dedicated protein machinery (see Lee and Schneewind, 2001, for review). Protein secretion through the inner membrane is similar in gram-negative and gram-positive bacteria using the Sec pathway machinery. For gram-positive organisms this allows proteins, such as exotoxins (see chapter 15 for various exotoxins found in the methicillin-resistant *S. aureus* [MRSA] genome), access to the external medium via traverse of the peptidoglycan meshwork. In gram-negative organisms passage across the outer membrane involves several kinds of specialized protein export machinery, termed type I-IV secretion systems, as well as assembly of transmembrane pili involved in attachment of *E. coli* to host cells. These are supramolecular complexes that are assembled in the inner and outer membranes.

Various enteric pathogenic bacteria initiate disease by expression of adherence and sometimes invasin proteins on the outer membranes, assembled via the secretion systems noted below. Adherent but noninvasive pathogens such as *Vibrio cholerae* and the enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) make protein-protein contacts to epithelial cells in the small intestine and then turn on signaling pathways in the host cells that activate

chloride ion channels for chloride and water efflux to create the watery diarrheal syndromes (Nataro and Kaper, 1998; Prente and Finlay, 2001). *Salmonella* strains and *Shigella dysenteriae* can induce uptake by epithelial cells (see chapter 15) and subsequent passage into extracellular spaces, crossing the epithelial barrier. In typhoid fever this produces bacteria in the blood (bacteremia and septicemia), while *Shigella* invasions are localized to the colonic and rectal mucosa, leading to destructive inflammatory tissue responses and the characteristic dysentery (Sansonetti et al., 2001).

The pore-forming hemolysin protein (HlyA) of uropathogenic *E. coli* that cause 80% of urinary tract infections has multiple repeats of a calcium-binding sequence, inserts into membranes, and is part of a family of repeat toxins (Coote, 1992) that are secreted by the type I machinery. There are three other gene products required to secrete HlyA. HlyC adds C_{14} or C_{16} acyl groups to two lysine side chains in HlyA to increase hydrophobicity. HlyB and HlyD are inner membrane protein components of the type I secretion machinery and TolC, noted above in the Acr efflux system, is the outer membrane partner protein that allows HlyA secretion through the outer membrane into the external medium. HlyB is an ABC-type ATPase, perhaps related to the MsbA transporter noted above, and connects type I protein secretion to ABC-type multidrug resistance transporters. TolC is the 12-stranded outer membrane barrel (Koronakis et al., 2000) with C-terminal α-helices that fill the interior channel at rest to prevent leakage of periplasmic components. Presumably the channel is opened to its full 3.4-nm diameter when complexed with type I secretion partners.

Type II secretion machinery is also known as the general secretory pathway (Lee and Schneewind, 2001) and accounts for the export of some bacterial toxins such as cholera toxin, *E. coli* enterotoxin, the *Shigella dysenteriae* Shiga toxin, and *E. coli* Shiga-like toxins (e.g., in the O157:H7 strains), all with AB_5 oligomeric structures, where the A subunit is enzymatically active when taken up by host cells. The B_5 pentameric component self-assembles in the host membrane and can recognize different glycolipids, globoside Gb3 for the Shiga-like toxin of enterotoxigenic *E. coli* (ETEC) and ganglioside GM1 for cholera toxin. The A component is then dissociated and internalized by the host cell, wherein it carries out some enzymatic step. The Shiga toxin of *Shigella* and *E. coli* O157:H7 is a specific *N*-glycosidase, depurinating a particular residue in 23S rRNA and thereby blocking protein biosynthesis, while the A subunit of the ETEC and of the cholera toxin of *V. cholerae* leads to elevation of cyclic AMP and cyclic GMP, activation of chloride ion channels, and resultant diarrheas (Groisman, 2001).

Also secreted by the type II pathway are exoenzymes, including proteases, elastase, phosphatases, and pectate lysases by such plant pathogens as *Erwinia carotovora*. As many as 12 inner membrane proteins are found in this secretion machine (Russel, 1998), an ATPase at the cytoplasmic face of the inner membrane, a periplasmic chaperone, GspS, and one outer membrane protein, GspD, also known as secretin. Secretin oligomerizes into a dodecameric ring with an inner diameter of 7.6 nm (Nouwen et al., 1999) that is thought to be the channel for protein passage through the outer membrane.

Type III secretion machinery is of particular interest for its central role in the virulence and pathogenesis of *Yersinia* and *E. coli* infections and for invasion of *Salmonella* and *Shigella* into host cells (see Lee and Schneewind, 2001, for

review). This topic is discussed further in chapter 15. It is proposed that type III secretion of proteins is induced by physical contact between bacteria and host cells, for example, via specific ion gradients, and serves to directly inject bacterial proteins into the cytoplasm of animal and human cells. The resultant derangement of host cell metabolism can lead to bacterial entry (into epithelial cells by *Salmonella*), neutralization of macrophage killing (by extracellular *Yersinia pestis*), or cell destruction (epithelial cells by EPEC). The type III membrane protein components are analogous to components in assembly of flagella and also build around a secretin dodecamer in the outer membrane and a needle protein (SctF) which grows from the secretin and penetrates the host cell membrane. Proteins being secreted, including 14 *Yersinia* outer proteins (Yops), pass through the secretin channel and the needle directly into the host cell. The substrate recognition signals to determine which bacterial proteins are marked for type III secretion are not yet deciphered (Lee and Schneewind, 2001). The infectivity of *Y. pestis* in causing plague (Perry and Fetherston, 1997) is dramatically decreased when type III secretion is blocked, so this machinery would be a good target for reducing pathogenesis in gram-negative infections.