Chapter I

Introduction
1. INTRODUCTION

1.1. The genus Shigella

Shigella are Gram-negative, facultatively aerobic, non-motile, non-spore-forming bacilli that belong to the group Enterobacteriaceae (Gray, 1995). Based on differences in clinical symptoms the infectious diarrhoeal diseases can be broadly classified into two categories: secretory (watery) and invasive. Invasive diarrhoea is caused by a distinct group of Gram-negative enteric bacteria, the most important of which belong to the genus Shigella. These organisms are the causative agents of the diarrhoeal disease known as shigellosis, which is prevalent in the tropical countries and in places where poor standards of hygiene prevail. These facultative, intracellular parasites infect the colonic epithelial cells of humans and higher primates. The hallmark of virulence is the ability to invade epithelial cells, to multiply intracellularly and to spread from cell to cell expanding the focus of infection (Sansonetti, 1991). The extent of the illness depends on the species involved, namely, S. dysenteriae, S. flexneri, S. boydii and S. sonnei. The disease in its severe form is characterized by intense abdominal pain, fever and bloody diarrhoea. Children are particularly susceptible to it.

1.2. Drug resistance of Shigella

Many antibacterial drugs are effective against Shigella. Their clinical use is less certain since widespread use of any drug has invariably led to the emergence of resistance towards that drug. Sulfonamides were the first antimicrobial agents used in the treatment of shigellosis (Cooper et al., 1941), till development of
resistance limited their use in the therapy of the disease (Weissman et al., 1973). For the last twenty years, ampicillin and trimethoprim-sulfamethoxazole (TMP-SMZ) were the most useful drugs for the treatment of shigellosis based on their safety for use in children and *in vitro* activity against most strains of *Shigella* (Salam and Bennish, 1991). Unfortunately, serious outbreaks of the disease have been reported to have been caused by strains resistant to these drugs in diverse locations such as Bangladesh, India, Hongkong, the Netherlands, Africa, Saudi Arabia etc. (Al Eissa et al., 1992; Bennish et al., 1992; Brito-Alayon et al., 1994; Horrichi et al., 1993; Kolavic et al., 1997; Lima et al., 1995; Ling et al., 1988; Rajkumar et al., 1997). Nalidixic acid was the next drug of choice. However, strains multiply resistant to ampicillin, TMP-SMZ and nalidixic acid have emerged (Lima et al., 1995; Panhotra et al., 1985; Thisyakorn and Rienprayoon, 1992). Although some of these strains are susceptible to the newer fluoroquinolones (Gotuzzo et al., 1989), these are limited in their use by the fact that they cause arthropathy in immature animals, damage the cartilage and are costly drugs. Moreover, low-level quinolone resistance has already been reported in clinical isolates of *S. dysenteriae* type 1 (Rahman et al., 1994; Sack et al., 1997).

In South-East Asia, Bangladesh was the first country to be affected by epidemic shigellosis due to multidrug-resistant *S. dysenteriae*, followed by South India (1972-1973), Sri Lanka (1976), Maldives (1982), Eastern India (1982), Nepal (1984-1985) and the Andaman and Nicobar Islands (1986) (Pal et al., 1989; Palchoudhuri et al., 1985; Sen et al., 1986). An extensive epidemic of bacillary
dysentery swept through the district of West Bengal in 1984 (Pal, 1984). The estimated number of bacillary infections is over 200 million annually, of which 650,000 persons die (Lindberg and Pal, 1993).

1.3. Rationale of the present study

The beta-lactam antibiotics were once the drugs of choice for the treatment of shigellosis. These drugs do not have the adverse side effects associated with the fluoroquinolones, particularly among children who represent a large section of those afflicted with the disease. Effective reemployment of beta-lactams in the therapy of shigellosis needs to be considered carefully. This requires a clear idea of the mechanisms of development of resistance towards the beta-lactams in *Shigella*. This is the major aim of the present study.

1.4. Mechanisms of development of drug resistance in bacteria

In general there are four mechanisms by which a bacterial cell can develop resistance towards drugs (Fig. 1). A single bacterial cell can utilize one of these, a combination of two mechanisms or all of these at one time. One mechanism is the production of an enzyme that destroys the antimicrobial agent e.g. the beta-lactamases. A second mechanism is through a subtle change in the structure of the drug target so that it is no longer affected by the drug. Alternatively, production of the drug target may become amplified. These mechanisms are associated with beta-lactam resistance attributed to the penicillin-binding proteins (PBPs). The third mechanism is by presenting a barrier to entry of the drug into the cell. Bacteria such as *Mycobacteria* are intrinsically resistant to a
Fig. 1. Four ways in which microbial cells might resist antibiotics

(A) the antibiotic (e.g., penicillin) is inactivated by an enzyme (beta-lactamase) secreted by the cells; (B) the antibiotic (e.g., penicillin) no longer binds to its target (penicillin-binding protein), because the target has become altered; (C) the drug (e.g., gentamicin) cannot penetrate the outer layers of the cell because they have become thickened or non-porous; and (D) the drug (e.g., tetracycline) is pumped out (effluxed) from the cell as it gains entry (Taken from Jenkinson, 1996).
large variety of drugs largely due to the low permeability of the cell wall. The fourth mechanism of antibiotic resistance occurs by the process of drug efflux (Nikaido, 1996). Drug efflux is associated with the activity of a specific protein or protein complex within the membrane.

1.5. The cell envelope of Gram-negative bacteria

The cytoplasmic membrane of both Gram-positive and Gram-negative bacteria is supported by a rigid peptidoglycan layer that protects it from osmotic lysis (Beveridge, 1981). In Gram-negative bacteria, an outer membrane is located over the cytoplasmic membrane like an envelope. The components of the outer membrane are either covalently bound to the peptidoglycan or interact with it through ionic bridges producing a tight network (Lugtenberg and van Alphen, 1983). The outer membrane serves as an efficient permeability barrier that protects the bacteria against harmful compounds such as antibiotics (Nikaido and Vaara, 1985). The sieving properties of the outer membrane are attributed to the presence of channel-forming proteins known as porins that form transmembrane open, water-filled channels across the membrane. These proteins will be discussed in section 1.9. In addition, the outer membrane contains lipids and lipopolysaccharides (LPS). The major lipid component of the enteric bacteria *Escherichia coli* and *Salmonella typhimurium* is the zwitterionic phosphatidylethanolamine (Cronan and Vagelos, 1972). Small amounts of phosphatidylglycerol and cardiolipin are also present.
1.6. *Lipopolysaccharides* (LPS)

LPS constitute an important component of the outer membrane of Gram-negative bacteria. LPS is a complex, amphipathic molecule that can be subdivided into three distinct regions. These are: the O side chain (referred to hereafter as the "O antigen"), a long, polysaccharide composed of specific repeating units showing great variability, linked through a short oligosaccharide, the core region, to a glucosamine-containing lipid, Lipid A. The sugars making up the core region, which can be further subdivided into the outer core oligosaccharide and the backbone region (inner core) include L-glycero-D-mannoheptose and 3-deoxy-D-manno-2-octulosonate (KDO), components apparently unique to LPS. Attached to these are phosphorylethanolamine and phosphorylethanolamine substituents. The O-antigenic polysaccharide chain of the LPS of *Shigella* consists of polymerized repeating units composed of di- to hexasaccharides (Lindberg *et al.*, 1991). The diversity of the structure of the repeating units is used for O-antigen-based serotyping (Lindberg, 1984). Smooth strains of enteric bacteria which tend to be the more virulent forms of the organism, possess complete LPS. Large numbers of rough mutants have been isolated that contain varying amounts of altered LPS. These range from a reduction in the amount of O antigenic side chains to those having only one or two sugar residues in the inner core region.

Lipid A is the component of LPS that is responsible for eliciting the non-specific pathophysiological reactions known as endotoxic reactions. The only mutants deficient in Lipid A biosynthesis are temperature-sensitive and fail to grow at the
restrictive temperature. The ketoside linkage between the terminal KDO residue of the core and Lipid A is acid-labile, hence the two parts of the molecule can be readily separated by mild acid hydrolysis and solvent extraction. The structure of Lipid A from a variety of enteric bacteria and *Pseudomonas* species is conserved. It is a glucosaminyl-\(\beta\)-1,6-glucosamine disaccharide substituted with amide or ester-linked hydroxy fatty acids, particularly \(\beta\)-hydroxy myristic acid, straight chain fatty acids, phosphate, ethanolamine and 4-aminoarabinose phosphate (Takayama *et al.*, 1983)

One of the characteristics of LPS is its microheterogeneity. Polysaccharide separated from Lipid A by mild acid hydrolysis has been shown to be heterogeneous with respect to size, with molecules differing in the number of saccharide repeating units from zero to approximately forty (Goldman *et al.*, 1980; Kropinski *et al.*, 1982). Changes in the amount of LPS present have been shown to alter antibiotic sensitivity presumably by affecting the permeability characteristics of the outer membrane (Leying *et al.*, 1991; Tzouvelekis *et al.*, 1994).

### 1.7. The bacterial cell wall peptidoglycan

*Shigella*, like all other bacteria, have a peptidoglycan cell wall. The peptidoglycan is a macromolecule surrounding the bacterial cell. It is composed of linear glycan chains, crosslinked by short peptides. The glycan chains consist of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues, which are linked to each other by \(\beta(1,4)\)-glycosidic bonds;
the muramic acid residues contain a side chain, initially a pentapeptide, which can be turned into tetra-or tripeptide (Park, 1987).

1.7.1. Biosynthesis of the peptidoglycan

The synthesis of the peptidoglycan (reviewed by Holtje and Schwarz, 1985) starts in the cytoplasm through production of the activated precursors : UDP N-acetylg glucosamine and UDP-N-acetylmuramyl pentapeptide. The transport of these hydrophilic precursors across the inner membrane is facilitated through coupling to a hydrophobic carrier, called undecaprenylphosphate or bactoprenol (Umbreit and Strominger, 1972) in reactions catalyzed by the Mra Y and Mur G transferases (Fig. 2) (Bupp and van Heijenoort, 1993). The third and final stage, i.e. the insertion of the disaccharide-pentapeptide precursor into the peptidoglycan layer, consists of transglycosylation (which lengthens the saccharide chain) and transpeptidation (which forms the peptide crossbridges) reactions which occur in the extracellular space and are catalyzed by the PBPs. In E. coli, the close interactions between PBPs and the cell division proteins such as Fts Z, Fts W, Rod A, Fts A and Fts Q are regulated in a cell-cycle dependent manner (Ghuysen, 1994).

1.8. The penicillin-binding proteins (PBPs)

Synthesis of peptidoglycan on the outer surface of the cytoplasmic membrane requires two enzymatic activities, a transglycosylase which polymerizes the glycan strands and a transpeptidase which crosslinks these strands via their
Fig. 2. The three last stages of cell-wall-peptidoglycan synthesis

(1) MrAY catalyzes the transfer of phospho-N-acetylmuramyl-pentapeptide (P-MurNAC-pentapeptide) (or L-Ala-γ-D-Glu-L-Xaa tripeptide) from UDP to the lipid carrier. (2) MurG catalyzes the transfer of N-acetylglucosamine (GlcNAc) from UDP to the lipid-linked MurNAC-pentapeptide (or tripeptide). (3) Cell-wall-peptidoglycan assembly. The point at which the components cross the plasma membrane is not known. Assembly is catalyzed by the penicillin-binding proteins (PBPs) and is temporally and topologically controlled by cell-cycle regulatory proteins. The size and cellular location of the Escherichia coli proteins, FtsW-RodA, FtsQ, FtsA and FtsZ, and the structure of penicillin are also shown. M, N-acetylmuramyl; G, N-acetylglucosaminyl. (Taken from Ghysen, 1994).
peptide strands (Ghuysen, 1991). DD-carboxypeptidase activity is required for maturation of the peptidoglycan and may be involved in regulation of the degree of crosslinking. The PBPs which possess these activities have been grouped into three classes on the basis of protein sequence similarities, the class A and class B high molecular mass PBPs and the low molecular mass PBPs (Ghuysen, 1994). The class A high molecular mass PBPs 1a and 1b of *E. coli* possess transglycosylase and transpeptidase activities (Ishino *et al.*, 1980; Nakagawa *et al.*, 1979; Suzuki *et al.*, 1980). Class B high molecular mass PBPs possess transpeptidase activity, and the low molecular mass PBPs generally have DD-carboxypeptidase activity.

1.8.1. Mechanism of interaction of the PBPs with the beta-lactams

Beta-lactam antibiotics inhibit peptidoglycan synthesis by acting as structural analogs of the terminal D-ala-D-ala residues of the peptide linkage that is cleaved during transpeptidation (Tipper and Strominger, 1965). The reactive-CO-N= bond of the beta-lactam ring of penicillin is in the same relative orientation as the bond between these two D-ala residues; enabling penicillin to form an acyl-enzyme complex with the penicilloyl serine transferase family of enzymes which include the PBPs and the beta-lactamases (Fig. 3). The acyl-enzyme complex is enzymatically inactive, and hydrolyzed very slowly in the case of the PBPs. The beta-lactam moiety is released by hydrolysis much more rapidly in the case of the beta-lactamases. The penicillin-interactive proteins fall into three groups, namely the low molecular mass PBPs, the high molecular
Fig. 3. Reactions catalysed by penicilloyl serine transferases

(a) Kinetics: $E,D,E,D$, $K$, $k_{+2}$, $k_{+3}$ are defined in the text; $P$, reaction product penicilloate. With the penicillin-binding proteins (PBPs), $k_{+3}$ is very small. (b) Molecular mechanics: entities bound to the enzyme active site along the reaction pathway. E-NH, backbone amino group of the enzyme cavity. The charge relay of the beta-lactamases performs steps 1 and 2 of the proton shuttle, whereas that of the PBPs is immobilized after step 1. (Taken from Ghuysen, 1994).
mass PBPs and the beta-lactamases. The membrane-spanning penicillin sensor BlaR is similar in structure to the class D beta-lactamases (Zhu et al., 1992). This dissertation will discuss only the PBPs and not dwell upon the beta-lactamases or the penicillin sensors such as BlaR.

The minimal motif for beta-lactam action is the 2-azetidinone (beta-lactam) ring and an ionizable acidic function within 3.5 Å of the amide nitrogen (Fig. 4). The bicyclic ring systems of penicillin and cephalosporin consist of a 2-azetidinone ring fused with thiazolidine or dihydrothiazine, respectively. The beta-lactam antibiotics bear structural analogy to acyl-D-ala which is involved in the transpeptidation reaction, and acylate the PBPs forming stable acyl enzyme complexes. A mixture of steric, conformational and electronic properties of the beta-lactam interacting with the target site must come into play in order to achieve acylation. These antibiotics act as mechanism-based inhibitors (Frere and Joris, 1985; Waxman and Strominger, 1983). The key to the success of these suicide substrates is that the potential leaving group remains covalently attached to the active-site serine preventing its regeneration. Acylation and deacylation are determined by the binding-site reactivity of the beta-lactam and the reactivity of the acyl enzyme, respectively.

1.8.2. Structural motifs of the PBPs

The beta-lactamases, the low molecular mass PBPs and the high molecular mass PBPs have unique sequence motifs in their primary structures which serve as "signatures" for this family of proteins. Three conserved amino acid
Fig. 4a. Minimal beta-lactam structure

Fig. 4b. Diagrammatic representation of binding of the acyl-D-Ala-D-Ala substrate (A) and penicillin (B) to the enzyme target subsites. (Taken from Neuhaus and Georgopapadakou, 1992)
groupings: \( S X X K, S X N \) or \( Y X N \) and \( K (H, R), T (S) G \) occur at equivalent places along their amino acid sequences (Ghuysen and Dive, 1994; Joris et al., 1988); \( S \) being the active site serine. These enzymes consist of an all \( \alpha \) domain and an \( \alpha/\beta \) domain, at the junction of which is the active site serine with the above structural motifs (Herzberg and Moult, 1987; Lamotte-Brasseur et al., 1991; Moews et al., 1990; Oefner et al., 1990; Strydnaka et al., 1992).

1.8.3. The low molecular mass PBPs

In general, the low molecular mass PBPs are relatively abundant. They catalyze acyl transfer reactions from D-alanyl-D-alanine-terminated peptides and depsipeptide substrates (Ghuysen et al., 1986; Nguyen-Disteche et al., 1986). By reference to the \textit{Streptomyces} K15 DD-transpeptidase as the putative peptidase ancestor, evolution resulted in increased preference for water as attacking nucleophile of the serine ester-linked acyl (R-L-X-D-alanyl) enzyme. Similar to the reactions that these DD-peptidases perform \textit{in vitro}, they probably help in controlling the extent of peptidoglycan crosslinking \textit{in vivo} (Begg et al., 1990) and play important roles in cell morphogenesis and sporulation (Begg et al., 1990; Schuster et al., 1990; Severin et al., 1992; Stoker et al., 1983). \textit{S. pneumoniae} PBP-negative mutants are affected in cell division and morphology (Schuster et al., 1990).

1.8.4. The high molecular mass PBPs

The high molecular mass PBPs illustrate the principle of multi-modular proteins where distinct catalytic/binding modules are linked together in a single
polypeptide chain (Ghuysen et al., 1996). The molecular organization of the
PBPs of known primary structure such as PBP1a (Broome-Smith et al., 1985)
and PBP3 (Nakamura et al., 1983) of *E. coli*, PBP1a (Martin et al., 1992), PBP2x
(Laible et al., 1989) and PBP2B (Dowson et al., 1989) of *Streptococcus
pneumoniae*, PBP5 (EI Kharroubi et al., 1991) and PBP3 (Piras et al., 1993) of
*Enterococcus hirae*, PBP2' of *S. aureus* (Song et al., 1987) and PBP2 of
*Neisseria meningitidis* and *N. gonorrhoeae* (Zhang and Spratt, 1989) has
evolved from a pairwise comparison of amino acid sequences and identification
of modules having similar patterns of distribution of hydrophobic clusters and
bearing conserved amino acid groupings (Englebert et al., 1993). Depending on
the motifs present in the non-penicillin-binding (n-PB) modules, the PBPs can be
classified into class A and class B types. Both transglycosylase and
transpeptidase activity can be assigned to *E. coli* PBP1a and *E. coli* PBP1b on
the basis of the reactions that they catalyse on lipid-linked disaccharide-peptide
precursors *in vitro*. Moenomycin, which inhibits the transglycosylase of PBP1b,
prevents peptide cross-linking (Van Heijenoort et al., 1987).
The high molecular mass PBPs are involved in important physiological events
such as cell elongation, septation or shape determination (Matsuhashi et al.,
1990; Wientjes et al., 1991). In many cases, these PBPs represent the lethal
targets for beta-lactam action, e.g. PBP1 is probably the key target in *S. aureus*,
since staphylococci with only this single PBP remaining functional, can survive
(Beise et al., 1988, 1988a; Labischinski, 1992; Reynolds, 1988). Deletion of the
genes encoding PBP1a and PBP1b is fatal in *E. coli*, but deletion of either one of them is tolerated (Yousif *et al.*, 1935).

The PBP*{}s* of class B appear to be essential. These contain a carboxy terminal transpeptidase domain, and an amino terminal domain of as yet unclear function. The 633-amino acid PBP2 is inactivated by mecillinam which causes *E. coli* to grow and divide as round coccal cells.

In addition to the PBP2-encoding *pbpA* gene, *rodA* and *dacA* (encoding PBP5) are also required to maintain the rod shape of *E. coli* (Matsuzawa *et al.*, 1989).

Inactivation of the PBP3*{}* gene by the monobactam, aztreonam, leads to filamentous growth of the cells. In addition to the PBP 3 gene *pbpB*, the 3 min region of the chromosome map of *E. coli* contains genes for the synthesis of peptidoglycan precursors and for cell division and septum formation (Taschner *et al.*, 1988). Wientjes and Nanninga (1991) have reported that in *E. coli*, PBP1 provides primers for PBP2 during cell elongation and for PBP3 during cell division to act upon. PBP2 has been found to be essential for the integrity of growing *E. coli* Pon B strains (del Portillo and Pedro, 1991).

The function of the n-PB modules of the high molecular mass PBPs of class B, is not clear. These modules together with the carboxy-terminal regions of the proteins, confer an active, penicillin-binding conformation to the PB modules through interactions with specific recognition sites. Truncation of the n-PB module up to the start of the first conserved motif is tolerated in *E. coli* PBP3 (Fraipont *et al.*, 1994). However, deletion of even the first conserved motif yields
an unstable PBP3 (Goffin et al., 1996), and the n-PB module of PBP3 acts as a 
non-cleavable intramolecular chaperone. Soluble forms of the *S. aureus* PBP2' 
in which the n-PB module has been truncated extensively, do not bind penicillin 
(Wu et al., 1994). In the low-affinity class B PBP5 of *E. hirae*, which is 
responsible for beta-lactam resistance, the E(46)-D(160) insert in the n-PB 
module is essential for the proper folding of this multimodular protein (Mollerach 
et al., 1996).

1.8.5. **Beta-lactam resistance associated with altered PBPs**

PBP-mediated resistance is rare. One major reason may be because beta-
lactams have multiple killing targets, and reduction of affinities of each of these 
targets for the antibiotics is necessary for the development of high-level 
resistance. In pneumococci, PBP-mediated resistance involves subtle 
restructuring of the active centre of the transpeptidase domain of the high 
molecular mass PBPs so that they decrease their affinity for penicillin without 
impairing their ability to recognize the normal substrate. The development of 
resistance is a gradual process involving multiple amino acid substitution into 
multiple high molecular mass PBPs (Spratt, 1994a). Antigenic variations of PBPs 
from penicillin-resistant and sensitive strains has been reported in *S. 
pneumoniae* (Hakenbeck et al., 1991 a). PBPs1A, 2A and 2B (but not PBP3) 
each show stepwise decrease in penicillin affinity in parallel with increasing 
levels of antibiotic resistance in clinical isolates of pneumococci (Jabes et al., 
1989). A *S. pneumoniae* PBP2B mutant exhibiting a greater than 1000-fold 
increased resistance to penicillin has several amino acid substitutions within the
W231-S249 sequence (Dowson et al., 1989). PBP2x of *S. pneumoniae* is affected in resistant mutants (Laible and Hakenbeck, 1987; Laible et al., 1991). A number of point mutations resulting in decreased penicillin affinity have been characterized in a number of cefotaxime-resistant mutants (Laible and Hakenbeck, 1991). Alteration in the *pbp2x* gene of *S. pneumoniae* has been suggested to be responsible for low-level penicillin resistance in South African isolates, while alterations in both PBP2B and PBP2x are required for high-level resistance (Smith et al., 1993). PBP2B and PBP2x of *S. pneumoniae* are the primary resistance determinants for different classes of beta-lactam antibiotics (Grebe and Hakenbeck, 1996; Krauss et al., 1996; Reichmann et al., 1996). Cefotaxime resistance was associated with the mutation Thr550→Ala in PBP2x, while piperacillin resistance was associated with the mutation Thr446→Ala in PBP 2B. Directly repeated insertion of a 9-nucleotide sequence between the active-site serine (residue 385) and Ser-X-Asn (residues 442 to 444) was detected in 13 clinical strains of *S. pneumoniae* (Yamane et al., 1996). Extended-spectrum cephalosporin-resistant clinical isolates of *S. pneumoniae* with differing *pbp1a* fingerprints, but identical fingerprints for *pbp2b* and *pbp2x*, have also been reported in the United States (McDougal et al., 1995). In clinical isolates of *N. gonorrhoeae*, the PBP2 that has a decreased affinity for penicillin, differs from the PBP2 of the sensitive strains in the insertion of an additional aspartic acid (Asp 345A) between Arg 345 and Asp 346 (Brannigan et
Methicillin-resistant *S. aureus* (MRSA) provides an example of PBP-mediated resistance by a different mechanism. MRSA produces all the normal PBPs but contains an additional high molecular mass PBP (PBP2') with a very low affinity for essentially all beta-lactam antibiotics. This PBP is the only functional PBP in the presence of otherwise inhibitory concentrations of methicillin (de Jonge *et al.*, 1992; Fontana, 1985; Hartman and Tomasz, 1986; Matsuhashi *et al.*, 1986; Reynolds and Fuller, 1986; Song *et al.*, 1987; Ubukata *et al.*, 1989). The PBP2' gene (*mecA*) is absent from normal *S. aureus* isolates and is part of a transposon (Song *et al.*, 1987). Methicillin-resistant staphylococci shift to the production of a new peptidoglycan with an abnormal muropeptide composition which may be the synthetic product of PBP2' when grown in the presence of methicillin (de Jonge and Tomasz, 1993).

The staphylococcal PBP2 has two homologs, PBP5, (El Kharroubi *et al.*, 1991) and PBP3R (Piras *et al.*, 1990) in the penicillin-resistant *E. hirae* strains. These PBPs have acquired the ability to discriminate between penicillin and D-alanyl-D-alanine-terminated peptides and can take over the functions needed for wall peptidoglycan assembly when all the other PBPs are inactivated. Penicillin resistance in enterococci is associated with overproduction of PBP5 associated with deletion of a genetic element 1 kb upstream of the *pbp5* gene (Fontana *et al.*, 1996). High-level ampicillin resistance was associated with amino acid substitutions in the region between the SDN and KTG motifs (Ligozzi *et al.*, 1996). Substitution of Met-485 located in the third position after the SDN triad in
E. faecium, by Thr or by Ala result in lowered affinities of PBP5 for benzylpenicillin (Zorzi et al., 1996).

Reduced susceptibilities of clinical isolates of E. coli to carbapenems have been attributed to mutations in PBP2 of these strains (Sumita and Fukasawa, 1995).

1.8.6. Resistance through horizontal transfer of genes: mosaic genes encoding PBPs

Resistance to beta-lactam antibiotics may emerge through horizontal transfer of genes from related species creating mosaic genes encoding hybrid PBPs. Such horizontal gene transfer has been demonstrated in Streptococcus (Dowson et al., 1989, 1990) and Neisseria species (Spratt, 1988; Spratt et al., 1989).

Interspecies recombinational events have led to the replacement of parts of the PBP2 -encoding genes (pen A) of N. meningitidis and N. gonorrhoeae with the pen A genes of the commensal species N. flavescens and N. cinerea (Bowler et al., 1994; Spratt et al., 1992).

Resistance in the gram-positive pathogen S. pneumoniae appears to be due entirely to the development of low-affinity PBPs. Genes for PBP1a of S. pneumoniae isolated from different clones of penicillin-resistant S. pneumoniae from South Africa and Spain are related. Mosaic pbp genes formed in S. pneumoniae has subsequently been transferred to other viridans streptococci, such as S. sanguis and S. oralis (Coffey et al., 1993; Dowson et al., 1990).

Genes homologous to pneumococcal pbp1a and 2b genes are present in viridans streptococci (Chalkley et al., 1991) and this argues strongly in favor of
transfer of penicillin resistance determinants from viridans streptococci into the pneumococcus.

The mecA gene for methicillin resistance is harbored by different phylogenetic lineages of S. aureus probably due to multiple episodes of horizontal transfer and recombination (Musser and Kapur, 1992).

PBP-mediated resistance to beta-lactam antibiotics is far less widespread than resistance due to beta-lactamases. However, it is becoming a cause of growing concern in pneumococci as well as methicillin-resistant S. aureus.

1.9. Porins

Porins are a family of bacterial and mitochondrial outer membrane proteins that form pores that are accessible to solutes with M, of <1000 (Schulz, 1996) These form large, open, water-filled channels that allow the influx of small, hydrophilic nutrient molecules and therapeutic agents, and efflux of waste products. The "general" porins show diffusional barriers for nonpolar solutes and some selectivity for either cations or anions. In addition, "specific" pores select for certain molecules, for example linear malto-oligosaccharides. Trimeric porins of high permeability are widespread among Gram-negative bacteria (Benz, 1988, Nakae, 1986; Nikaido, 1992, 1994a). High-affinity, energy-dependent transport systems differ from the simple, specific channels mentioned above, because the proteins bind to the ligands with much higher affinity, and because the systems apparently carry out uphill transport through energy coupling via TonB protein.
The transport of iron-chelator complexes and vitamin B\textsubscript{12} is carried out by such systems in \textit{E. coli}.

Besides serving as channel-forming proteins, the porins have been identified as carriers of foreign epitopes, as possible antigens (Klebba \textit{et al.}, 1990; Nikaido, 1993) and as phage receptors (Nikaido, 1983).

### 1.9.1. Structure

The X-ray structures of some of these porins have been solved (Schulz, 1994) including the porin from \textit{Rhodobacter capsulatus} (1.8 Å) (Weiss \textit{et al.}, 1990) and \textit{R. blastica} (Kreusch \textit{et al.}, 1994), the \textit{E. coli} porins OmpF and PhoE (Cowan \textit{et al.}, 1992). Each subunit of these homotrimers contains 250-450 amino acid residues that form a completely antiparallel beta barrel consisting of 16 and 18 beta strands, in which all strands are hydrogen-bonded to their next neighbours along the chain (Schulz, 1996). Polypeptide loops lining the inner barrel restrict the channel width over a length of \(\approx 10\)Å to a small eyelet (Schulz, 1996). This eyelet defines the diffusion properties of a porin channel.

### 1.9.2. The trimeric porins OmpF, OmpC and PhoE of \textit{E. coli}

The OmpF channel has an estimated diameter of 1.2 nm. The penetration rates of solutes are very strongly influenced, inversely, by their size. The OmpC channel is only slightly narrower than the OmpF channel (1.1 nm). OmpF and OmpC prefer cations over anions. The permeation rates of fairly large (M, \(>300\)), or hydrophobic, or anionic compounds are greatly decreased in the OmpC channel (Nikaido, 1992). Since enteric bacteria live in an environment full of bile
salts, inhibitors which are large, hydrophobic and anionic, they would benefit from the production of OmpC rather than OmpF, in such an environment. Indeed, in such an environment, the synthesis of OmpC rather than OmpF is promoted. The molecular mechanism of this regulation, involving the prototype two-component regulatory system EnvZ-OmpR has been studied extensively (Mizuno and Mizushima, 1990).

When enteric bacteria become starved for phosphate, the production of PhoE becomes derepressed. PhoE is quite homologous to OmpC and OmpF, but favors diffusion of anions rather than cations. In the PhoE sequence, several additional lysine residues occur in places occupied by neutral or acidic amino acids in the OmpC or OmpF porins. Site-directed mutagenesis showed that replacing lysine-125 by glutamic acid alone changes the PhoE porin from an anion-selective one to a cation-selective one (Bauer et al., 1989).

### 1.9.3. Other porin channels

In contrast to *E. coli*, *Pseudomonas aeruginosa* totally lacks the trimeric porins, and contains only low-efficiency porins that allow the diffusion of small molecules at about one-hundredth the rate of the rate of the trimeric porins (Hancock et al., 1990). The best studied example of a monomeric porin from *Pseudomonas* is the protein OprF. The OprF sequence (Duchenne et al., 1988) is homologous to the *E. coli* OmpA protein, which exists as monomers and has a porin function similar to that of the OprF protein (Sugawara and Nikaido, 1992).
Examples of specific channels include, in *E. coli*, LamB (facilitating the diffusion of maltose and maltodextrins) and Tsx (nucleoside transporter). The *P. aeruginosa* outer membrane has many specific channels including D1 (OprB) (glucose transporter), D2 (OprD) (basic amino acid and carbapenem transporter) and P (OprP) (phosphate transporter) (Hancock *et al.*, 1990). These proteins can be thought of as modified porins, having specific binding sites within the channels. LamB exists as a tightly associated trimer reminiscent of the OmpF porin, but for many others there is little evidence of an oligomeric structure.

1.9.4. **Biogenesis and insertion into the outer membrane**

Although several questions still remain unanswered, it has been suggested that the porins are secreted into the periplasm, interact with LPS, become inserted into the outer membrane and are finally assembled into the trimeric form (de Cock and Tommassen, 1996; Nikaido, 1992).

1.9.5. **Porins as channels for beta-lactam antibiotics**

Permeability of beta-lactam antibiotics through the porin channels of *E. coli* varies according to the size, hydrophobicity and charge of the beta-lactams (Yoshimura and Nikaido, 1985). Among the “classical” monoanionic beta-lactams, the higher the hydrophobicity, the lower is the rate of permeation through the outer membrane porin channels. The presence of a bulky side chain like the 7-α-methoxy-side chain present in cefmetazole or the substituted ureido groups on the α-carbon of the side chain at position 7 (e.g. cefoperazone, mezlocillin and piperacillin) reduce rates of permeation across the outer membrane. The presence of an additional cationic group accelerates penetration
rates through the porin channels. Imipenem shows a much higher permeation rate than traditional penicillins and cephalosporins.

1.9.6. Beta-lactam resistance associated with alterations in porins
The absence or reduced expression of porins is associated with multiple drug resistance (Lee et al., 1991; Masuda et al., 1995; Trias et al., 1989). In the family Enterobacteriaceae, resistance to new beta-lactam compounds has been frequently associated with alterations in envelope permeability (Bush et al., 1985; Charrel et al., 1996; Cornaglia et al., 1995, 1996; Hopkins and Towner, 1990; Lee et al., 1991; Nikaido, 1989; Nikaido and Rosenberg, 1990; Pangon et al., 1989; Rice et al., 1993; Tzouvelekis et al., 1992).

1.10. Drug efflux systems
Recent studies have shown that multiple drug efflux pumps, many with unusually broad substrate specificities, present a clinical threat by causing resistance to a broad spectrum of chemotherapeutic drugs (Jenkinson, 1996; Lewis, 1994). The best-characterized multidrug efflux pump is the P-glycoprotein encoded by the human or rodent mdr gene, which mediates resistance to a broad range of cytotoxic drugs by an ATP-dependent export (Endicott and Ling, 1989; Gottesman and Pastan, 1993). mdr1-encoded P-glycoprotein and related proteins are members of the ATP-binding cassette (ABC) superfamily of transporters (Higgins, 1992, 1995). Several ABC transporters sharing homology with MDR1 have been identified in microorganisms such as the yeast ABC proteins (Balzi et al., 1994; Decottignies et al., 1995; McGrath and Varshavsky,
1989), and the *Plasmodium* MDR1 protein. Multidrug resistance mediated by a bacterial homolog of the human multidrug transporter MDR1 has recently been demonstrated in *Lactococcus lactis* (Bolhuis et al., 1996).

The multidrug extrusion systems of bacteria that have been most widely studied use the proton motive force (pmf) rather than ATP as the driving force and act as drug/H⁺ antiporters (Nikaido, 1994b). Proton-dependent multidrug efflux systems of bacteria have been extensively reviewed (Paulsen et al., 1996). Computer-based sequence analyses (Saier, 1994) have enabled grouping of the pmf-dependent multidrug efflux systems into three distinct families of proteins: the major facilitator superfamily (MFS) (Griffith et al., 1994; Marger and Saier, 1993), the resistance/nodulation/cell division (RND) family (Dinh et al., 1994; Saier et al., 1994) and the small multidrug resistance (SMR) family (Grinius et al., 1992; Paulsen et al., 1993, 1995, 1996). In Gram-negative bacteria, some multidrug efflux systems apparently require the function of additional auxiliary proteins. These auxiliary proteins belong to the membrane fusion protein (MFP) (Dinh et al., 1994; Saier et al., 1994) and outer membrane factor (OMF) families (Dong and Mergeay, 1994) and enable the efflux of drugs across the outer membrane permeability barrier. Examples of multidrug efflux systems are diagrammatically represented in (Fig. 5). Only a few of the better studied multidrug efflux systems are discussed in the following paragraphs.
Fig. 5. Diagrammatic representation of the cytoplasmic membrane (CM) showing examples of multidrug efflux systems.

The *Staphylococcus aureus* QacA and *E. coli* EmrB protein (MSF), the *S. aureus* Smr protein (SMR family), and the *Pseudomonas aeruginosa* MexB (RND family) all appear to utilize the transmembrane proton gradient ($\Delta \mu_{H^+}$) as the driving force for multidrug efflux. In contrast, the mammalian multidrug efflux pump P-glycoprotein (Pgp) is driven by ATP hydrolysis. The PMF-dependent multidrug efflux proteins EmrB and MexB, both of which are found in Gram-negative bacteria, probably function with the auxiliary constituents of the MFP family, EmrA/MexA, respectively. In the case of the MexAB system, an additional outer membrane (OM) protein from the OMF family, OprM, which enables drug efflux across both the CM and OM of Gram-negative bacterial cells, has been identified. A similar OMF family protein is likely to be associated with the EmrAB system but has yet to be identified and is indicated (?) accordingly (Taken from Paulsen et al., 1996).
1.10.1. Major facilitator superfamily

The MFS consists of five distinct families of membrane proteins involved in (a) drug resistance, (b) sugar uptake, (c) uptake of Krebs cycle intermediates, (d) phosphate ester/phosphate antiport and (v) oligosaccharide uptake. The first of these clusters consists of pmf-dependent efflux proteins, such as the well-characterized tetracycline exporter, TetB (Levy, 1992). Hydropathy and phylogenetic analyses suggest that the resistance-conferring drug efflux proteins of group (a) can be divided into two major groups consisting of proteins with 12 or with 14 transmembrane segments (TMS) (Paulsen and Skurray, 1993). The 14-TMS family includes (a) a cluster with several yeast proteins, including a probable multidrug efflux protein, Sge1 and a possible toxin exporter, ToxA, (b) a small cluster containing the yeast multidrug resistance protein Atr1; (c) a small cluster containing two Streptomyces resistance proteins; (d) a cluster of Gram-positive bacterial tetracycline efflux proteins such as TetK and TetL; (e) a large cluster of various bacterial drug resistance efflux proteins (mostly from Gram-positive bacteria), including the multidrug efflux proteins LfrA, Ptr, QacA and SmvA, and (f) a cluster of Gram-negative bacterial proteins including the multidrug efflux protein EmrB. Within the 12-TMS family the following clusters can be identified (a) a cluster of fungal and yeast proteins, including the multidrug efflux protein CaMDR1 (b) a cluster of two hypothetical yeast proteins and several bacterial proteins including Bcr and EmrD, (c) a cluster of vesicular monoamine and acetylcholine transporters from higher eukaryotes; (d) a cluster of bacterial proteins, including two chloramphenicol resistance proteins CmlA.
and CmlB; (e) a cluster including various tetracycline efflux proteins from Gram-negative bacteria and three multidrug efflux proteins, Blt, Bmr, and NorA from Gram-positive bacteria; and finally a number of distant members of the family, such as the multidrug efflux protein LmrP.

14-TMS Family of Multidrug Resistance Proteins

The S. aureus qacA gene was the first gene encoding a pmf-dependent multidrug efflux pump to be described and sequenced (Rouch et al., 1990; Tennent et al., 1989). It specifies resistance to a range of structurally disparate organic cations, including monovalent cations, such as ethidium, benzalkonium, and cetrimide, and divalent cations such as chlorhexidine and pentamidine. The emr locus was identified at 57.5 min of the E. coli chromosome and confers resistance to hydrophobic uncouplers such as carbonyl cyanide m-chlorophenylhydrazone and tetrachlorosalicylanilide, to organomercurials and to some hydrophobic antibiotics, such as nalidixic acid and thiolactomycin (Furukuwa et al., 1993; Lomovskaya and Lewis, 1992). The locus consists of three cotranscribed genes, emrR, emrA and emrB. The emrR gene codes for a regulator of the emrRAB operon. EmrA and EmrB function cooperatively, with EmrB enabling drug extrusion across the inner membrane of E. coli cells and EmrA playing a role in drug efflux across the outer membrane (Lewis, 1994; Lomovskaya and Lewis, 1992). EmrA and EmrB may also function cooperatively with a member of the OMF family of proteins in a manner analogous to RND family proteins in Gram-negative bacteria.
Other 14-TMS family multidrug resistance proteins include the *Mycobacterium smegmatis* *ifrA* gene that mediates resistance to hydrophilic fluoroquinolones and organic cations, such as ethidium, acridine and some quaternary ammonium compounds (Liu *et al*., 1996; Takiff *et al*., 1996).

12-TMS Multidrug Efflux Proteins

The three proteins Bmr, Bft and NorA form a phylogenetically related cluster and also display functional similarity. The *Bacillus subtilis* multidrug efflux protein Bmr mediates resistance to structurally diverse compounds including rhodamine 6G and acridine dyes, ethidium bromide, tetraphenylphosphonium compounds (TPP), puromycin, chloramphenicol, doxorubicin and fluoroquinolones (Neyfakh *et al*., 1991). Bmr-mediated ethidium export is dependent on the pmf and is probably driven by the ΔpH, suggesting an electroneutral drug/proton antiport mechanism. The *S. aureus* *norA* gene confers resistance to a similar range of substrates to that encoded by *bmr* (Paulsen *et al*., 1996). *norA* is potentially regulated by a divergently encoded open reading frame *norR* whose product shares sequence similarity with repressor proteins such as the QacR and TetT repressors (Kaatz *et al*., 1993).

1.10.2. Smr multidrug efflux family

The smallest known proton-dependent efflux proteins belong to the SMR family. These proteins are typically around 110 amino acid residues in length with 4 predicted TMS and they do not exhibit sequence homology with the 12- or 14-TMS family discussed above. Since these proteins are very small, it has been
proposed that they may function as oligomeric complexes (Paulsen et al., 1993). The best-characterized member of this family is a staphylococcal multidrug efflux protein known variously as Smr, QacC, QacD or Ebr (Grinius et al., 1992; Littlejohn et al., 1991; Lyon and Skurray, 1987; Sasatsu et al., 1989). This protein will be referred to hereafter as Smr. Other members of this family that mediate multidrug efflux include the chromosomally encoded *E. coli* resistance protein EmrE, previously known as MvrC and Ebr (Lewis, 1994; Morimyo et al., 1992; Purewal, 1991), and the QacE protein (Paulsen et al., 1993). The *smr* gene is typically located on both conjugative and nonconjugative plasmids in clinical isolates of *S. aureus* and other staphylococci (Leelaporn et al., 1994, 1995; Littlejohn et al., 1991, 1992) and encodes resistance to a variety of organic cations, including quaternary ammonium compounds, dyes, such as ethidium, and other compounds such as TPP (Grinius et al., 1992; Littlejohn et al., 1992).

EmrE of *E. coli* confers resistance to monovalent cations such as ethidium, proflavine, pyronin Y, safranin O and methyl viologen (Morimyo et al., 1992; Purewal et al., 1990) as well as to erythromycin, sulfadiazine, TPP and tetracycline (Yerushalmi et al., 1995).

The multidrug resistance *qacE* gene was identified first on the *Klebsiella aerogenes* plasmid R751 (Paulsen et al., 1993), a potentially mobile element found in Gram-negative bacteria. *qacE* confers a drug resistance phenotype similar to the staphylococcal *smr* gene.
1.10.3. Resistance/Nodulation/Cell division family

These proteins probably mediate proton-dependent export across the cytoplasmic membrane and their proposed structure consists of 12 TMS with two large loops between TMS 1 and 2 and TMS 7 and 8 (Saier et al., 1994). Comparative sequence analyses have indicated that the N-terminal and C-terminal halves of RND proteins share sequence similarity, implying that they may have evolved via tandem intragenic duplication in a manner analogous to that proposed for the MFS (Saier et al., 1994).

AcrAB Multidrug efflux system

The *E. coli* chromosomal *acrA* locus is involved in determining resistance to acriflavine and other cationic dyes, as well to detergents and antibiotics (Nakamura 1965, 1968). Cloning, sequencing and characterization of this locus (Ma et al., 1993) identified an operon with two genes *acrA* and *acrB*, encoding members of the MFP and RND families, respectively. Both these genes are required for drug resistance (Ma et al., 1995). The TolC channel may represent the OMF protein associated with the AcrAB system (Ma et al., 1994).

MexAB/OprM MULTIDRUG EFFLUX SYSTEM

*Pseudomonas aeruginosa* has at least two distinct pmf-dependent multidrug efflux systems (Li et al., 1994, 1995). The *mexAB oprM* operon (Gotoh et al., 1995; Poole et al., 1993a, 1993b) confers resistance to a range of antimicrobial agents. Mutations in *mexA, mexB* or *oprM* result in enhanced sensitivity to tetracycline, chloramphenicol, ciprofloxacin and iron-binding compounds (Poole...
et al., 1993b) as well to other quinolones and beta-lactam compounds (Gotoh et al., 1995). Mutations in \textit{mexA} or \textit{oprM} lead to increased accumulation of tetracycline, norfloxacin and benzylpenicillin, and, conversely, overproduction of MexAB/OprM leads to decreased accumulation of tetracycline or chloramphenicol and increased resistance to a range of compounds (Li et al., 1995). The \textit{mexA} and \textit{mexB} genes code for members of the MFP and RND families, respectively. The \textit{oprM} gene codes for a member of the OMF family.

Recently, a divergently encoded open reading frame upstream of the \textit{mexAB oprM} operon has been identified and named \textit{mexR} (Poole et al., 1996). This encodes a protein similar to MarR and appears to function both as a repressor and as an activator. A second operon consisting of the \textit{mexC}, \textit{mexD} and \textit{oprJ} confers resistance to quinolones, tetracycline, chloramphenicol and newer cephems (Poole et al., 1996).

\textit{MtrCDE MULTIDRUG EFFLUX SYSTEM}

Mutations in the \textit{mtr} locus of \textit{Neisseria gonorrhoeae} confer resistance to hydrophobic antibiotics, detergents and dyes, as well as to bile salts and fatty acids typically found on mucosal surfaces (Maness et al., 1973; Sparling et al., 1975). The \textit{mtr} locus consists of \textit{mtrR} encoding a transcriptional repressor protein related to AcrR and AcrS (Pan and Spratt, 1994) and an operon containing the \textit{mtrC}, \textit{mtrD} and \textit{mtrE} genes (Hagman et al., 1995) which encode members of the MFP, RND and OMF families. The \textit{mtrC} gene encodes a lipoprotein (Hagman et al., 1995) and disruption of \textit{mtrC} causes increased susceptibility to a range of hydrophobic drugs. Deletion of the \textit{mtrR} gene results
in increased multidrug resistance, increased production of the MtrC protein (Pan and Spratt, 1994) and increased transcription of mtrC and probably of mtrD and mtrE (Hagman and Schafer, 1995). High-level resistance to multiple hydrophobic drugs in N. gonorrhoeae is due to a single base pair deletion in a 13-bp inverted repeat located within the mtrR and mtr promoters. This mutation apparently decreases mtrR expression while increasing the expression of the mtr operon, suggesting that it may be a cis-acting regulatory element (Paulsen et al., 1996). Mutations in mtrR, resulting in intermediate levels of drug resistance, and in the 13-bp inverted repeat, resulting in high-level drug resistance, have been observed in clinical isolates of N. gonorrhoeae.

For Gram-negative bacteria RND proteins typically function in conjunction with MFP and OMF proteins to mediate transport across both membranes of the cell envelope (Fig. 5). RND proteins have also been identified in Gram-positive bacteria where they do not seem to be associated with MFP or OMF proteins. In Gram-negative bacteria, the genes for RND family proteins are frequently found in association with genes encoding members of a second family of proteins, the MFP family (Dinh et al., 1994; Saier et al., 1994). Dinh et al. have hypothesized that the MFP proteins are involved in enabling substrate transport across the bacterial outer membrane, possibly by inducing the fusion of the inner and outer membranes of the cell. MFP proteins are also associated with other classes of transport proteins such as ABC or MFS transporters, where they similarly play a role in enabling substrate transport across the outer membrane of gram-negative
bacteria. The MFP proteins are apparently tethered to the inner membrane either by a single N-terminal TMS, e.g. HlyD (Schulein et al., 1992) or by a lipid moiety e.g. AcrE (Seiffer et al., 1993). The MFP proteins probably span the periplasmic space and interact with constituents in both membranes. In some cases, MFP proteins and their respective transport proteins interact with members of a third protein family, namely the OMF family. This family consists of outer membrane proteins. Ma et al. (1994) have suggested that they act as outer membrane channels and function cooperatively with RND and MFP proteins.

1.10.4. Other pmf-dependent multidrug efflux systems

Biochemical and physiological studies have identified other pmf-dependent multidrug efflux systems whose genes have not been characterized. Charvalos et al. (1995) have obtained mutants of Campylobacter jejuni that are resistant to multiple drugs, such as pefloxacin, erythromycin, chloramphenicol, tetracycline and beta-lactams. Accumulation assays suggest that these strains extrude pefloxacin, ciprofloxacin and minocycline in a protonophore-sensitive manner.

1.10.5. Multidrug resistance due to regulatory loci

The E. coli chromosomal marRAB locus confers resistance to tetracycline, chloramphenicol, fluoroquinolones, nalidixic acid, rifampin, penicillin and other compounds (Cohen et al., 1989). marRAB does not encode a multidrug efflux system. It is a global regulatory locus that controls the expression of multiple genetic loci, such as the porin gene ompF and the acrAB drug efflux genes (Cohen et al. 1993; Sparling et al., 1975). Similarly, the Klebsiella pneumoniae
multidrug resistance ramA gene is another regulatory locus that codes for a transcriptional activator homologous to MarA (George et al., 1995).

1.10.6. Pmf-dependent drug efflux systems are widespread and of overlapping substrate specificities

Pmf-dependent multidrug pumps are widespread among both prokaryotic and eukaryotic microorganisms. In most cases these are chromosomally encoded, but particularly in clinical isolates of some pathogenic bacteria, these are encoded by resistance plasmids (Paulsen et al., 1996). A number of multidrug efflux systems of overlapping specificities may be present in a single organism. For example, at least six of the multidrug efflux systems of E. coli can transport ethidium cations. Each of the following pairs of proteins, EmrE and QacE, EmrA/B and EmrD, and AcrA/B and AcrE/F, shares a high degree of overlap with regard to their substrate specificities.