

# Molecular Properties of Bacterial Multidrug Transporters

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<b>INTRODUCTION</b> .....	<b>672</b>
<b>SECONDARY MULTIDRUG TRANSPORTERS</b> .....	<b>672</b>
<b>Major Facilitator Superfamily</b> .....	<b>673</b>
<b>The 12-TMS cluster of multidrug transporters</b> .....	<b>673</b>
<b>The 14-TMS cluster of multidrug transporters</b> .....	<b>675</b>
<b>Small Multidrug Resistance Family</b> .....	<b>675</b>
<b>Resistance-Nodulation-Cell Division Family</b> .....	<b>677</b>
<b>Multidrug and Toxic Compound Extrusion Family</b> .....	<b>678</b>
<b>Enterogenic Drug/Proton Antiport</b> .....	<b>679</b>
<b>Conserved Sequences in Secondary Multidrug Transporters</b> .....	<b>679</b>
<b>Résumé</b> .....	<b>679</b>
<b>ATP-DEPENDENT MULTIDRUG TRANSPORTERS</b> .....	<b>680</b>
<b>REGULATION</b> .....	<b>682</b>
<b>RECONSTITUTION</b> .....	<b>683</b>
<b>SUBSTRATE BINDING</b> .....	<b>683</b>
<b>MECHANISM OF TRANSPORT</b> .....	<b>684</b>
<b>ANTIBIOTIC RESISTANCE</b> .....	<b>686</b>
<b>PHYSIOLOGICAL FUNCTION</b> .....	<b>686</b>
<b>CONCLUDING REMARKS</b> .....	<b>686</b>
<b>REFERENCES</b> .....	<b>688</b>

## INTRODUCTION

The development and use of antibiotics has been one of the most important steps towards controlling infectious diseases in the 20th century. However, the subsequent appearance and spread of antibiotic resistance in pathogenic organisms have made many currently available antibiotics ineffective (160, 169). As a consequence, tuberculosis and other infectious diseases are reemerging, causing a serious public health problem (32, 38). To successfully fight the increasing numbers of drug-resistant and multidrug-resistant (MDR) bacteria, extensive knowledge of the molecular mechanisms underlying microbial antibiotic resistance is required.

Microorganisms have developed various ways to resist the toxic effects of antibiotics and other drugs (Fig. 1) (83, 169). One of these mechanisms involves the production of enzymes that inactivate antibiotics by hydrolysis or the formation of inactive derivatives (Fig. 1A) (40). Well-known examples are  $\beta$ -lactamases (29, 206) and enzymes that phosphorylate, adenylate, or acetylate aminoglycoside antibiotics (234). A second mechanism of resistance is target alteration (Fig. 1B). Cellular targets can be altered by mutation or enzymatic modification in such a way that the affinity of the antibiotic for the target is reduced (95, 239, 265, 277). A third, more general mechanism of resistance is the inhibition of drug entry into the cell (Fig. 1C). Due to the low permeability of the outer membrane of gram-negative bacteria (175, 176) and the exceptionally efficient barrier of the gram-positive mycobacteria (101), drug

diffusion across the cell envelope is reduced. The permeability of the outer membrane can be further decreased by the loss of porins (77, 157, 177). These barriers, however, cannot prevent the drugs from exerting their toxic action once they have entered the cell, and the active efflux of drugs (Fig. 1D) is essential to ensure significant levels of drug resistance (122, 177).

Some transporters, such as the tetracycline efflux proteins (209, 237), are dedicated systems which mediate the extrusion of a given drug or class of drugs. In contrast to these specific drug transporters, the so-called multidrug transporters can handle a wide variety of structurally unrelated compounds (127, 178, 190, 257). On the basis of bioenergetic and structural criteria, multidrug transporters can be divided into two major classes (Fig. 2). Secondary multidrug transporters utilize the transmembrane electrochemical gradient of protons or sodium ions to drive the extrusion of drugs from the cell. ATP-binding cassette (ABC)-type multidrug transporters use the free energy of ATP hydrolysis to pump drugs out of the cell.

Here we present a comprehensive review describing the currently known multidrug transporters in bacteria. Important issues addressed are (i) the functional reconstitution of purified multidrug transporters into proteoliposomes, which allows detailed molecular characterization of the proteins, (ii) the transcriptional regulation of genes encoding multidrug efflux systems, (iii) the structure-function relationships that dictate drug recognition and transport, (iv) the presence of multiple drug-binding sites, and (v) the mechanism of transport of both ATP-driven and secondary multidrug transporters.

## SECONDARY MULTIDRUG TRANSPORTERS

Most bacterial multidrug efflux systems known to date are sensitive to agents that dissipate the proton motive force (PMF), indicating that they mediate the extrusion of toxic compounds from the cells in a coupled exchange with protons

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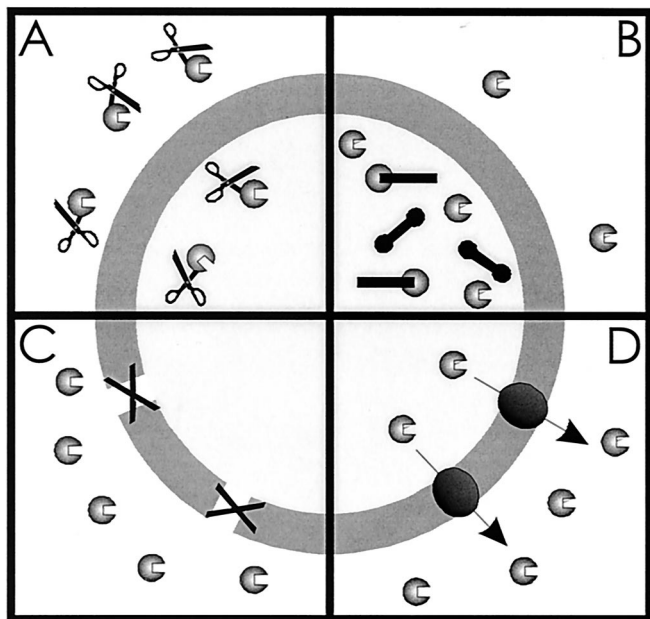


FIG. 1. Resistance mechanisms in bacteria comprise (A) drug inactivation, (B) target alteration, (C) prevention of drug influx, and (D) active extrusion of drug from the cell.

(15, 42, 84, 133, 190). On the basis of size and similarities in the primary and secondary structure, these secondary multidrug transporters can be subdivided into distinct families of transport proteins: the major facilitator superfamily (MFS) (146),

the small multidrug resistance (SMR) family (191), the resistance-nodulation-cell division (RND) family (216), and the multidrug and toxic compound extrusion (MATE) family (28). These families are not solely associated with multidrug export but include proteins involved in other PMF-dependent transport processes or other functions.

**Major Facilitator Superfamily**

The MFS consists of membrane transport proteins which are found from bacteria to higher eukaryotes and are involved in the symport, antiport, or uniport of various substrates, such as sugars, Krebs cycle intermediates, phosphate esters, oligosaccharides, and antibiotics (146). Hydropathy analysis and alignment of conserved motifs of the resistance-conferring drug efflux proteins revealed that these proteins can be divided into two separate clusters, with either 12 or 14 transmembrane segments (TMS) (Fig. 3 and 4) (188). Table 1 summarizes the multidrug transporters from both the 12-TMS and 14-TMS clusters found in gram-positive and gram-negative bacteria.

**The 12-TMS cluster of multidrug transporters.** The *Staphylococcus aureus* NorA protein was first discovered in a quinolone- and methicillin-resistant clinical isolate (251). Sequencing and characterization of the *norA* gene revealed that NorA is a 388-amino-acid membrane protein that confers resistance to hydrophilic compounds and only low or no resistance to hydrophobic drugs (271, 278). Studies on the substrate specificity demonstrated that NorA is a true multidrug transporter, mediating resistance to a range of structurally dissimilar drugs (171, 172).

A structural and functional homolog of NorA has been found in a *Bacillus subtilis* strain selected with increasing concentrations of rhodamine 6G and named Bmr for bacterial

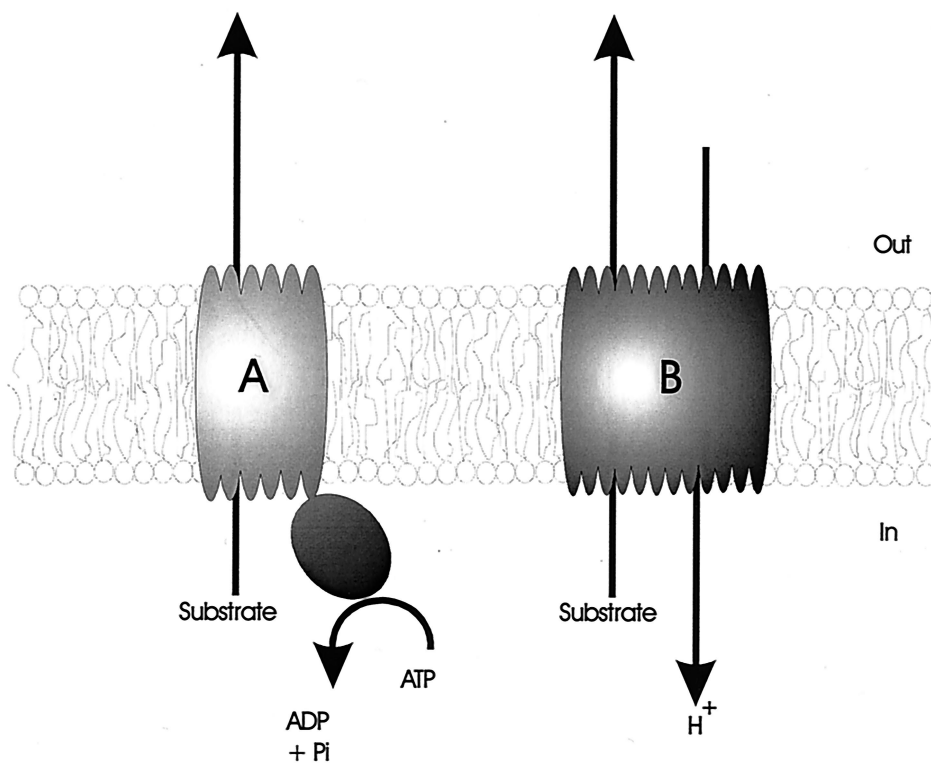


FIG. 2. Schematic representation of the two major classes of multidrug transporters. (A) ABC-type multidrug transporters utilize the free energy of ATP hydrolysis to pump drugs out of the cell. (B) Secondary multidrug transporters mediate the extrusion of structurally unrelated drugs in a coupled exchange with protons or sodium ions.

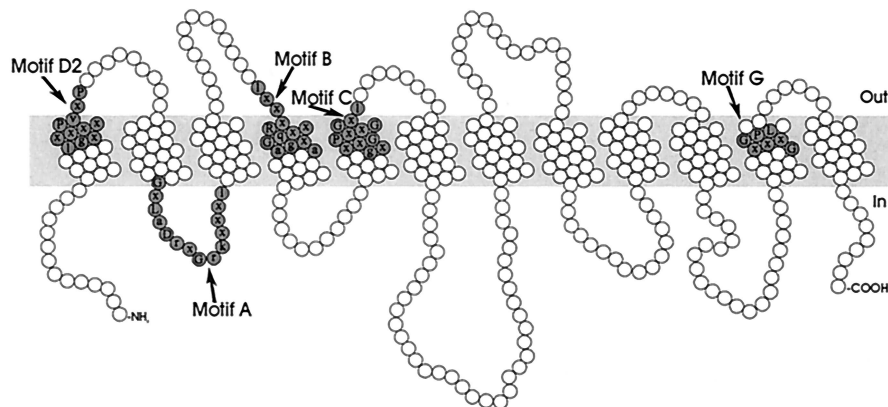


FIG. 3. Structural model for the 12-TMS multidrug transporters of the MFS. The residues constituting the conserved sequence motifs are shaded.

multidrug resistance (170). Ahmed et al. (4) described a second multidrug transporter of *B. subtilis*. This protein, named Blt, is highly homologous to Bmr and confers resistance to a similar range of drugs. Despite the similarities between Bmr and Blt, their expression patterns are different. Under standard cultivation conditions, Bmr is expressed while the expression of Blt is undetectable. The differences in regulation of transcription and the operon organization suggest that Bmr and Blt have distinct physiological functions and that the two transporters may be involved in the transport of different natural substrates (4).

PMF-dependent drug extrusion in *Lactococcus lactis* was first observed in strains exposed to increasing stepwise concentrations of daunomycin and rhodamine 6G (20). The gene responsible for this activity, *lmrP*, encodes an integral membrane protein that shows 19 and 24% homology with the multidrug transporters Bmr and NorA, respectively (21). Transport studies in whole cells and inside-out membrane vesicles demonstrated that LmrP mediates the electrogenic export of lipophilic drugs from the inner leaflet of the membrane to the aqueous phase (23, 203) (see below).

The *Escherichia coli* transporter Bcr confers resistance to the diketopiperazine antibiotic bicyclomycin (18). Vedantam-Gayatri et al. (261) revealed that the sulfathiazole resistance determinant *sur* is identical to *bcr*, indicating that Bcr is involved

in resistance to structurally unrelated antibiotics. EmrD, an *E. coli* homolog of Bcr, is involved in a low-energy shock adaptive response. Expression of EmrD protects the cell against the uncouplers CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) and tetrachlorosalicylanilide, but does not affect resistance to bicyclomycin, quinolones, or chloramphenicol, suggesting that EmrD has a fairly narrow substrate spectrum (167). Another MDR transporter found in *E. coli* is MdfA (49), also known as Cmr (179) and CmlA (208), which was initially described as a membrane-associated efflux pump for chloramphenicol. However, detailed analysis of the substrate spectrum revealed that MdfA confers resistance to a wide range of unrelated neutral and positively charged drugs. Substitutions of E<sup>26</sup> in the first transmembrane segment demonstrated that a single membrane-embedded negatively charged residue is critical for the recognition of positively charged drugs by MdfA (50). Surprisingly, overexpression of MdfA causes decreased resistance to spectinomycin (19). Thus, the activity of a multidrug pump can result in resistance to several toxic compounds and increased sensitivity to certain others.

The multidrug transporter Cmr of the nonpathogenic organism *Corynebacterium glutamicum* significantly increases resistance to several structurally unrelated antibiotics when expressed in *E. coli* but does not affect antibiotic resistance when expressed in *C. glutamicum*. The inability of Cmr to confer

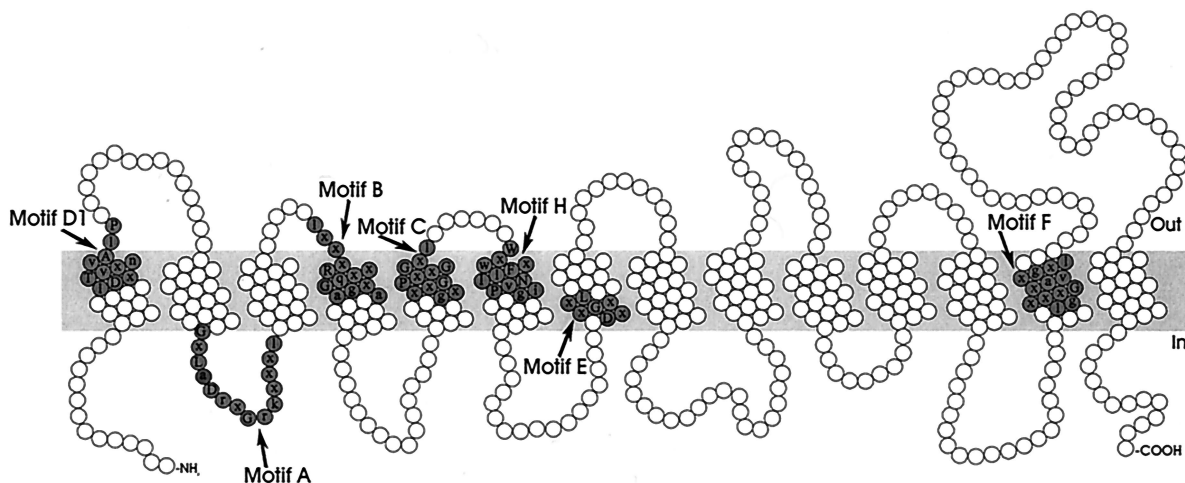


FIG. 4. Structural model for the 14-TMS multidrug transporters of the MFS. The residues constituting the conserved sequence motifs are shaded.

TABLE 1. MFS multidrug transporters

Multidrug transporter	Organism	Accession no(s).	Reference(s)
<b>12-TMS cluster</b>			
Bcr	<i>Escherichia coli</i>	X63703	18
Blt	<i>Bacillus subtilis</i>	L32599	4
Bmr	<i>Bacillus subtilis</i>	M33768	170
Cmr	<i>Corynebacterium glutamicum</i>	U43535	98
EmrD	<i>Escherichia coli</i>	P31442	168
LmrP	<i>Lactococcus lactis</i>	X89779	21
MdfA (Cmr/CmlA)	<i>Escherichia coli</i>	Y08743, U44900	49, 179
NorA	<i>Staphylococcus aureus</i>	D90119	278
PmrA	<i>Streptococcus pneumoniae</i>	AJ007367	63
Tap	<i>Mycobacterium fortuitum</i> , <i>Mycobacterium tuberculosis</i>	AJ000283	5
<b>14-TMS cluster</b>			
Bmr3	<i>Bacillus subtilis</i>	D50098	181
EmrB	<i>Escherichia coli</i>	P27304	135
LfrA	<i>Mycobacterium smegmatis</i>	U40487	244
QacA	<i>Staphylococcus aureus</i>	X56628	213
QacB	<i>Staphylococcus aureus</i>	U22531	191
VceB	<i>Vibrio cholerae</i>	AF012101	37

resistance in its original host could result from a lower expression level of Cmr (98).

Mycobacteria cause several diseases, including tuberculosis and leprosy. The treatment of these infections is often difficult because mycobacteria are intrinsically resistant to most commonly used antibiotics. The Tap multidrug efflux pump in *Mycobacterium fortuitum* and *Mycobacterium tuberculosis* confers resistance to aminoglycosides and tetracycline and makes it more difficult to control mycobacterial infections (5). The number of TMS could not be unambiguously determined from the primary sequence and was estimated to be close to 10. However, Ainsa et al. (5) suggested, on the basis of the homology between Tap and other multidrug transporters of the MFS, that Tap is a 12-TMS transporter.

Several studies suggested that a reserpine-sensitive multidrug transporter is involved in fluoroquinolone resistance in *Streptococcus pneumoniae* (15, 25, 26, 279). Recently, Gill et al. (63) cloned and characterized the efflux pump PmrA of *S. pneumoniae*. Analogous to NorA, Bmr, and LmrP, PmrA is inhibited by the plant alkaloid reserpine. Expression of PmrA increases resistance to acriflavine, ethidium, and the fluoroquinolones ciprofloxacin and norfloxacin.

**The 14-TMS cluster of multidrug transporters.** Resistance to antiseptic and disinfectant compounds, including intercalating dyes, quarternary ammonium compounds, and diamines, is widespread among multidrug-resistant strains of *Staphylococcus aureus*. One of the energy-dependent export mechanisms responsible for this resistance is encoded by the *qacA* gene (248). Hydrophathy analysis (213) and topological analysis using alkaline phosphate and  $\beta$ -galactosidase fusions (192) indicated that the QacA protein contains 14 TMS. Whereas QacA confers resistance to monovalent and divalent organic cations, the closely related QacB only confers resistance to monovalent organic cations (192). It has been speculated that the extensive use in hospital environments of bacteriostatic divalent cations, such as chlorhexidine, resulted in the evolution of *qacA* from *qacB* (193).

Sequencing of an *E. coli* locus for multidrug resistance revealed the presence of two open reading frames, designated *ermA* and *emrB* (135). The *emrB* gene encodes a 513-amino-acid protein which is homologous to QacA. ErmA, a 42.7-kDa membrane fusion protein with a single TMS and a large C-terminal periplasmic domain, is thought to form a continuous

channel between the inner and outer membrane (126, 135). EmrAB confers resistance to hydrophobic uncouplers and antibiotics (59, 135). Tanabe et al. (246) characterized the *E. coli* *emrKY* operon. Transcription of *emrKY* is growth phase dependent and induced by tetracycline. EmrK and EmrY are 50 and 63% identical to EmrA and EmrB, respectively, suggesting a similar function. However, the involvement of EmrKY in multidrug efflux remains to be demonstrated.

Bmr3, a third *B. subtilis* multidrug transporter, shows moderate homology to EmrB (25% identity). Bmr3 confers resistance to the antibiotics puromycin, tosufloxacin, and norfloxacin. Northern hybridization analysis revealed that expression of Bmr3 is decreased in the late log phase (181).

Shortly after the introduction of fluoroquinolones for the treatment of tuberculosis, resistant clinical isolates of *M. tuberculosis* were described. From the rapidly growing *Mycobacterium smegmatis*, which was used as a model genetic system for *M. tuberculosis*, the *lfrA* gene, which confers low-level fluoroquinolone resistance when present on a multicopy plasmid, was isolated. LfrA confers resistance to hydrophilic quinolones but not to the more hydrophobic quinolones (134). Hybridization analysis showed that LfrA homologs are present in *M. tuberculosis* and *Mycobacterium avium* (244).

*Vibrio cholerae*, an important gram-negative enteric pathogen, is the causative agent of the severe diarrheal disease cholera. Colmer et al. (37) identified a multidrug resistance pump, VceAB, that provides *V. cholerae* with resistance to several toxic compounds, including deoxycholate and the antibiotics nalidixic acid and chloramphenicol. VceA and VceB show significant homology to EmrA and EmrB, respectively.

### Small Multidrug Resistance Family

Multidrug transporters of the SMR family (Table 2, Fig. 5), the smallest secondary drug efflux proteins known, are typically about 107 amino acid residues in length. Hydrophathy analysis (191), gene fusions with alkaline phosphatase and  $\beta$ -galactosidase (179),  $\alpha$ -periodicity analysis (51), cysteine accessibility scanning (242), and Fourier transform infrared spectroscopy (11) support a model of a tightly packed four-helix antiparallel bundle. Due to the small size of the multidrug transporters of the SMR family, it has been proposed that they may function as homooligomeric complexes (187, 189).



TABLE 2. SMR multidrug transporters

Multidrug transporter	Organism	Accession no(s).	Reference(s)
EbrA	<i>Bacillus subtilis</i>	AB029306	154
EbrB	<i>Bacillus subtilis</i>	AB029306	154
EmrE (MvrC)	<i>Escherichia coli</i>	Z11877, M62732	162, 202
Mmr	<i>Mycobacterium tuberculosis</i>	Z83866	42
QacE	Gram-negative bacteria	X68232	187
QacEΔ1	Gram-negative + gram-positive bacteria	X68232	187
QacG	<i>Staphylococcus</i> spp.	Y16944	85
QacH	<i>Staphylococcus saprophyticus</i>	Y16945	84
Smr (Ebr/QacC/QacD)	<i>Staphylococcus aureus</i>	X15574, M37888, M37889, M33479	71, 132, 221
YkkC	<i>Bacillus subtilis</i>	P49856	97
YkkD	<i>Bacillus subtilis</i>	AJ002571	97

The first gene encoding a multidrug transporter of the SMR family was detected on both conjugative and nonconjugative plasmids from clinical isolates of *S. aureus* and other staphylococci (120, 121, 132). This gene, first described as *qacC* and also known as *qacD* (132) and *ebr* (221), has been renamed *smr* (71). Grinius and Goldberg (72) demonstrated that the purified and reconstituted Smr protein mediates electrogenic drug/proton antiport. In staphylococci isolated from the food industry, plasmids pST94 and p2H6 were found. Sequence analysis of these plasmids revealed two new members of the SMR family, designated QacG and QacH (84, 85). QacG and QacH have 69 and 76% identity to Smr, respectively. Both transporters confer resistance to ethidium bromide and quaternary ammonium compounds.

The *E. coli* multidrug transporter EmrE (also known as MvrC) has been identified and cloned on the basis of its ability to confer resistance to ethidium and methyl viologen (162, 202). Yerushalmi et al. (275) demonstrated that resistance is caused by PMF-dependent extrusion of drugs. Negative dominance studies indicated that EmrE functions as a homooligomer, probably composed of three monomers (276). Studies using single cysteine mutants of EmrE showed that none of the residues in the putative transmembrane helices react with *N*-ethyl-maleimide (NEM), a small membrane-permeant sulfhydryl reagent. As the reaction with NEM requires water, the results imply very tight packing of the protein without a continuous aqueous domain. Based on these findings, it was proposed that EmrE translocates its substrates through a hydrophobic pathway (242).

The antiseptic resistance genes *qacE* and *qacEΔ1* are located on an integron, a potentially mobile element. As a consequence, these genes are widely spread among gram-negative bacteria (108). The *qacEΔ1* gene but not the *qacE* gene was

detected in clinical isolates of gram-positive bacteria (109). The *qacE* gene was originally found on the *Klebsiella aerogenes* plasmid R751 (187). The *qacEΔ1* gene probably represents a disrupted form of *qacE* that evolved as a result of the insertion of a DNA segment near the 3' end of the *qacE* gene. Ethidium transport experiments indicated that the mechanism of resistance mediated by QacE is active export driven by the PMF. Neither QacE nor QacEΔ1 is inhibited by reserpine, a potent inhibitor of multidrug transporters such as Bmr, NorA, and LmrP (187).

The multidrug transporter Mmr of *M. tuberculosis* confers resistance to tetraphenyl phosphonium (TPP<sup>+</sup>), ethidium bromide, erythromycin, acriflavine, safarin O, and pyronin Y (42). TPP<sup>+</sup> accumulation experiments showed that Mmr actively extrudes TPP<sup>+</sup>, using the PMF as the driving force. The presence of *mmr*-like genes in other *Mycobacterium* species (*M. simiae*, *M. goodnae*, *M. marinum*, and *M. bovis*) was demonstrated by Southern hybridization (42).

The *Bacillus subtilis* genome encodes seven SMR-type proteins (194). Surprisingly, six of these proteins are encoded from gene pairs in three distinct operons, *ebrAB*, *ykkCD*, and *yvdRS*. Masaoka et al. (154) demonstrated that neither EbrA nor EbrB is sufficient for conferring drug resistance. However, coexpression of the two proteins causes an MDR phenotype in both *E. coli* and *B. subtilis*. A similar result was obtained for the YkkCD pair. Only when both the *ykkC* and *ykkD* genes were expressed together was resistance observed against a broad range of drugs, including cationic dyes and neutral and anionic antibiotics (97). In contrast to the SMR-type multidrug transporters, which appear to function as homooligomers, EbrAB and YkkCD are composed of two dissimilar but homologous subunits. One member of each pair is short (105 to 106 amino

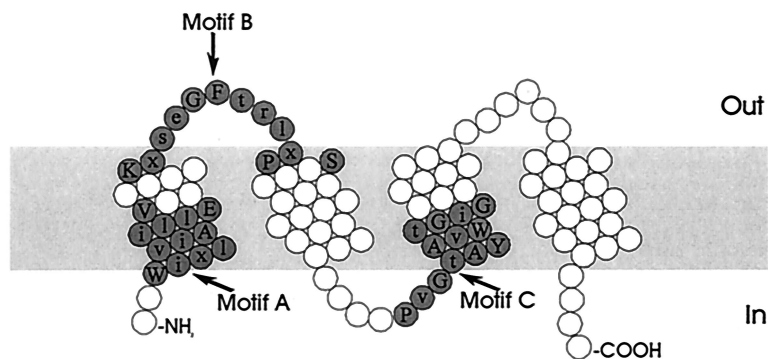


FIG. 5. Structural model for multidrug transporters of the SMR family. The residues constituting the conserved sequence motifs are shaded.

TABLE 3. RND multidrug transporters

Multidrug transporter	Organism	Accession no.	Reference
AmrB	<i>Burkholderia pseudomallei</i>	AF072887	161
AcrB	<i>Escherichia coli</i>	U00734	141
AcrF (EnvD)	<i>Escherichia coli</i>	X57948	110
HI0895	<i>Haemophilus influenzae</i>	L42023	57
MexB	<i>Pseudomonas aeruginosa</i>	L11616	198
MexD	<i>Pseudomonas aeruginosa</i>	U57969	200
MexF	<i>Pseudomonas aeruginosa</i>	X99514	115
MexY	<i>Pseudomonas aeruginosa</i>	AB015853	158
MtrD	<i>Neisseria gonorrhoeae</i>	U60099	81
YhiV	<i>Escherichia coli</i>	U00039	143

acids), while the other is longer (111 to 117 amino acids). This difference is due to a C-terminal hydrophilic extension (97).

The *tehA* gene of *E. coli*, which was initially identified as part of an operon associated with resistance to potassium tellurite, encodes a 36-kDa protein with 10 TMS (247). Surprisingly, a region of TehA which includes TMS 2 through 5 was found to be homologous to members of the SMR family (250). Expression of TehA increased the resistance to monovalent cations, such as tetraphenylarsonium and ethidium bromide, but decreased the resistance to the divalent cations dequalinium and methyl viologen (250). Thus, like MdfA of *E. coli*, TehA confers resistance to one group of toxic compounds and hypersensitivity to another group. Since deletion of the C-terminal region of TehA (TMS 8 to 10) did not decrease transport significantly, TMS 2 to 5 may be primarily responsible for the activity of TehA (250). Although TehA contains only one of the three SMR signature sequences (191) and is larger than the reported SMR-type multidrug transporters, it may represent a distantly related member of the SMR family.

### Resistance-Nodulation-Cell Division Family

Multidrug transporters belonging to the RND family (Table 3) interact with a membrane fusion protein (MFP) and an outer membrane protein to allow drug transport across both the inner and outer membrane of gram-negative bacteria. The secondary structure of RND-type efflux proteins (Fig. 6) was proposed to consist of 12 TMS, with two large loops between TMS 1 and 2 and TMS 7 and 8 (190, 216). Membrane topology analysis of the *Pseudomonas aeruginosa* RND protein MexB recently verified this model (75). The MFP proteins, which contain a single N-terminal TMS and a large C-terminal periplasmic domain, are thought to induce fusion of the inner and outer membrane (45, 216) or form a channel-like structure that spans the periplasmic space (281).

The *acrA* locus of *E. coli* has long been known to be involved in resistance to basic dyes, detergents, and antibiotics (166, 167). Characterization of this locus revealed the presence of two genes, *acrA* and *acrB* (formerly *acrE*), which are located within a single operon (141). AcrA is a highly asymmetric protein that belongs to the MFP family, while AcrB is a member of the RND family (45, 281). Insertion mutations showed that both AcrA and AcrB are required for drug resistance (143). A study by Fralick (58) suggested that the outer membrane protein TolC is the third component of the AcrAB efflux system. Recently, the 2.1-Å crystal structure of TolC has been determined (118). Three TolC promoters assemble to form a continuous, solvent-accessible "tunnel" that spans the outer membrane and periplasmic space. This tunnel may transiently open up upon the interaction with an energized inner mem-

brane transporter and establish a conduit between the cytosol and the external environment. *E. coli* contains two AcrAB homologs, AcrEF (formerly known as EnvCD [110]) and YhiUV (formerly called OrfAB), as well as an AcrB homolog, AcrD, which is not associated with a gene for an MFP (142). Inactivation of AcrEF decreased the resistance to a broad range of drugs, including dyes, detergents, and various classes of clinically important antibiotics, indicating that AcrEF functions as a multidrug transporter (141). Mutations in the *yhiUV* genes result in drug hypersusceptibility, suggesting that YhiUV also plays a role in antibiotic resistance (142, 143). Direct multidrug transport, however, has not yet been demonstrated.

*P. aeruginosa* is a clinically important opportunistic pathogen characterized by relatively high intrinsic resistance to a variety of antimicrobial agents. This property is now recognized to result from the synergy between low outer membrane permeability and at least four RND efflux systems. The *mexAB-oprM* operon was first thought to play a role in the export of the siderophore pyoverdine (198). Further characterization of the operon, however, showed that overexpression of MexAB-OprM increased the resistance of *P. aeruginosa* to various antibiotics (199). A direct assay of drug accumulation showed that MexAB-OprM indeed pumps out various antibacterial agents in an energy-dependent fashion (129). MexA and MexB are members of the MFP and RND families, respectively. The third gene of the operon was originally identified as *oprK*, but Gotoh et al. (64) demonstrated that the outer membrane component of this efflux system is OprM rather than OprK. Results obtained by Evans et al. (52) suggested that the quorum-sensing-related homoserine lactone PAI-1 is a substrate for MexAB-OprM and that exclusion of PAI-1 limits the production of virulence factors, such as the blue-green pigment pyocyanin. Examination of *P. aeruginosa* strains showing widely different levels of intrinsic resistance suggested the presence of more than one endogenous multidrug efflux system (128). Indeed, Poole and coworkers (200) identified a second efflux operon in *P. aeruginosa*, designated MexCD-OprJ. The third antibiotic efflux system of *P. aeruginosa* is composed of the MFP MexE, the RND protein MexF, and the outer membrane protein OprN (115). Similar to MexAB-OprM, MexEF-OprN expression correlates inversely with the production of the virulence factor pyocyanin (115). In contrast to MexAB-OprM, MexCD-OprJ and MexEF-OprN do not confer resistance to  $\beta$ -lactam antibiotics (65, 115). Subunit swapping experiments demonstrated that the inner membrane efflux components of the MexAB-OprM transporter are responsible for the  $\beta$ -lactam specificity of the multidrug efflux pumps in *P. aeruginosa* (66, 155, 240). Recently, two new genes mediating resistance to quinolones, aminoglycosides, and macrolide antibiotics were cloned from the chromosome of *P. aeruginosa* (6, 158). These genes, designated *mexX* and *mexY*, show significant similarity to *mexAB*, *mexCD*, and *mexEF*. No open reading frame corresponding to an outer membrane protein was found downstream of *mexXY*. Mine et al. (158) demonstrated that TolC or OprM is required for MexXY activity in *E. coli*. It has been reported that OprM contributes to antibiotic resistance in *P. aeruginosa* independent of MexAB (283). The substrate specificity of this OprM-dependent and MexAB-independent system corresponds to that of the MexXY system, suggesting that OprM functions as the outer membrane protein of the MexXY drug efflux pump in *P. aeruginosa* (158).

The resistance of *Neisseria gonorrhoeae* to hydrophobic agents, including detergent-like fatty acids and bile salts, is conferred by the MtrRCDE efflux system. The *mtrRCDE* region of the gonococcal chromosome contains a transcriptional regulator gene (*mtrR*) and three tandemly linked genes (*mtrC*,

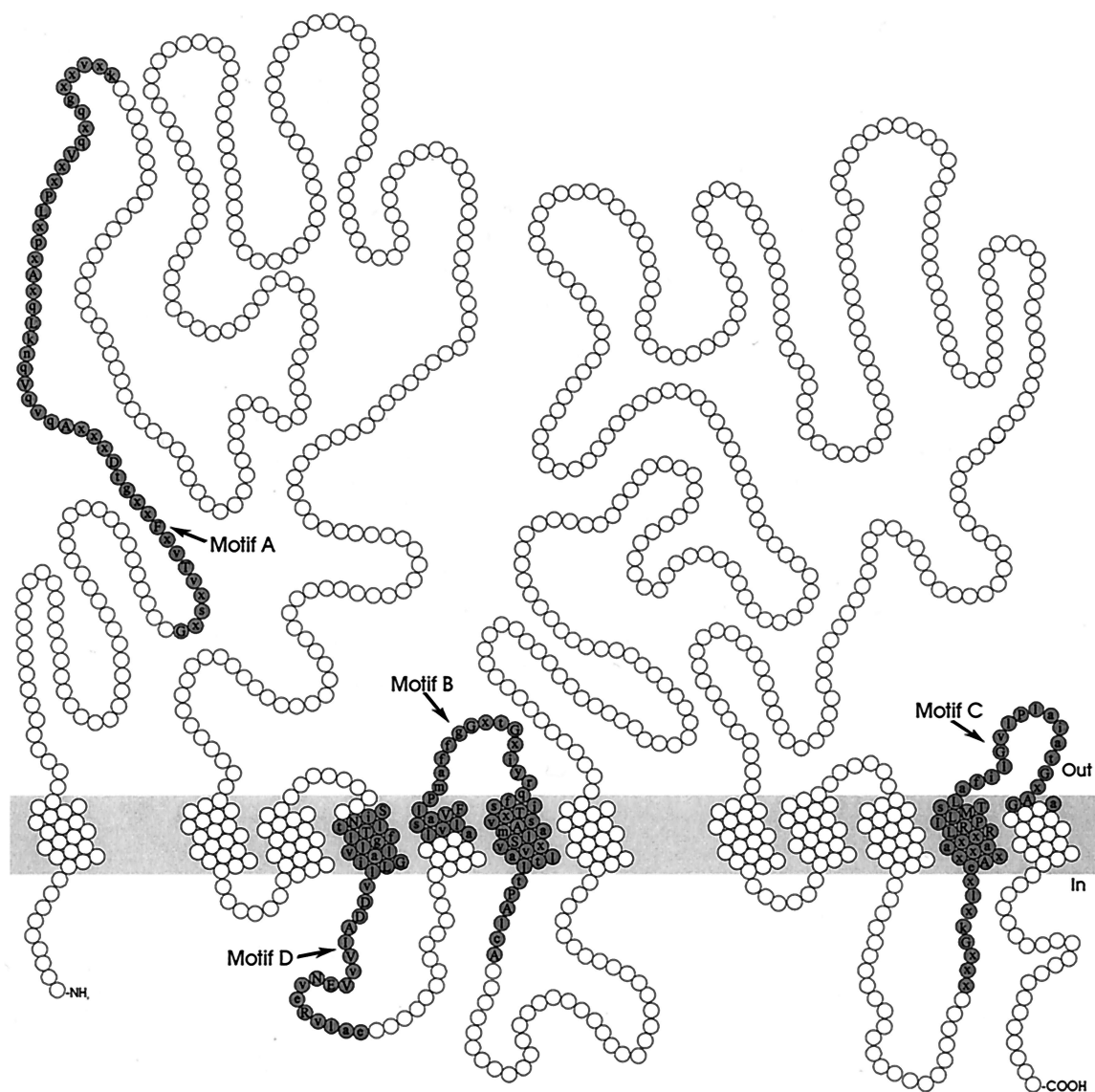


FIG. 6. Structural model for multidrug transporters of the RND family. The residues constituting the conserved sequence motifs are shaded.

*mtrD*, and *mtrE*), encoding proteins of the MFP, RND, and outer membrane protein families, respectively (80). Insertional inactivation of either the *mtrC* (80) or *mtrE* (41) gene resulted in a significant decrease in resistance to erythromycin, penicillin, Triton X-100, and fatty acids, suggesting that both proteins are required in the efflux process.

Genome sequencing of *Haemophilus influenzae* has identified a three-gene cluster, consisting of HI0893, HI0894, and HI0895, which is homologous to the *acrRAB* cluster of *E. coli* (57). Disruption of either HI0894 (an *acrA* homolog) or HI0895 (an *acrB* homolog) caused hypersusceptibility to erythromycin, rifampin, novobiocin, sodium dodecyl sulfate, and cationic dyes. Erythromycin accumulation experiments indicated that the HI0894 and HI0895 proteins contribute to the resistance of *H. influenzae* through PMF-dependent drug efflux (220).

*Burkholderia pseudomallei*, a motile gram-negative rod, is the causative agent of melioidosis. Successful treatment of melioidosis patients is difficult because *B. pseudomallei* is in-

trinsically resistant to a variety of antibiotics, including  $\beta$ -lactams, aminoglycosides, and macrolides. Recently, the *amrAB-OprA* operon was identified, which encodes a multidrug efflux system in *B. pseudomallei* which confers resistance to both aminoglycoside and macrolide antibiotics. AmrA and AmrB show strong homology to MexC and MexB, respectively, of *P. aeruginosa* (161).

#### Multidrug and Toxic Compound Extrusion Family

Norfloxacin accumulation experiments revealed that *Vibrio parahaemolyticus* contains an energy-dependent efflux system, designated NorM (163). NorM and its *E. coli* homolog YdhE mediate resistance to dyes, hydrophilic fluoroquinolones, and aminoglycosides. NorM contains 12 putative TMS, and on this basis it has been suggested to be a member of the MFS (163). NorM and YdhE, however, do not have sequence similarity with any members of the MFS and do not exhibit any of the signature sequences (see below). Brown et al. (28) found that



NorM and YdhE are members of a previously unidentified family which contains more than 30 proteins, termed the multidrug and toxic compound extrusion (MATE) family. Phylogenetic analysis of the MATE family revealed the presence of three distinct clusters, of which the first includes NorM, YdhE, and hypothetical multidrug efflux proteins from *H. influenzae* and *B. subtilis* (28).

### Electrogenic Drug/Proton Antiport

Drug transport by multidrug transporters of the MFS, SMR, RND, and MATE families is driven by the transmembrane electrochemical gradient of protons or possibly sodium ions. The PMF is composed of a chemical proton gradient ( $\Delta\text{pH}$ , inside alkaline) and an electrical potential ( $\Delta\Psi$ , inside negative). The ionophores nigericin and valinomycin, which can selectively dissipate the  $\Delta\text{pH}$  and  $\Delta\Psi$ , respectively, are valuable tools for the study of the energetics of secondary multidrug transporters. Ng et al. (174) demonstrated that NorA-mediated norfloxacin uptake in inside-out membrane vesicles was completely abolished by nigericin. In contrast, addition of valinomycin resulted in a slight increase in the uptake of norfloxacin. These results indicate that the  $\Delta\text{pH}$  is the major driving force for NorA. Grinius et al. (73) reported that NorA-mediated drug transport in proteoliposomes is an electrogenic drug/proton antiport process. Transport experiments in the presence of valinomycin and/or nigericin showed that both the  $\Delta\text{pH}$  and the  $\Delta\Psi$  are driving forces of LmrP-mediated transport of trimethyl ammonium-diphenyl hexatriene (TMA-DPH) (22) and Hoechst 33342 (203), consistent with an electrogenic exchange of one lipophilic cation for two or more protons. Mitchell et al. (159) investigated which component of the PMF is involved in energizing QacA-mediated drug efflux. It was found that both the  $\Delta\text{pH}$  and the  $\Delta\Psi$  drive the electrogenic transport of monovalent and divalent cations by QacA. The involvement of both components of the PMF as the driving force in Smr-mediated drug transport was demonstrated by studies using purified Smr reconstituted into proteoliposomes. A  $\Delta\Psi$  (positive inside) superimposed on  $\Delta\text{pH}$  (acidic inside) resulted in additional ethidium uptake via Smr (72). An electrogenic drug/proton antiport reaction by Smr was further supported by the observation that Smr increased the resistance of *E. coli* cells to ethidium in alkaline medium, when the PMF predominantly consists of a  $\Delta\Psi$  (72). The electrogenic drug/ $n\text{H}^+$  ( $n \geq 2$ ) antiport mechanism observed for LmrP, NorA, QacA, and Smr appears to be a general feature of multidrug transporters of the MFS and SMR families and may also be valid for the RND- and MATE-type multidrug transporters. The substrates used in the studies on the energetics of secondary multidrug transporters are monovalent cations. However, several multidrug transporters, such as QacA, MdfA, and YkkCD, also mediate resistance to divalent cations, neutral drugs, and anionic compounds. An analysis of the relative contribution of the  $\Delta\Psi$  and the  $\Delta\text{pH}$  to the driving force for transport of these cations can provide further insight into the drug/proton ratio of the export process.

### Conserved Sequences in Secondary Multidrug Transporters

Multiple-sequence analysis of transporters within the various clusters of solute and drug transporters of the MFS revealed a substantially larger sequence similarity in the N-terminal halves of these proteins than in their C-terminal halves (70, 146, 188, 213). It has been hypothesized that the N-terminal domain is involved in proton translocation, while the C-terminal domain of the transporters is primarily involved in determining the substrate specificity (70, 213). On the other

hand, the significant sequence similarity observed between the N- and C-terminal halves of transporters of the MFS suggests that they have evolved from a duplication of a common ancestor with six TMS (122, 145, 215, 274). The 14-TMS cluster of the MFS may have evolved via a similar gene duplication event and the acquisition of two additional TMS (70, 188, 217). The homology between the first and second halves of RND proteins suggests that these proteins also may have arisen via an intragenic duplication event during evolution (75, 216).

The multiple alignments of amino acid sequences further revealed a number of strongly conserved amino acid sequence motifs (Table 4 and Fig. 3, 4, 5, and 6) throughout the multidrug transporters of the MFS, SMR, and RND families (188, 190, 191, 213, 216). The conservation of these motifs suggests that they play an important structural or functional role in the transporters. Motif A in the cytoplasmic loop between TMS 2 and 3 in transporters of the MFS, which is predicted to contain a  $\beta$ -turn structure, may be involved in the reversible conformational change required for the opening and closing of a transport channel (102, 188, 272, 273). The absolute conservation of the basic arginine residue suggests that motif B in TMS 4 of MFS transporters may be involved in proton transfer (188). Interestingly, motif C in TMS 5 is found in multidrug transporters and specific drug efflux pumps, but not in symporters of the MFS. This suggests that motif C dictates the direction of transport (70, 188). The GP dipeptide of this motif causes a bend in helix 5, and the repeating pattern of glycine residues forms a pocket devoid of side chains. These structures may determine the orientation of the unoccupied substrate-binding site(s) and, consequently, the direction of transport (261). The C-terminal motifs F and G represent partial duplications of motif C and may have a similar function. Site-directed mutagenesis studies can provide valuable information on the structure and mechanism of multidrug transporters of the MFS, RND, and SMR families. The conserved motifs may be directly involved in drug or proton translocation. The charged residue in motif B (Arg) and in motif D1 (Asp) in members of the MFS, in motif A (Glu) in SMR-type multidrug transporters, and in motif C (two Arg) in drug efflux systems of the RND family are predicted to be located in TMS. The presence of positively or negatively charged amino acid residues in the membrane is energetically unfavorable, suggesting that these motifs play a functional role in the binding and transport of charged drugs and/or translocation of protons. Alternatively, they may play a structural role in the interaction between the transporters of the RND family (motif A) and proteins of the MFP family, or in the formation of homo- and heterooligomers of SMR-type multidrug transporters. The conserved sequences are characteristic of proteins of the MFS, SMR, and RND families and can be used to identify new members of these families in bacterial genomes.

### Résumé

The secondary multidrug transporters identified in bacteria (Tables 1 to 3) belong to one of four distinct families of proteins, MFS, SMR, RND, and MATE. Whereas multidrug transporters of the MFS and SMR families have been found in both gram-positive and gram-negative bacteria, RND- and MATE-type multidrug transporters have so far only been identified in gram-negative organisms. Table 5 indicates that bacteria can contain several multidrug transporters from different families. The presence of a variety of multidrug transporters with an overlapping substrate spectrum would seem redundant. However, the availability of an array of multidrug transporters, which are controlled by both global and specific reg-



TABLE 4. Consensus sequences of conserved motifs in transporters of the MFS, SMR, and RND families

Motif	Consensus sequence <sup>a</sup>	Location
MFS, both 12- and 14-TMS cluster		
A	G x L a D r x G r k x x (x) l	Loop between TMS 2 and 3
B	l x x x R x x q G x g a a	TMS 4
C	g x x x G P x x G G x l	End of TMS 5
MFS, 12-TMS cluster		
D2	l g x x x x P v x P	End of TMS 1
G	G x x x G P L	End of TMS 11
MFS, 14-TMS cluster		
D1	l D x T v x n v A l P	End of TMS 1
E	D x x G x x L	TMS 7
F	l g x x x G x a v x g x l	TMS 13
H	W x w x F l l N v P i g	TMS 6
SMR family		
A	W i x l v i A i l l E V	TMS 1
B	K x s e G F t r l x P S	Loop between TMS 1 and 2
C	P v G t A Y A v W t G l G	Start of TMS 3
RND family		
A	G x s x v T v x F x x g t D x x x A q v q V q n k L q x A x p x L P x x V q x q g x x v x k	Loop between TMS 1 and 2
B	a l v l s a V F l P m a f f g G x t G x i y r q f s i T x v s A m a l S v x v a l t l t P A l c A	TMS 6
C	x x x G k x l x e A x x x a x x R L R P l L M T s L a f i l G v l P l a i a t G x A G a	TMS 11
D	S i N t l T l f g l v l a i G L l v D D A l V v V E N v e R v l a e	TMS 4

<sup>a</sup> The motifs were identified by alignment of amino acid sequences (190, 191). The consensus sequences of the motifs are displayed as follows: x, any amino acid; capital letters, amino acid occurs in >70% of the examined sequences; lowercase letters, amino acid occurs in >40%; (x), amino acid not always present.

ulatory proteins, enables the bacterium to fine tune the response to different environmental signals.

The most striking difference between multidrug transporters of the SMR family and drug efflux systems from the other families is their size. Whereas members of the MFS, RND, and MATE families consist of 12 or 14 TMS, SMR-type multidrug transporters only contain 4 TMS. These small multidrug efflux pumps are thought to function as homooligomers, presumably composed of three monomers. However, two new SMR-type multidrug transporters were recently identified in *B. subtilis* which require two homologous but dissimilar subunits for drug efflux. This observation raises the question of what molecular determinants dictate whether an SMR transporter functions as a homo- or heterooligomer.

Secondary multidrug transporters extrude drugs in exchange with protons (or sodium ions). Both components of the PMF can drive the extrusion of drugs by multidrug transporters from the MFS, SMR, and RND families, indicating that an electrogenic antiport mechanism is a general feature of secondary multidrug transporters.

TABLE 5. Number of sequenced and functionally characterized multidrug transporters of various families in selected bacteria

Organism	MFS		SMR	RND	MATE
	12-TMS cluster	14-TMS cluster			
<i>Bacillus subtilis</i>	2	1	2	0	0
<i>Escherichia coli</i>	3	1	1	3	1
<i>Staphylococcus aureus</i>	1	2	3	0	0
<i>Mycobacterium tuberculosis</i>	1	1	1	0	0
<i>Pseudomonas aeruginosa</i>	0	0	0	4	0

Multiple sequence alignments of transporters within one family revealed the presence of three or more conserved motifs in multidrug transporters of the MFS, SMR, and RND families (Table 4). Hydrophathy analysis, the recognition of family-specific conserved motifs, and the availability of the complete genomic sequences of 28 bacteria and 8 archaea (<http://www.tigr.org/tdb/mdb>) enable the identification of putative multidrug transporters. However, as multidrug transporters are frequently more homologous to substrate-specific transporters than to other multidrug transporters, the activity of newly identified proteins needs to be characterized.

#### ATP-DEPENDENT MULTIDRUG TRANSPORTERS

Although most bacterial multidrug drug transporters utilize the PMF (or sodium) for the extrusion of cytotoxic compounds, some drug efflux systems are driven by the free energy of ATP hydrolysis (Fig. 7). All ATP-dependent drug efflux proteins known to date are members of the ABC superfamily (87), also referred to as traffic ATPases (8). In general, ABC transporters require four distinct domains: two highly hydrophobic membrane domains, which usually consist of six putative transmembrane  $\alpha$ -helices each, and two hydrophilic nucleotide-binding domains (NBDs), containing the Walker A and B motifs (263) and the ABC signature (96). The individual domains can be expressed as separate proteins or may be fused into multidomain polypeptides in a variety of ways (55, 87, 96).

Most bacterial ABC drug transporters mediate the export of specific antibiotics (17, 55, 76, 130, 183, 197, 210, 212). The first true bacterial ABC multidrug transporter was found in *Lactococcus lactis* (20). The gene encoding this transporter, designated *lmrA*, encodes a 590-amino-acid membrane protein with an N-terminal hydrophobic domain with six putative transmembrane helices and a C-terminal hydrophilic domain, con-

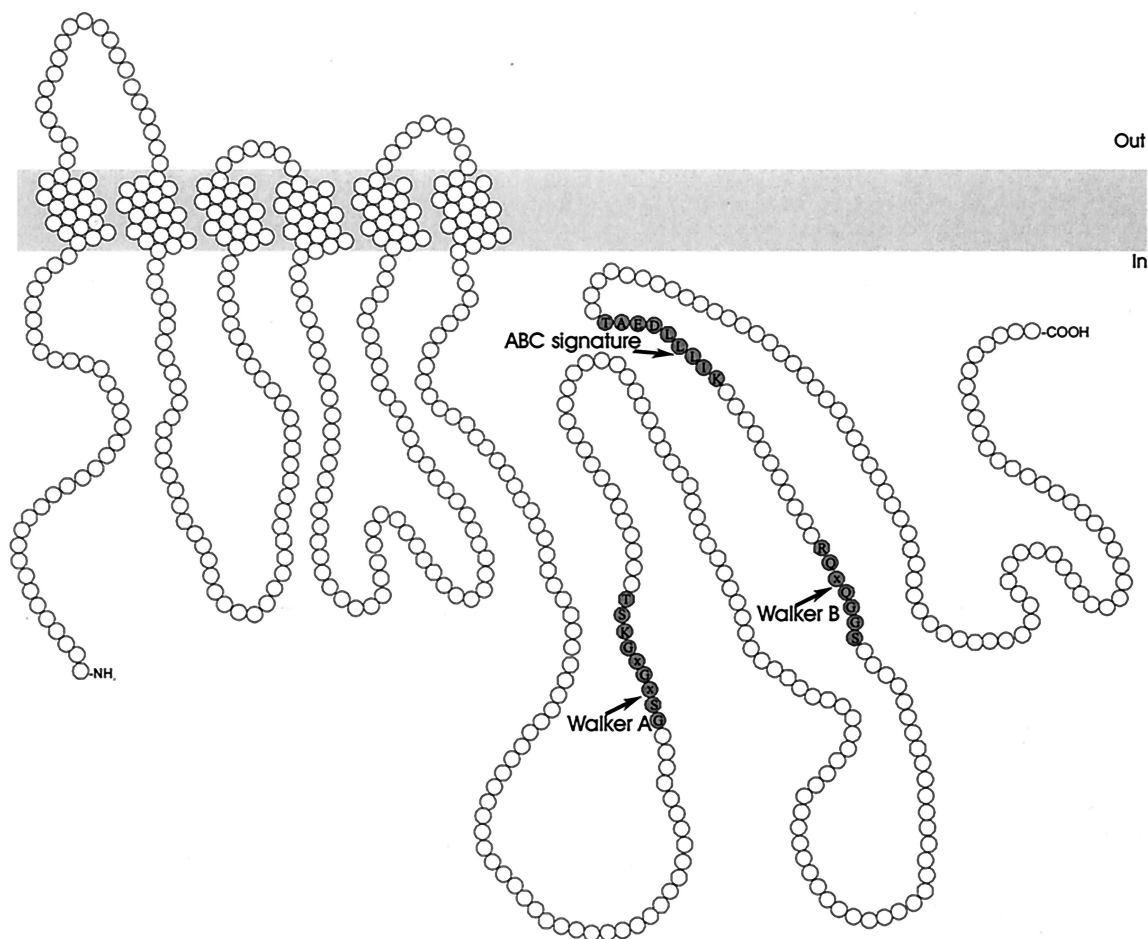


FIG. 7. Structural model for multidrug transporters of the ABC superfamily. Shown is the multidrug transporter LmrA, with six transmembrane helices and an NBD, containing the Walker A and B motifs and the ABC signature sequence. In view of the general four-domain organization, it may function as a homodimer. The Walker A and B motifs and the ABC signature sequence are shaded.

taining the ATP-binding cassette (256). LmrA is homologous to each of the two halves of the human multidrug transporter P-glycoprotein (256), suggesting that LmrA is functional as a homodimer. This suggestion was confirmed by studies on covalently linked dimers composed of wild-type LmrA units (designated KK), mutant LmrA units containing an inactivated NBD (designated MM), and combinations of wild-type and mutant units (designated KM and MK) (258). The ethidium transport activity and substrate-stimulated ATPase activity of the KK dimeric form of LmrA was comparable to that of wild-type LmrA. However, the KM, MK, and MM mutant forms of dimeric LmrA had lost all LmrA-associated ATPase activity and the ability to transport ethidium from the cell. These results are supported by reconstitution experiments which demonstrate the negative dominance of M units over K units in proteoliposomes and suggest that homodimeric LmrA is the minimal functional unit. Hence, functional crosstalk between two LmrA monomers is essential for activity (258).

When expressed in human lung fibroblast cells, LmrA is targeted to the membrane and confers multidrug resistance on these human cells (257). The pharmacological characteristics of LmrA and the human multidrug transporter P-glycoprotein (67) and the affinities of both proteins for vinblastine and  $Mg^{2+}$ -ATP are very similar. Blockers of P-glycoprotein also inhibited LmrA-mediated resistance. These results demon-

strate that LmrA and the human multidrug transporter P-glycoprotein are functionally interchangeable, indicating that this type of multidrug transporter is conserved from bacteria to humans (258).

Kinetic analysis of vinblastine dissociation from LmrA and characterization of transport of Hoechst 33342 or vinblastine in the presence of low concentrations of a second substrate revealed that LmrA contains at least two allosterically linked drug-binding sites with overlapping substrate specificities (see below) (257, 258). Purified LmrA reconstituted in proteoliposomes mediates ATP-dependent transport of Hoechst 33342 (147). Certain ABC transporters, including the LmrA homolog MsbA of *E. coli* (285), are able to translocate lipids. The specificity of reconstituted LmrA for  $C_6$ -NBD-labeled phosphatidylethanolamine and phosphatidylcholine was investigated. Interestingly, LmrA catalyzes the transport of  $C_6$ -NBD-labeled phosphatidylethanolamine but not of  $C_6$ -NBD-labeled phosphatidylcholine. These results indicate that LmrA exhibits specificity for phospholipid headgroups and that LmrA may be involved in the translocation of specific lipid or lipid-linked precursors in *L. lactis* (147).

Hop-resistant lactobacilli can cause serious spoilage of beer. Sami et al. (218, 219) demonstrated that plasmid pRH45 of *Lactobacillus brevis* is involved in the high resistance to hop compounds (iso- $\alpha$ -acids). pRH45 contains the *horA* gene,

which encodes a protein of the ABC superfamily. HorA is a structural homolog of LmrA, containing six putative TMS and a C-terminal ATP-binding domain. In addition to conferring hop resistance, HorA confers resistance to the structurally unrelated drugs novobiocin and ethidium (219). HorA has been heterologously expressed in *Lactococcus lactis*, purified, and functionally reconstituted in proteoliposomes. HorA-mediated Hoechst 33342 transport in proteoliposomes is inhibited by hop compounds (K. Sakamoto et al., submitted for publication).

Kaidoh et al. (105) described an ATP-dependent drug efflux system in the archaeon *Haloferax volcanii*. This efflux system mediates the transport of doxorubicin, vinblastine, vincristine, ethidium, and monensin. Expression of the transporter is induced in media containing a large excess of essential amino acids, glucose, or fructose. These observations suggest that this efflux system plays a role in the active extrusion of cytotoxic compounds or toxic metabolites generated in the cell. The gene encoding this ABC-type multidrug transporters remains to be cloned and sequenced (105). Genome sequencing revealed the presence of putative LmrA homologs in *E. coli*, *B. subtilis*, and the pathogens *Helicobacter pylori*, *Mycobacterium genitalium*, *H. influenzae*, and *S. aureus* (259). Functional characterization of these proteins is required to demonstrate their involvement in the ATP-dependent transport of structurally unrelated drugs.

Mutant analysis and photoaffinity labeling experiments have been used to identify essential residues and putative substrate-binding sites in the human multidrug transporter P-glycoprotein. The results demonstrated that, although some mutations leading to changes in specificity are located in other regions of the protein, TMS 5, 6, 11, and 12 and the loop between TMS 2 and 3 and TMS 11 and 12 are most important in drug binding and/or transport (39, 43, 68, 69, 82, 106, 137, 139, 164, 282). Interestingly, sequence conservation between the lactococcal multidrug transporter LmrA and P-glycoprotein includes parts of TMS 5 and 6 and the loop between TMS 2 and 3 (256), indicating that these particular regions may be important for substrate binding in both human and bacterial ABC-type multidrug transporters.

## REGULATION

Several studies demonstrated that the expression levels of multidrug transporters such as Bmr, NorA, QacA, and P-glycoprotein can be enhanced by the addition of structurally diverse compounds which are exported by these pumps (3, 74, 104, 107). These observations indicate that the expression of multidrug transporters is controlled by regulatory proteins which, like the transporters, are capable of recognizing structurally diverse compounds. The activator and repressor proteins involved in the transcriptional regulation of multidrug transporter genes are summarized in Table 6.

The best-studied regulator of multidrug transport is BmrR, the transcriptional activator of the *B. subtilis* multidrug transporter Bmr. BmrR, which is encoded by a gene immediately downstream of *bmr*, belongs to the MerR family of regulators and has strong homology with other members of the family only in the N-terminal helix-turn-helix DNA-binding domain. Binding of one rhodamine 6G molecule to a dimer of BmrR increases the affinity of BmrR for the *bmr* promoter, resulting in enhanced transcription of the *bmr* gene (3). The individually expressed C-terminal region of BmrR forms dimers and binds a broad range of drugs with the same affinity as full-length BmrR (148, 150). High-resolution crystal structures of the BmrR C-terminal region and its complex with TPP<sup>+</sup> revealed

TABLE 6. Reported transcriptional regulators that control the expression of multidrug transporters in bacteria

Multidrug transporter(s)	Regulator	Type	Reference
Blt	BltR	Activator	4
Blt, Bmr	Mta	Global activator	16
Bmr	BmrR	Activator	3
EmrB	EmrR	Repressor	136
QacA	QacR	Repressor	74
AcrB	MarA	Global activator	182
AcrB	AcrR	Repressor	144
AmrB	AmrR	Repressor	161
MexB	MexR	Repressor	201
MexD	NfxB	Repressor	200
MexF	MexT	Activator	116
MexZ	MexX	Repressor	6
MtrD	MtrA	Activator	214
MtrD	MtrR	Repressor	185

drug-induced unfolding and relocation of an  $\alpha$ -helix, which exposes an internal drug-binding pocket. Residue Glu-134, which is completely buried in the unliganded structure, is the key to cation selectivity, while a number of nonpolar and aromatic side chains impose specific requirements on the size and shape of the substrate (284). The transcription of the *blt* gene is regulated by another member of the MerR family, BltR. The C-terminal domains of BmrR and BltR have no sequence similarity, which is consistent with the observation that Bmr and Blt are expressed in response to different inducers. This difference in inducer specificity may explain why Bmr and not Blt is normally expressed in wild-type *B. subtilis* (4).

The expression of the *S. aureus* multidrug efflux gene *qacA* is regulated by the divergently transcribed repressor protein QacR. QacR binds specifically to the *qacA* promoter, preventing transcription of the gene. Substrates of QacA, such as ethidium and rhodamine 6G, interact with QacR directly and inhibit the binding of QacR to the *qacA* promoter, resulting in derepression of *qacA* expression (74).

The EmrAB multidrug pump of *E. coli* is induced in the presence of CCCP, the weak acid salicylate, and a number of other structurally unrelated drugs. The derepression is controlled by the EmrR, a MarR type of repressor protein (136). EmrR was found to bind putative substrates of the EmrAB pump, such as 2,4-dinitrophenol, CCCP, and carbonyl cyanide *p*-(trifluoro-methoxy)-phenylhydrazone (FCCP), with micromolar affinity. Equilibrium dialysis experiments suggested one bound ligand per EmrR dimer (27).

Analysis of the sequence upstream of the *mtrCDE* multidrug efflux system of *N. gonorrhoeae* revealed the presence of the divergently transcribed repressor gene *mtrR*. MtrR binds to the 13-bp inverted repeat sequence between the *mtrR* and *mtrC* promoters, thereby inhibiting expression of the *mtrCDE* gene complex (80, 185). Studies by Hagman and Shafer (79), however, showed that the MtrCDE efflux system is subject to both MtrR-dependent and MtrR-independent regulation. Recently, the transcriptional activator MtrA was shown to be involved in the modulation of *mtrCDE* gene expression (214).

The transcription of the *acrAB* operon in *E. coli* is regulated by the repressor AcrR (144). Gel mobility shift assays and *lacZ* transcriptional fusions suggested that the general-stress-enhanced transcription of *acrAB* (144) is primarily mediated by global regulatory pathways such as the *mar* regulon (see below) and that a major function of AcrR is that of a specific secondary modulator, which fine tunes the level of *acrAB* transcription (144).



The transcriptional repressor AmrR of *Burkholderia pseudomallei* shows strong similarity to the regulator MtrR. The expression of the AmrAB-OprA efflux system was not affected by the substrate streptomycin or by stress conditions (4% ethanol or 0.5% NaCl), and further studies are required to identify the inducers of this system (161).

The expression of the *P. aeruginosa* multidrug transporter MexAB-OprM is regulated by MexR, a MarR-type regulator (53, 201). The transcriptional repressor NfxB regulates a second MDR in *P. aeruginosa*, MexCD-OprJ, as well as its own expression (200). The *mexEF-oprN* efflux operon differs from the other *P. aeruginosa* efflux systems in that it is positively regulated by MexT, a protein belonging to the LysR family of transcriptional activators (115, 116). A third MDR in *P. aeruginosa*, MexXY, may be regulated by MexZ, which is homologous to the repressor proteins MtrR and AmrR (6).

In addition to the specific regulatory mechanisms that affect the expression of single multidrug transporters, induction of multidrug efflux systems can also result from global regulatory mechanisms. Multiple-antibiotic resistance (Mar) mutants of *E. coli* express elevated levels of resistance to a wide range of antibiotics. The alleles that affect the Mar phenotype are located in the *marRAB* operon (34). Expression of this operon is normally repressed by the transcriptional repressor MarR (9, 225) but can be derepressed by various compounds, such as tetracycline, chloramphenicol, and salicylates (35, 78). The *marA* gene encodes an AraC-type activator that is structurally and functionally similar to two other global regulators of *E. coli*, SoxS and Rob (60, 235, 270). The MarA, SoxS, and Rob proteins are transcriptional activators of at least a dozen promoters, the *mar* and *soxRS* regulons, that confer resistance to a variety of antibiotics and superoxide-generating agents (10, 62, 99, 100, 226). The observed resistance is partly due to increased *micF* transcription and the consequent decrease in expression of the outer membrane porin OmpF (33, 60). The RND-type multidrug transporter AcrAB-TolC plays a major role in the antibiotic and organic solvent resistance induced by MarA, SoxS, or Rob (182, 266). The *mar* operon is widespread among enteric bacteria, including the genera *Salmonella*, *Shigella*, *Klebsiella*, *Citrobacter*, *Hafnia*, and *Enterobacter* (36). Expression of *E. coli* MarA in *Mycobacterium smegmatis* resulted in increased resistance to multiple antimicrobial agents, suggesting that MarA functions in *M. smegmatis* and that a *mar*-like regulatory system exists in mycobacteria (156). Recently, Baranova et al. (16) identified a global MerR-type regulator in *B. subtilis*, termed Mta. The individually expressed N-terminal DNA-binding domain of Mta mimics the inducer-bound form and interacts directly with the promoters of *bmr* and *blt*, thereby inducing transcription of these genes. Additionally, this domain stimulates the expression of the *mta* gene and at least one more gene, *ydfK*, which encodes a hypothetical membrane protein (16).

### RECONSTITUTION

Multidrug transporters have predominantly been studied in whole cells and membrane vesicles, in which other proteins and cellular components or processes may complicate the interpretation of the data. In order to elucidate the structure, function, and mechanism of multidrug transporters at the molecular level, methods have been developed for overexpression, purification, and functional reconstitution into liposomes (72, 73, 147, 203, 227, 275, 280). Although the overexpression of membrane proteins is often deleterious for the cells (48, 119, 203, 243), drug transporters could be overexpressed up to 5 to 30% of total membrane protein using homologous expression sys-

tems and inducible promoters, such as the T7 polymerase promoter (275), the *lac* promoter (236), and the *nisA* promoter (147, 203). The first step in the purification of membrane proteins involves extraction of the protein from the membrane. The *E. coli* multidrug transporter EmrE can be quantitatively and functionally extracted with a mixture of organic solvents, such as chloroform-methanol (275). However, solubilization of most other membrane proteins requires the use of detergents (115). The choice of detergent is crucial, since some detergents can irreversibly inactivate the protein (227; J. Knol, R. H. E. Friesen, and B. Poolman, submitted for publication). In addition, detergents are potential substrates for multidrug efflux proteins and can affect the transport activity (46, 203, 268). Retention of detergent during the reconstitution procedure is a serious problem which further restricts the choice of detergent for the solubilization and reconstitution of multidrug transporters (115). Affinity chromatography, a highly specific and efficient technique, allows the purification of several multidrug transporters to a high degree of purity (>90%) in a single step (72, 147, 203, 227, 280). After reconstitution in liposomes, Smr, P-glycoprotein, EmrE, NorA, LmrA, LmrP, AcrB, and HorA mediated the transport of multiple drugs in response to the imposition of an artificial proton gradient or the addition of ATP (72, 73, 147, 203, 227, 275, 280; K. Sakamoto, H. W. van Veen, and W. N. Konings, unpublished data). Taken together, these results demonstrate that multidrug transporters of the MFS, SMR, RND, and ABC families are capable of mediating drug transport independent of accessory proteins or cytosolic components.

### SUBSTRATE BINDING

Both secondary and ATP-dependent multidrug transporters extrude a wide variety of toxic compounds. Although the substrates have very different structures, they share physical characteristics, such as high hydrophobicity, an amphiphilic nature, and a positive or neutral charge. In the absence of three-dimensional structural information, the identification of structure-function relationships in multidrug transporters has relied on amino acid sequence alignments and site-directed mutagenesis. The localization of a charged residue in the membrane-embedded part of the protein is normally considered energetically unfavorable, yet many multidrug transporters of the MFS and SMR families, such as MdfA, QacA, EmrE, and Smr, contain an acidic amino acid residue in the middle of TMS 1 (50). Furthermore, Guan et al. (75) identified three highly conserved negatively charged amino acid residues in transmembrane domains of efflux proteins of the RND family. Since the majority of the MDR substrates are positively charged, the negative charge of an intramembranous acidic residue may be involved in substrate binding. The role of the conserved Glu in TMS 1 of Smr has been investigated using site-directed mutagenesis. Even conservative substitutions, such as the replacement of Glu with Asp, effectively abolished the activity of the efflux system, indicating that this residue may be directly involved in the multidrug efflux process, potentially in substrate binding and/or the exchange of drug molecules for protons (72). Edgar and Bibi (50) demonstrated that a single membrane-embedded negative charge is critical for the recognition of positively charged drugs by the multidrug transporter MdfA of *E. coli* but not for transport activity. Replacement of the Glu residue in TMS 1 of EmrE abolished binding activity, but replacement with a negatively charged Asp created a mutant protein that retained its ability to bind substrate (165). Whereas QacA transports both monovalent and divalent cations, the closely related multidrug transporter QacB only

transports monovalent cations. Sequence analysis and mutagenesis revealed that the presence of an acidic residue in TMS 10 (position 322 or 323) of these transporters is crucial for the recognition of divalent cations (192). Altogether, these results suggest that charge-charge interactions play an important role in the recognition and export of lipophilic cations by multidrug transporters.

Based on the observation that aromatic amino acid residues in transmembrane regions of the human multidrug transporter P-glycoprotein are highly conserved, Pawagi et al. (195) hypothesized that the side chains of these residues can participate in the initial binding and subsequent transport of a variety of ring-containing compounds. Indeed, Dougherty (47) indicated that cation- $\pi$  interactions between cations, such as quaternary ammonium compounds, and aromatic side chains from the amino acids Phe, Tyr, and Trp are important in a variety of proteins that bind cationic substrates. The potential role of the conserved aromatic residues Tyr<sup>59</sup> and Trp<sup>62</sup> in the staphylococcal multidrug transporter Smr has been investigated by site-directed mutagenesis (189). Substitutions at these positions abolished the multidrug efflux capacities of the protein, indicating that these residues play a critical structural or functional role. Mutations of residues Phe<sup>143</sup>, Val<sup>286</sup>, and Phe<sup>306</sup> of Bmr altered the sensitivity to reserpine and changed the substrate specificity, indicating that these residues are likely involved in substrate recognition (2, 111). Furthermore, mutations of Phe, Trp, and Tyr residues in TMS 6, 11, and 12 drastically altered the drug resistance profile of P-glycoprotein (82, 137). These results illustrate the importance of aromatic and nonpolar amino acid residues in drug binding.

The three-dimensional structure of the multidrug-binding domain of BmrR, the transcriptional activator of Bmr, supports a model in which a negatively charged amino acid residue as well as aromatic and nonpolar residues is involved in drug binding (284). The binding of TPP<sup>+</sup> to Bmr induces the displacement of a short amphipathic  $\alpha$ -helix to allow the drug to enter the drug-binding pocket. Inside the binding pocket, TPP<sup>+</sup> forms a number of van der Waals' forces and stacking interactions with the surrounding hydrophobic and aromatic residues. The bottom of the binding pocket contains a buried glutamate residue, which makes an electrostatic interaction with the positively charged substrate (284). The substrate-binding site(s) of a multidrug transporter may have a similar organization. Most secondary multidrug transporters contain an essential membrane-embedded negatively charged amino acid residue. This residue could act like a magnet to attract positively charged substrates from the inner leaflet of the membrane. The surrounding hydrophobic residues would impose requirements on the size and shape of the substrate. In contrast to secondary multidrug transporters, the ABC multidrug transporters LmrA, P-glycoprotein, and others do not contain negatively charged residues in predicted TMS. It should also be noted that the binding of neutral or negatively charged substrates cannot be explained by the model described above.

There is increasing evidence for the presence of multiple drug-binding sites in both secondary and ATP-dependent multidrug transporters, rather than a single binding site, as suggested previously (126, 195). Tamai and Safa (245) indicated for the first time that the human multidrug transporter P-glycoprotein may contain at least two kinetically distinguishable drug-binding sites. Kinetic analysis of the drug-stimulated ATPase activity of P-glycoprotein showed that cyclosporin A competitively inhibits the verapamil-stimulated ATPase activity, whereas daunorubicin, gramicidin D, vinblastine, and colchicine give an allosteric inhibition. Interestingly, cooperative stimulation of the verapamil-induced ATPase activity was

found with progesterone (131). Several other studies demonstrated a similar complex effect of pairs of substrates or modulators on the drug-induced ATPase activity of P-glycoprotein (30, 61, 153, 186). Noncompetitive inhibition of drug binding (44, 56, 153, 264) and drug transport by P-glycoprotein (13, 196, 230, 238) further support a model with multiple cooperating drug interaction sites.

Noncompetitive inhibition of rhodamine 6G transport by drugs such as quinidine, puromycin, and colchicine also suggested multiple cooperating drug interaction sites in the yeast multidrug transporter Pdr5p (117). Van Veen et al. (257) demonstrated that nicardipin interacts with the lactococcal multidrug transporter LmrA at a binding site distinct from but allosterically linked to the vinca alkaloid-binding site. Competition studies showed that QacA-mediated transport of ethidium is inhibited competitively by the monovalent cations benzalkonium and tetraphenylarsonium and noncompetitively by the divalent cations chlorhexidine and propamide. These observations also suggest that the *S. aureus* multidrug transporter QacA contains distinct binding sites for monovalent and divalent cations (159). Furthermore, kinetic analysis of LmrP-mediated Hoechst 33342 transport in inside-out membrane vesicles revealed a competitive inhibition by verapamil and quinine, a noncompetitive inhibition by nicardipin and vinblastine, and a noncompetitive inhibition by TPP<sup>+</sup>, implying that LmrP must contain at least two drug interaction sites (204). These results indicate that the presence of two or more interacting substrate-binding sites may be a general feature of multidrug efflux systems, including those of the SMR and RND families.

## MECHANISM OF TRANSPORT

The common ability of MDR substrates to intercalate in the membrane led to the suggestion that multidrug transporters transport their substrates from the lipid bilayer (88, 207, 252) rather than from the cytoplasmic aqueous phase (7). Drug recognition within the membrane is suggested by a number of observations: (i) site-directed mutagenesis has identified specific residues implicated in substrate specificity, which are predominantly located within predicted TMS (2, 50, 65, 103, 184, 192), (ii) photoactive substrates label the human multidrug transporter P-glycoprotein in or near transmembrane helices (69, 207), and (iii) nonfluorescent acetoxymethyl esters of various fluorescent indicators, such as 2',7'-bis-(2-carboxymethyl)-5-(and 6)-carboxy-fluorescein (BCECF) and calcein, are extruded from the membrane before they can be converted into fluorescent carboxylates by nonspecific esterases in the cytoplasm (23, 89, 91).

The most convincing evidence for drug efflux from the membrane to the aqueous phase, however, is provided by transport studies using the fluorescent compounds TMA-DPH, Hoechst 33342, and 2-[4-[4-(dimethylamino)phenyl]-1,3-butadienyl]-3-ethylbenzo-thiazolium perchlorate (LDS-751) which are strongly fluorescent in a hydrophobic environment but essentially nonfluorescent in an aqueous environment. Transport of these compounds from the membrane to the aqueous phase can be monitored as a decrease in fluorescence over time (22, 23, 180, 228, 229, 231). Shapiro et al. (229) demonstrated that the initial rate of P-glycoprotein-mediated Hoechst 33342 transport is directly proportional to the amount of Hoechst 33342 in the membrane, demonstrating that P-glycoprotein removes the drug from the lipid membrane. The mechanism of transport of LmrA (23), LmrP (22), MexAB-OprM (180), and QacA (159) was studied with TMA-DPH. TMA-DPH displays a biphasic interaction with bacterial membranes; a fast parti-

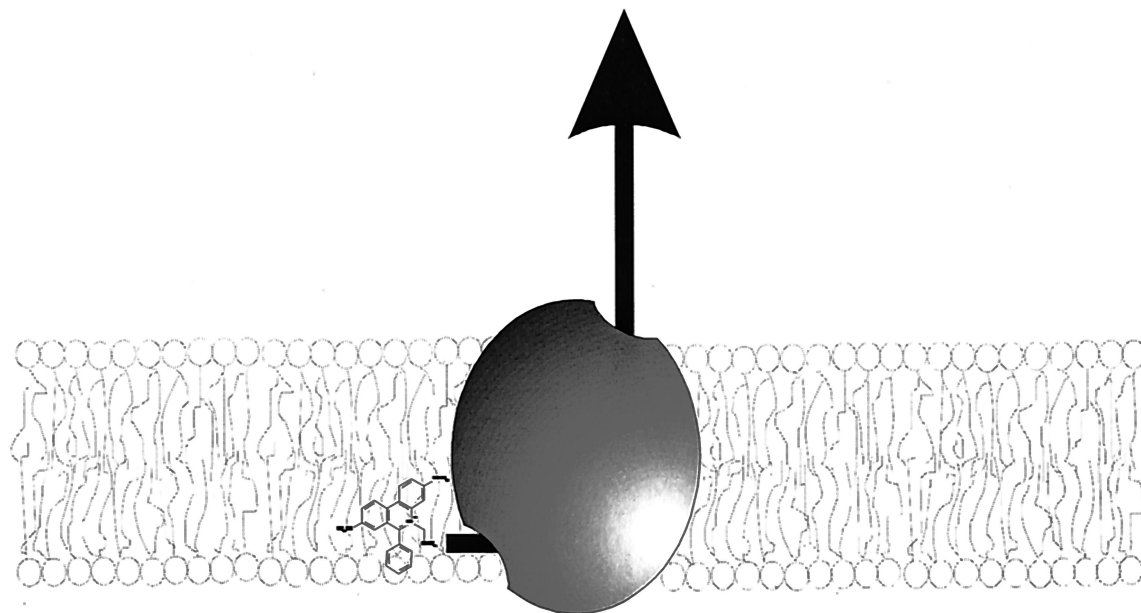


FIG. 8. Extrusion of hydrophobic drugs by multidrug transporters. Drugs are expelled from the cytoplasmic leaflet of the membrane to the external medium.

tioning of the probe in the outer leaflet of the phospholipid bilayer, followed by a slower transbilayer movement of the probe from the outer to the inner leaflet (23, 159). The initial rate of TMA-DPH extrusion by the lactococcal multidrug transporter LmrA increases with the concentration of TMA-DPH in the inner leaflet, demonstrating that LmrA transports drugs from the inner leaflet of the membrane (23). A similar relationship between the initial rate of transport and the concentration of substrate in the inner leaflet of the membrane was observed for LmrP (22) and P-glycoprotein (229, 231). Also, the MDR transporters MexAB-OprM and QacA are capable of strongly reducing the accumulation of TMA-DPH in the inner leaflet of the bilayer (159, 180). Altogether, these results demonstrate that the multidrug transporters of the MFS, RND, and ABC families extrude toxic compounds from the inner leaflet of the cytoplasmic membrane to the aqueous extracellular medium (Fig. 8). Thus, despite differences in energy coupling, ATP- and PMF-dependent multidrug efflux pumps display a general mechanism of transport for hydrophobic drugs.

Drug transport from the inner leaflet of the cytoplasmic membrane to the extracellular aqueous medium requires a high-affinity substrate-binding site that is accessible from the inner leaflet of the phospholipid bilayer. This high-affinity site is converted to a low-affinity outward-facing drug-binding site at the expense of ATP hydrolysis or proton translocation. Recently, drug transport cycles were described for the lactococcal ABC-type multidrug transporter LmrA (258) and the *E. coli* SMR-type multidrug transporter EmrE (165). Equilibrium binding experiments, photoaffinity labeling studies, and drug transport assays support the notion that homodimeric LmrA mediates multidrug transport by an alternating two-site (two-cylinder engine) mechanism. The transporter possesses two drug-binding sites: a transport-competent site on the inner membrane surface and a drug release site on the outer membrane surface. The interconversion of these two sites is driven by the hydrolysis of ATP. Indeed, a combination of total-

reflection Fourier transform infrared spectroscopy,  $^2\text{H}/\text{H}$  exchange, and fluorescence-quenching experiments indicated that LmrA undergoes a secondary-structure change and passes through three different conformational states during its drug transport cycle (262). The mechanism proposed for LmrA may also be relevant for P-glycoprotein and other ABC transporters (258). For P-glycoprotein, evidence has been obtained which supports a model of alternating catalytic sites (223). Both NBDs are able to bind and hydrolyze ATP (92, 138). The two nucleotide-binding sites interact strongly and cannot hydrolyze  $\text{Mg}^{2+}$ -ATP simultaneously, suggesting that the two NBDs may alternate in catalysis (93, 224, 253, 254).

EmrE, which contains a membrane-embedded glutamate residue that is required for ligand binding and is proposed to function as a homotrimer, binds 1 mol of  $\text{TPP}^+$  per  $\sim 3$  mol of EmrE. From the observation that both binding and release of  $\text{TPP}^+$  from EmrE are strongly influenced by pH, Muth and Schuldiner (165) proposed a mechanism of transport by EmrE. As the substrate approaches the hydrophobic binding pocket, two protons are released from the negatively charged glutamate triplet. The positively charged substrate is bound through electrostatic interactions with the negatively charged carboxylate groups of the glutamates. Following a currently unknown conformational transition, the opening to the substrate-binding pocket becomes accessible to the external face of the membrane while being closed off from the internal face. The subsequent movement of two protons towards the binding pocket catalyzes the release of the bound substrate. EmrE then undergoes a conformational transition that converts the binding pocket accessibility back to the original membrane face. As most secondary multidrug transporters contain one or more negatively charged amino acid residues in TMS, a similar mechanism may be valid for other transporters. The available techniques for purification and functional reconstitution of the secondary multidrug transporters Smr, EmrE, NorA, LmrP, and AcrB enable structural studies that can reveal conformational changes during the catalytic cycle.



## ANTIBIOTIC RESISTANCE

Bacterial multidrug efflux systems are a serious problem in the pharmacological treatment of patients with infectious diseases, since the substrate spectra of many multidrug transporters include clinically relevant antibiotics. Multidrug transporters are associated with both intrinsic and acquired resistance to antibiotics. Disruption of the genes encoding AcrAB, MexAB-OprM, HI0894/HI0895, NorA, and others causes increased sensitivity to several antibiotics, demonstrating that these multidrug transporters are expressed in wild-type strains and contribute to the intrinsic antibiotic resistance of *E. coli*, *P. aeruginosa*, *H. influenzae*, and *S. aureus*, respectively (143, 199, 220, 271). In addition, it has been shown that the expression of several genes encoding multidrug efflux pumps is inducible by the drugs to which they offer protection and that the expression of some multidrug transporters is modulated in response to environmental conditions. Acquired MDR can arise via three mechanisms: (i) amplification and mutations of genes encoding multidrug transporters, which change the expression level (174) or activity (113), (ii) mutations in specific or global regulatory genes which lead to the increased expression of multidrug transporters, exemplified by the *mexR* mutation in the OCR1 strain of *P. aeruginosa* (201), and (iii) intercellular transfer of resistance genes on transposons or plasmids, such as *qacE* (108).

The clinical importance of multidrug transporters strongly depends on the antibiotic spectrum to which they confer resistance. The antibiotic resistance profiles of the multidrug transporters known to date are summarized in Table 7. The data in Table 7 clearly show that the drug resistance profiles of the multidrug transporters vary strongly and family-specific profiles are not found. Resistance is observed against all important classes of antibiotics. MexAB-OprM of *P. aeruginosa* and the ABC-type multidrug transporter LmrA of *L. lactis* display very broad antibiotic specificity, demonstrating that both secondary and ATP-dependent multidrug transporters can seriously affect the efficacy of many antibiotics. LmrA and the human multidrug transporter P-glycoprotein are functionally interchangeable (257), suggesting that P-glycoprotein may also be involved in export of antibiotics. Indeed, several *Streptomyces* macrolide antibiotics have been reported to inhibit P-glycoprotein function (140). The plasmid-encoded multidrug transporter HorA confers resistance to a smaller range of antibiotics than the chromosomally encoded LmrA. However, since few antibiotic resistance data are available for other plasmid-encoded multidrug transporters, such as QacA, QacB, and Smr, it is not known whether a more restricted antibiotic resistance profile is a general feature of plasmid-encoded multidrug transporters. NorA and Bmr confer higher levels of resistance to hydrophilic quinolones, such as norfloxacin and ciprofloxacin, than to hydrophobic quinolones, such as sparfloxacin, ofloxacin, and nalidixic acid (104, 171, 271, 278). Similarly, norfloxacin transport by LfrA is inhibited by hydrophilic and not by hydrophobic quinolones (134). This specificity for hydrophilic quinolones was also observed for PmrA and NorM (63, 163). However, EmrB and VceB confer resistance only to hydrophobic quinolones, whereas LmrA and the multidrug transporters of the RND family transport both hydrophilic and hydrophobic quinolones (37, 66, 135, 158, 200, 205). In addition to conferring resistance, the overexpression of multidrug transporters can also cause increased drug sensitivity. The *E. coli* multidrug transporter MdfA confers resistance to the aminoglycosides neomycin and kanamycin but causes hypersensitivity to spectinomycin (19, 49). Overexpression of MexCD-OprJ results in hypersusceptibility to gentamicin and carbenicillin but

not to ampicillin and penicillin (200, 240, 241). The mechanism of this increased drug susceptibility remains to be studied.

## PHYSIOLOGICAL FUNCTION

The efflux of a broad range of structurally unrelated toxic compounds can be the primary physiological function of multidrug transporters or, alternatively, can be merely a fortuitous side effect of the transport of an unidentified specific physiological substrate (173). The latter role of MDRs would be consistent with the observation that multidrug transporters belong to four distinct families which are frequently more homologous to substrate-specific transporters than to each other (126). More convincing evidence for a role of multidrug transporters in the efflux of a specific substrate comes from the *B. subtilis* multidrug transporter Blt. Woolridge et al. (269) demonstrated that overexpression of Blt results in an increased efflux of spermidine in the medium. The *blt* gene is cotranscribed with a downstream gene, *bltD*, which encodes a spermidine acetyltransferase (4, 269). This genetic arrangement suggests that the efflux of spermidine is the natural function of the Blt transporter, whereas multiple drugs may be recognized merely opportunistically. The ability of some MDR transporters to mediate lipids or fluorescent lipid derivatives may indicate that the physiological function of these multidrug transporters involves phospholipid translocation or transport of lipid-linked precursors of peptidoglycan (24, 90, 147, 254a, 280).

Alternatively, multidrug transporters may have evolved to protect cells from diverse environmental toxins. Schinkel et al. (222) demonstrated that disruption of the mouse *mdr1a* P-glycoprotein gene leads to increased sensitivity to drugs such as ivermectin and vinblastine. Similarly, increased antibiotic susceptibility was found in *norA* knockout mutants (94, 271). P-glycoprotein and the *E. coli* multidrug transporter AcrAB are induced in response to cellular damage (31) and stress conditions (143), respectively. Furthermore, the substrate spectra of several multidrug transporters include detergents (46, 203, 268), bile salts (80, 143, 249), organic solvents (266), and ionophores (54, 218, 233), suggesting that multidrug transporters play a role in protection of the membrane integrity or energy state of the cell.

## CONCLUDING REMARKS

Multidrug transporters are a serious problem in the treatment of patients with hospital- or community-acquired infectious diseases, as they confer resistance to various of the antibiotics employed. A major force in the overexpression of endogenous multidrug transporters and spread of plasmid-encoded multidrug transporters is the huge consumption of antibiotics in human therapy, animal husbandry, and agriculture (12, 123). To restrain the evolution of antibiotic resistance, several strategies have been proposed to reduce the selective pressure of the presence of antibiotics (14, 123, 124, 267). The inappropriate use of antibiotics in household products, for the treatment of viral diseases, and as growth promoters in farm animals should be reduced. In addition, the development of new therapeutic agents is essential to fight the increasing number of multidrug-resistant microorganisms. One approach is to search for antibiotics with novel modes of action, which will not face previously selected resistance determinants. Alternatively, resistance-blocking agents can be identified and developed, allowing existing antibiotics to remain effective (125, 149). Several inhibitors of the multidrug transporters NorA and PmrA have been shown to increase the



activity of fluoroquinolones against *S. aureus* and *S. pneumoniae*, respectively. Furthermore, these inhibitors dramatically suppress the in vitro emergence of resistant strains (1, 151, 152). However, since the known inhibitors, such as reserpine, are toxic to humans, the development of nontoxic inhibitors is required. Detailed information about the structure-function relationships that determine drug binding and transport by multidrug transporters should provide us with a rationale for these strategies.

Considerable progress has been made towards the identification of amino acid residues implicated in drug binding. Mutants of both ATP-dependent and secondary multidrug transporters demonstrated that aromatic, nonpolar, and negatively charged amino acid residues in predicted TMS are involved in substrate binding by multidrug transporters. Increasing evidence suggests the presence of multiple drug-binding sites, but the exact number and their three-dimensional organization are not known. Understanding the molecular mechanism of multidrug transport will ultimately require high-resolution structures of MDR proteins. As integral membrane proteins are generally very difficult to crystallize, structures of MDR regulator proteins which can bind a similar broad range of drugs may provide useful information on the structure-function relationships that dictate drug recognition. The low-resolution structure of P-glycoprotein (211) and the high-resolution (2.7 Å) three-dimensional structure of the drug-binding region of the regulator protein BmrR (284) are the first steps towards resolving the mystery of the broad substrate specificity of multidrug transporters.

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