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Proton-Dependent Multidrug Efflux Systems

IAN T. PAULSEN,[1,2] MELISSA H. BROWN,[1] AND RONALD A. SKURRAY[1]*

School of Biological Sciences, University of Sydney, Sydney, New South Wales 2006, Australia,[1]
and Department of Biology, University of California at San Diego,
La Jolla, California 92097-0116[2]

INTRODUCTION

Both bacterial and eukaryotic cells typically contain an array of cytoplasmic membrane transport systems involved in vital roles such as the uptake of essential nutrients, the excretion of toxic compounds, and the maintenance of cellular homeostasis. Increasing numbers of such transport systems are being identified, primarily because of the explosion in the use of cloning and sequencing technology over the last 15 years. Comparative amino acid sequence analysis of various transport proteins has enabled the identification of a number of distinct families and superfamilies of transporters (90, 248).

Many membrane transport systems have been demonstrated to play an important role in both bacteria and eukaryotes by conferring resistance to toxic compounds. For instance, in human cancer cells, resistance to antitumor chemotherapeutic agents is commonly mediated by the P-glycoprotein efflux pump (87), and in bacterial pathogens, resistance to antibiotics and antiseptics is frequently due to extrusion of the drug (148). These resistance efflux systems are characteristically energy dependent and may be either primary or secondary active transport systems (148, 196).

Most efflux systems, and indeed most transport systems, typically deal with a narrow range of structurally related substrates; for example, the *Escherichia coli* tetracycline exporter TetB is capable of extruding tetracycline and a narrow range of close structural analogs (148). However, export systems which can apparently handle a wide range of structurally dissimilar compounds have also been identified, and these have become known as multidrug exporters or multidrug efflux pumps (149). These multidrug efflux systems present a disturbing clinical threat, since the acquisition of such a single system by a cell may decrease its susceptibility to a broad spectrum of chemotherapeutic drugs.

The best-characterized multidrug efflux pump is P-glycoprotein, encoded by the human or rodent *mdr1* gene, which mediates resistance to a broad range of cytotoxic drugs via ATP-dependent export (62, 87). P-glycoprotein is a member of the ATP-binding cassette (ABC) superfamily of transporters, and homologs within this family have also been proposed to be involved in ATP-dependent export-mediated multidrug resistance to antimalarial agents in *Plasmodium falciparum* (71); to emetine, iodoquinol, and diloxanide in *Entamoeba histolytica* (253, 254); and to leptomycin B and other cytotoxic drugs in *Schizosaccharomyces pombe* (197). Homologs of *mdr* have also been identified by sequence analysis in such diverse organisms as *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *E. coli*, *Haemophilus influenzae*, *Saccharomyces*

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* Corresponding author. Mailing address: School of Biological Sciences, Macleay Building A12, University of Sydney, NSW 2006, Australia. Phone: 61 2 9351-2376. Fax: 61 2 9351-4771. Electronic mail address: skurray@extro.ucc.su.oz.au

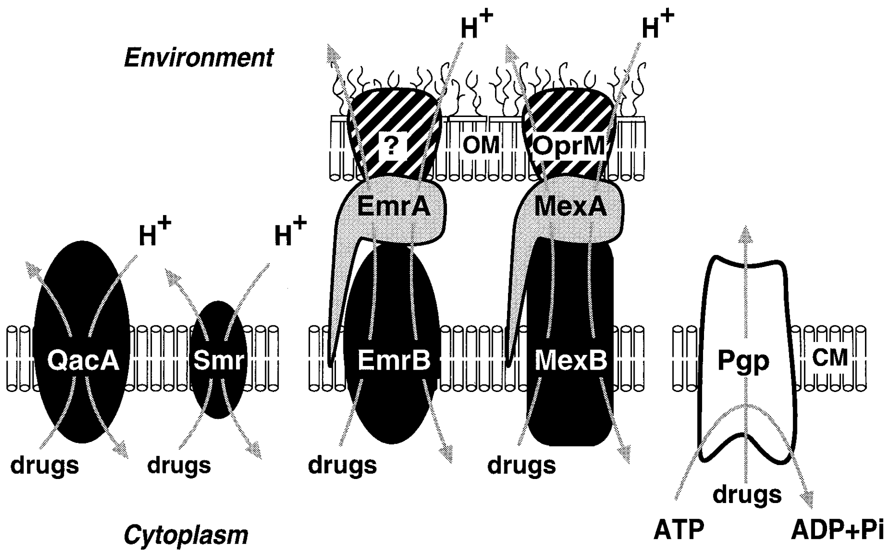


FIG. 1. Diagrammatic representation of the cytoplasmic membrane (CM) showing examples of multidrug efflux systems. The *Staphylococcus aureus* QacA and *E. coli* EmrB proteins (MFS; large solid ovals), the *S. aureus* Smr protein (SMR family; small solid oval), and the *Pseudomonas aeruginosa* MexB (RND family; solid rectangle) all appear to utilize the transmembrane proton gradient ($\Delta\mu_{H^+}$) as the driving force for multidrug efflux (92, 153, 159, 212). In contrast, the mammalian multidrug efflux pump P-glycoprotein (Pgp; white) is driven by ATP hydrolysis (62). 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 (gray), respectively. In the case of the MexAB system, an additional outer membrane (OM) protein from the OMF family, OprM (hatched), 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 (see the text and tables for references and further details of these proteins).

cerevisiae, *Staphylococcus aureus*, and *Xenopus laevis* (11, 35, 57, 69, 105, 307, 308).

P-glycoprotein and its homologs have been the subjects of numerous studies and many reviews (for examples, see references 45, 62, 87, 107, 147, and 270). However, a growing number of multidrug efflux systems which are secondary transporters, driven by the proton motive force (PMF) of the transmembrane electrochemical proton gradient ($\Delta\mu_{H^+}$) rather than by ATP hydrolysis, are being identified (92, 156, 159, 166, 188, 208). These proton-dependent multidrug transporters share no detectable sequence similarity with P-glycoprotein, but they do share an analogous ability to transport a wide variety of structurally unrelated substrates, including, in many cases, a number in common with P-glycoprotein.

Computer-based sequence analyses have revealed that the PMF-dependent multidrug efflux systems identified to date belong to one of three distinct families of proteins: the major facilitator superfamily (MFS) (90, 169, 210), the resistance/nodulation/cell division (RND) family (54, 249), and the small multidrug resistance (SMR) family (91, 204, 208, 213). In addition to multidrug efflux proteins, each of these families includes proteins involved in other PMF-driven transport processes or other functions; i.e., these families are not solely associated with multidrug export.

Examples of each type of PMF- and ATP-dependent multidrug efflux systems are displayed diagrammatically in Fig. 1. The transmembrane proton gradient ($\Delta\mu_{H^+}$), is composed of a chemical gradient of hydrogen ions (ΔpH) and an electrical charge gradient ($\Delta\Psi$). Either or both of the ΔpH and $\Delta\Psi$ components of the PMF are capable of driving drug efflux depending on the particular system (see below for details). The PMF-dependent multidrug efflux proteins QacA and EmrB (MFS), Smr (SMR family), and MexB (RND family) all probably function via a multidrug/proton antiport mechanism. In contrast, the multidrug efflux pump P-glycoprotein (ABC superfamily) is driven by ATP hydrolysis. In gram-negative bacteria, some multidrug efflux systems (EmrB and MexB) apparently require the function of additional auxiliary proteins (Fig. 1; also see below). These auxiliary proteins belong to the membrane fusion protein (MFP) (54, 249) and outer membrane factor (OMF) families (56) and apparently enable the efflux of drugs across the outer membrane permeability barrier (Fig. 1).

We present here a comprehensive review describing the known PMF-dependent multidrug export systems. The following sections detail the salient features of each of these families and the multidrug efflux proteins within each family. These resistance-conferring efflux proteins appear to be very widespread in nature, because they have been identified in organisms ranging from bacteria to humans (see the tables [below]). Underlining their biological significance, it appears likely that most organisms encode several different multidrug export systems; e.g., in *E. coli*, at least nine different systems have now been identified. Major issues addressed in this review include the molecular basis of the ability of these export systems to recognize and transport structurally disparate drugs, the primary physiological roles of such multidrug systems, and their clinical significance.

MAJOR FACILITATOR SUPERFAMILY

The MFS consists of membrane transport proteins from bacteria to higher eukaryotes involved in the symport, antiport, or uniport of various substrates (90, 169). More than 300 individual proteins which belong to this superfamily have been identified (206). It includes well-known and much studied proteins, such as the *E. coli* lactose permease LacY (127, 129) and the human GLUT glucose transporters (88), which are often considered paradigms for secondary active transport and facilitative transport, respectively. Marger and Saier (169) identified five distinct clusters or families of membrane transport proteins within the MFS involved in (i) drug resistance, (ii) sugar uptake, (iii) uptake of Krebs cycle intermediates, (iv) phosphate ester/phosphate antiport, and (v) oligosaccharide uptake. The first of these clusters consisted of PMF-dependent drug efflux proteins (210), including a number of multidrug efflux proteins, in addition to other substrate-specific drug efflux proteins, such as the well-characterized tetracycline exporter, TetB (148).

Experimental analyses (7, 32, 114, 158, 268) of the membrane topologies of proteins within clusters ii to v have revealed that they share a common structure, each with 12 transmembrane segments (TMS). In contrast, hydropathy and phylogenetic analyses have suggested that the resistance-conferring drug efflux proteins within cluster i could be divided into two distinct families with 12 and 14 TMS (90, 210). This hypothesis has been confirmed experimentally with a representative member from each of these two families (7, 205) (see below). This has led to a revised phylogeny of this cluster as proposed by Paulsen et al. (205), such that the MFS consists of at least six separate families (Fig. 2).

Two further protein families which may be distantly related to the MFS have recently been identified. One of these families consists of yeast proteins of unknown function identified by genome sequencing (81). These proteins each contain 14 predicted TMS but are distinct from the efflux proteins within the 14-TMS family identified by Paulsen and Skurray (210). The second family consists of Na^+/P_i symporters (234).

Searches of the latest versions of the protein databases have indicated that the 12- and 14-TMS families within the MFS contain more than 100 members (206). The 14-TMS family (Table 1) contains a number of known or probable PMF-dependent multidrug efflux proteins from bacteria and fungi, other resistance-conferring efflux proteins, and a number of uncharacterized or hypothetical proteins identified by genome sequence analysis. The 12-TMS family (Table 2) includes known or probable multidrug efflux proteins, vesicular amine transporters from higher eukaryotes involved in neurotransmission which can also mediate multidrug resistance (see the section on VMAT1 and VMAT2, below) (261, 264), other PMF-dependent efflux proteins, such as the TetB tetracycline/H^+ antiporter (148), and various uncharacterized or hypothetical proteins. Phylogenetic analyses of these two families are presented in Fig. 3 and 4.

Within the 14-TMS family (Fig. 3), several distinct phylogenetic groupings can be discerned: (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 of 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. A model of a representative 14-TMS family protein, the QacA multidrug efflux protein, is presented in Fig. 5.

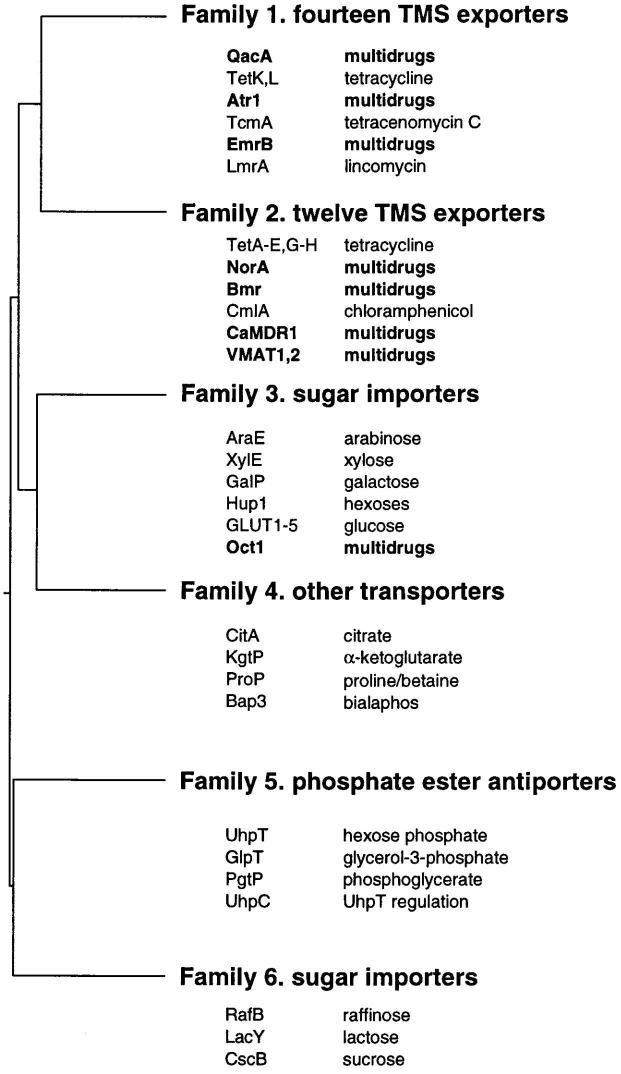


FIG. 2. Phylogenetic tree displaying the proposed evolutionary relationships among the six well-characterized families of the MFS. Phylogenetic analyses were performed with PILEUP (53). Branch points indicate the relative levels of similarity, which increases from left to right. Representative transporters within each family are shown with their substrate, putative substrate, or proposed function; multidrug exporters are in boldface type. This tree was adapted from the one presented by Paulsen et al. (205). The existence of two further families in the MFS, more distant than those shown in this figure, has been hypothesized (see the text for details) (81, 234).

Similarly, within the 12-TMS family (Fig. 4), several distinct 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 various bacterial proteins, including two multidrug efflux proteins, Bcr and EmrD; (c) a cluster of vesicular monoamine and acetylcholine transporters from higher eukaryotes, some of which appear capable of multidrug/proton antiport; (d) a cluster of bacterial proteins, including two chloramphenicol resistance proteins, Cml 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.

The clustering patterns within the 14- and 12-TMS families seem to mainly reflect functional differences between the proteins forming each cluster, e.g., clusters d and e in the 12-TMS family (Fig. 4), rather than the phylogenetic origins of the host organisms. However, in both families, there appears to be a cluster of yeast-specific proteins, which remain largely uncharacterized to date. Interestingly, in both families, multidrug efflux proteins are located within several different lineages. This fact correlates with the previous observation of Lewis that broad-substrate-specificity transporters in the MFS are gener-

TABLE 1. 14-TMS family export proteins of the MFS

Protein[a]	Organism	Representative substrate(s)[b]	Accession no.[c]	Reference(s)
Multidrug resistance				
Atr1	*Saccharomyces cerevisiae*	Aminotriazole, 4-nitroquinoline-*N*-oxide	GB Z49210	83, 131
EmrB[d]	*Escherichia coli*	CCCP, nalidixic acid, organomercurials, TCS, thiolactomycin	SW P27304	159
LfrA	*Mycobacterium smegmatis*	AC, BC, EB, fluoroquinolones	GB U40487	287
Ptr	*Streptomyces pristinaespiralis*	Pristinamycin I and II, rifampin	GB X84072	24
QacA	*Staphylococcus aureus*	Mono- and divalent organic cations, e.g., BC, CH, CT, EB, PE	EM X56628	240
Sge1	*Saccharomyces cerevisiae*	CV, EB	SW P33335	12
SmvA	*Salmonella typhimurium*	EB, MV	SW P37594	111
Other resistance				
ActII	*Streptomyces coelicolor*	Actinhordin	GB M64683	68
ActVa	*Streptomyces coelicolor*	Actinhordin	GB X58833	31
BsTet	*Bacillus subtilis*	TET	SW P23054	200, 250
CmcT	*Nocardia lactamdurans*	Cephamycin	SW Q04733	43
LmrA	*Streptomyces lincolnensis*	Lincomycin	EM X59926	325
Mmr	*Streptomyces coelicolor*	Methylenomycin A	GB M18263	183
MmrB	*Bacillus subtilis*	Methylenomycin A	SW Q00538	231
Pur8	*Streptomyces lipmanii*	*N*-Acetylpuromycin, puromycin	GB X76855	291
TcmA	*Streptomyces glaucescens*	Tetracenomycin C	GB M80674	94
Tet347	*Streptomyces rimosus*	TET	SW P14551	236
TetL	*Bacillus stearothermophilus*	TET	SW P07561	112
TetK	*Staphylococcus aureus*	TET	EM M16217	199
Hypothetical or uncharacterized				
EmrY[d]	*Escherichia coli*	Unknown	GB D78168	295
HI0852	*Haemophilus influenzae*	Unknown	SW P44903	69
HI0897[d]	*Haemophilus influenzae*	Unknown	SW P44927	69
Orf613	*Saccharomyces cerevisiae*	Unknown	GB X87941	296
Sc9852x	*Saccharomyces cerevisiae*	Unknown	GB Z49259	77
SvOrf4	*Streptomyces violaceoruber*	Unknown	GB L37334	17
ToxA	*Cochliobolus carbonum*	Unknown	GB L48797	220
Ybr293w[f]	*Saccharomyces cerevisiae*	Unknown	EM Z36162	74
Ycl69w[f]	*Saccharomyces cerevisiae*	Unknown	SW P25594	202
YieO	*Escherichia coli*	Unknown	SW P31474	29
Ym8021	*Saccharomyces cerevisiae*	Unknown	GB Z49704	215

[a] For sequences that are greater than 90% identical, only one representative protein is shown; e.g., TetL from *Bacillus stearothermophilus* (SW: P07561) (112) and TetL from *Streptococcus pneumoniae* (SW: P11063) (142) are 99.7% identical.

[b] Abbreviations: AC, acriflavin; BC, benzalkonium chloride; CH, chlorhexidine; CT, cetyltrimethylammonium bromide; CV, crystal violet; EB, ethidium bromide; MV, methyl viologen; PE, pentamidine isethionate; TCS, tetrachlorosalicylanilide; TET, tetracycline.

[c] Accession number: GB, GenBank; SW, SwissProt, EM, EMBL.

[d] EmrB functions in conjunction with EmrA, a member of the MFP (see text for details); similarly, EmrY has been postulated to function with EmrK, an MFP member; and HI0897 has been postulated to function with HI0898.

[e] It has been postulated that ToxA may function as a toxin pump (220).

[f] From our analyses, we predict that the sequence of these proteins may be incomplete and/or contain sequencing errors.

ally no more closely related to each other than to other members of these families (149). Thus, within the MFS, the phenomenon of multidrug resistance seems to have arisen independently on a number of occasions.

Multiple-sequence analysis of the MFS in general and of the 14- and 12-TMS families in particular (Fig. 6 and 7) has revealed that sequence similarity between these proteins is substantially greater in their N-terminal halves than in their C-terminal halves (90, 169, 210, 240), although some sequence similarity can be observed between their C-terminal halves (210) as is evident by the occurrence of conserved motifs in these regions (see below). Given the wide range of substrates recognized by members of the MFS (Fig. 2; Tables 1 and 2), it has been hypothesized that the C-terminal regions of MFS transporters are involved primarily in determining the substrate specificities of the proteins in the MFS and the N-terminal regions are involved primarily in the energization of transport (90, 240).

Significant sequence similarity has been observed between the N- and C-terminal halves of the 12-TMS proteins within the MFS (90, 148, 169, 210, 241). This internal homology would appear to indicate that the MFS evolved via a gene duplication event from an ancestral gene encoding a six-TMS protein (241). In the case of the 14-TMS family, sequence similarity between the N- and C-terminal regions of the proteins is less evident, but it seems likely that they also evolved via a gene duplication event and the acquisition of two additional TMS (90, 148, 210).

A number of highly conserved regions or motifs have been identified within members of the MFS (90, 169, 210). In particular, Paulsen and Skurray (210) identified motifs which were conserved throughout the MFS (motifs A and B), were found only in both the 12- and 14-TMS families (motif C), or were exclusive to either the 12- or 14-TMS family (motifs D to G). Multiple-sequence alignments of representative members from each of the main clusters of the 12- and 14-TMS families (Fig.

TABLE 2. 12-TMS family export proteins of the MFS

Protein[a]	Organism	Representative substrate(s)[b]	Accession no.[c]	Reference(s)
Multidrug resistance				
Bcr	*Escherichia coli*	Bicyclomycin, sulfathiazole	PR JN0659	20
Blt	*Bacillus subtilis*	AC, CML, CT, EB, fluoroquinolones, rhodamine 6G, TPP	EM L32599	4
Bmr	*Bacillus subtilis*	Similar range of substrates to Blt	SW P33449	188
EmrD	*Escherichia coli*	Hydrophobic uncouplers, e.g., CCCP	SW P31442	29, 182
LmrP	*Lactococcus lactis*	Daunomycin, EB, TPP	GB X89779	26
CaMDR1	*Candida albicans*	Benomyl, cycloheximide, methotrexate, 4 nitroquinolone-*N*-oxide	SW P28873	70
NorA	*Staphylococcus aureus*	Similar range of substrates to Blt	SW P21191	324
VMAT1	*Rattus norvegicus*[d]	Doxorubicin, EB, rhodamine 6G, isometamidium, MPP, TPP	GB M97380	157
VMAT2	*Bos taurus*[d]	Similar range of substrates to VMAT1	EM U02876	113
Other resistance				
Car1	*Schizosaccharomyces pombe*	Amiloride	SW P33532	120
CyhR	*Candida maltosa*	Cycloheximide	SW P32071	257
Cml	*Streptomyces lividans*	CML	SW P31141	55
CmlA	*Pseudomonas aeruginosa*	CML	SW P32482	23
CmlB	*Rhodococcus fascians*	CML	EM Z12001	52
OpdE	*Pseudomonas aeruginosa*	Unknown[e]	SW Q01602	115
Ppflo[f]	*Pasteurella piscicda*	Florfenical	GB D37826	117
TetA	*Escherichia coli*	TET	EM X00006	303
TetB	*Escherichia coli*	TET	EM J01830	191
TetC	*Pseudomonas aeruginosa*	TET	EM J01749	216
TetD	*Salmonella ordonez*	TET	EM X65876	9
TetE	*Escherichia coli*	TET	SW Q07282	8
TetG	*Vibrio anguillarum*	TET	GB S52437	326
TetH	*Pasteurella maltocida*	TET	GB U00792	99
Unc17	*Caenorhabditis elegans*	Acetylcholine	SW P34711	6
Hypothetical or uncharacterized				
CbOrf337	*Cloxiella burnetti*	Unknown	GB X78969	306
HI1242	*Haemophilus influenzae*	Unknown	SW P45123	69
P9584.7	*Saccharomyces cerevisiae*	Unknown	GB U28371	122
Slr0616	*Synechocystis* sp.	Unknown	GB D64004	133
SPAC11D3	*Schizosaccharomyces pombe*	Unknown	GB Z68166	16
TetHu	*Homo sapiens*	Unknown	EM L11669	59
YbdA[f]	*Escherichia coli*	Unknown	SW P24077	38, 271
Ybr008c	*Saccharomyces cerevisiae*	Unknown	SW P38124	67
Ybr043c	*Saccharomyces cerevisiae*	Unknown	SW P38227	67
Ybr180w	*Saccharomyces cerevisiae*	Unknown	SW P38125	63, 67
YceE	*Escherichia coli*	Unknown	SW P25744	293
YdhC	*Escherichia coli*	Unknown	SW P37597	60
YhfC	*Escherichia coli*	Unknown	SW P21229	19, 134
YhjX	*Escherichia coli*	Unknown	SW P37662	276
Yhr048w	*Saccharomyces cerevisiae*	Unknown	SW P38776	121
YidY	*Escherichia coli*	Unknown	SW P31462	29
Yil120w	*Saccharomyces cerevisiae*	Unknown	EM Z47047	15
Yil121w	*Saccharomyces cerevisiae*	Unknown	EM Z47047	15
YjiO	*Escherichia coli*	Unknown	SW P39386	30
YuxJ[f]	*Bacillus subtilis*	Unknown	SW P40760	227
YwfA	*Bacillus subtilis*	Unknown	SW P39637	80
YybF	*Bacillus subtilis*	Unknown	SW P37498	200

[a] For sequences that are greater than 90% identical, only one representative protein is shown.
[b] Abbreviations as for Table 1; CML, chloramphenicol.
[c] Accession numbers as for Table 1; PR, PIR.
[d] VMAT1 and VMAT2 have been cloned from a number of species (see the text for details); only one example is given here.
[e] OpdE has been postulated to function as a transcriptional regulator (115).
[f] From our analyses, we predict that the sequence of these proteins may be incomplete and/or contain sequencing errors.

6 and 7) have enabled refinement of these particular motifs and led to the identification of an additional family-specific motif (motif H in Fig. 6). The conservation of these motifs suggests that they play an important structural or functional role in these transporters, and this is discussed further below. The family-specific motifs defined provide a useful tool, in conjunction with hydropathy and other analyses, for allocating newly identified MFS proteins into their appropriate family group. Significantly, no conserved regions were identified that are found only in the putative multidrug exporters and are

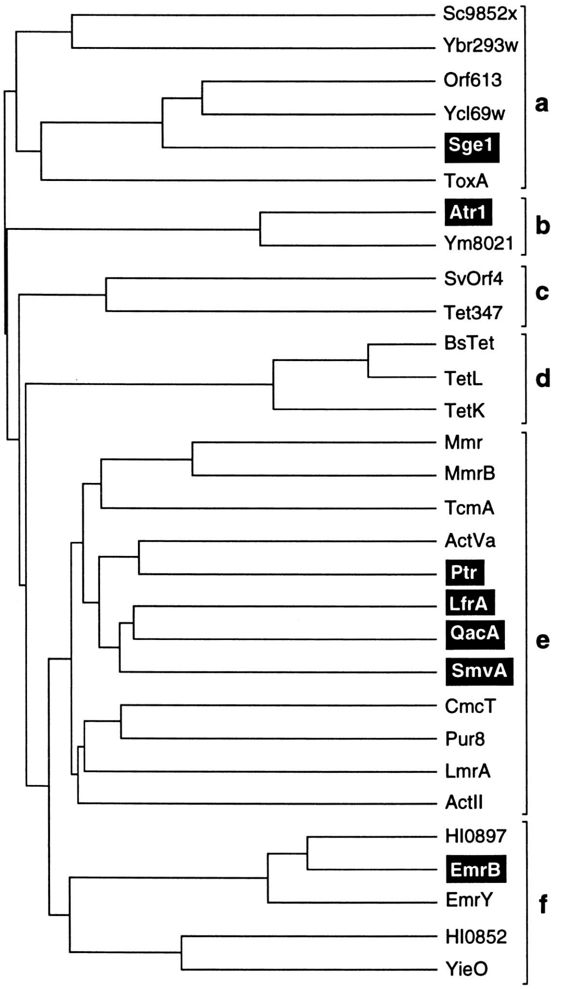


FIG. 3. Phylogenetic tree displaying the relationships among proteins within the 14-TMS family of the MFS. Phylogenetic analyses were performed as for Fig. 2. Multidrug efflux proteins are highlighted in reverse type. Clusters a to f, as described in the text, are indicated. See Table 1 and the text for further details about specific proteins in the family.

absent from other export proteins with a limited substrate specificity.

The following sections examine the known or probable multidrug efflux proteins which belong to the 14- and 12-TMS families, respectively.

QacA/B 14-TMS Multidrug Efflux Proteins

The *Staphylococcus aureus qacA* gene was the first gene encoding a PMF-dependent multidrug efflux protein to be described and sequenced (156, 240, 289, 290). *qacA* has characteristically been found on multiresistance plasmids from clinical strains of *S. aureus* and other staphylococci (79, 146, 156, 162), and 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 (156). Transport assays have indicated that *qacA* confers resistance to ethidium (156) and other organic cations (212) via PMF-dependent efflux. Studies with ionophores indicated that drug transport was driven by the ΔpH, suggesting an electroneutral drug cation/H$^+$ exchange mechanism (Fig. 1) (212).

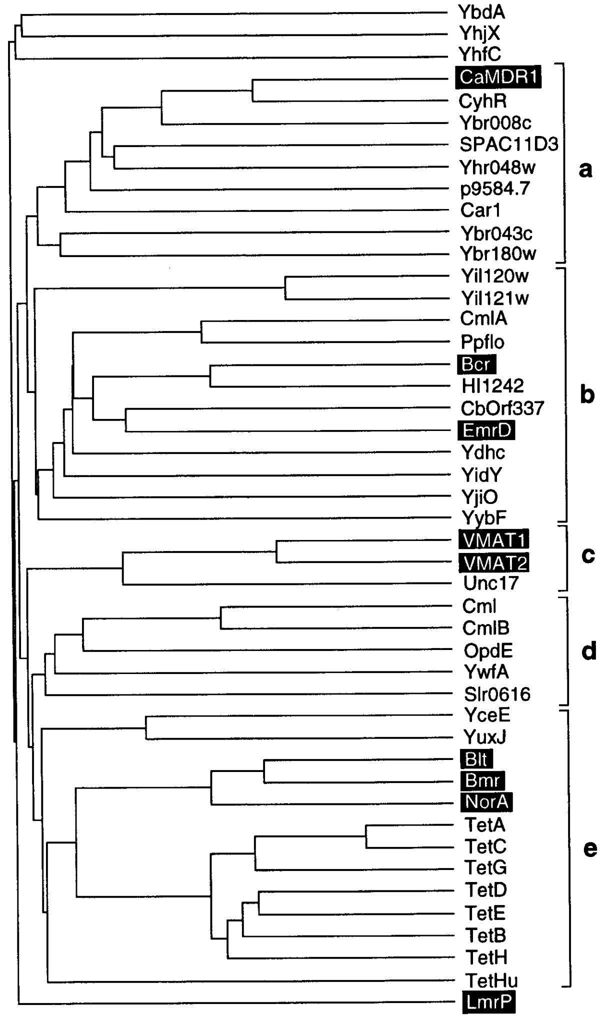


FIG. 4. Phylogenetic tree displaying the relationships among proteins within the 12-TMS family of the MFS. The tree was constructed as described in the legend to Fig. 2. Multidrug transporters are highlighted in reverse type. Clusters a to e, as described in the text, are indicated. See Table 2 and the text for further details about specific proteins in the family.

Rouch et al. (240) identified a divergently encoded gene upstream of *qacA*, *qacR*, previously known as *orf188* (Fig. 8) (28). The QacR protein shares sequence similarity with various transcriptional repressors, such as the TetR protein which regulates expression of the tetracycline resistance *tetB* gene (Fig. 8) (109). Expression of the *qacA* gene has been demonstrated to be induced by some substrates of the QacA efflux protein, e.g., ethidium and benzalkonium, and not by others, e.g., chlorhexidine (28). In the absence of *qacR*, *qacA* is expressed constitutively and overexpression of *qacR* prevents expression of *qacA*, suggesting that QacR acts as a transcriptional repressor of *qacA* expression (28).

A closely related multidrug resistance determinant, *qacB*, which confers resistance to monovalent organic cations but characteristically differs from *qacA* by conferring lower or no resistance to divalent cations, has been identified in *S. aureus* (156, 162). Sequencing of *qacB* indicated that there are only seven nucleotide differences between *qacA* and *qacB* (Fig. 5) (205). Generation of *qacB* mutants which conveyed resistance to divalent cations and site-directed mutagenesis of *qacA* have provided unequivocal evidence that the phenotypic differences

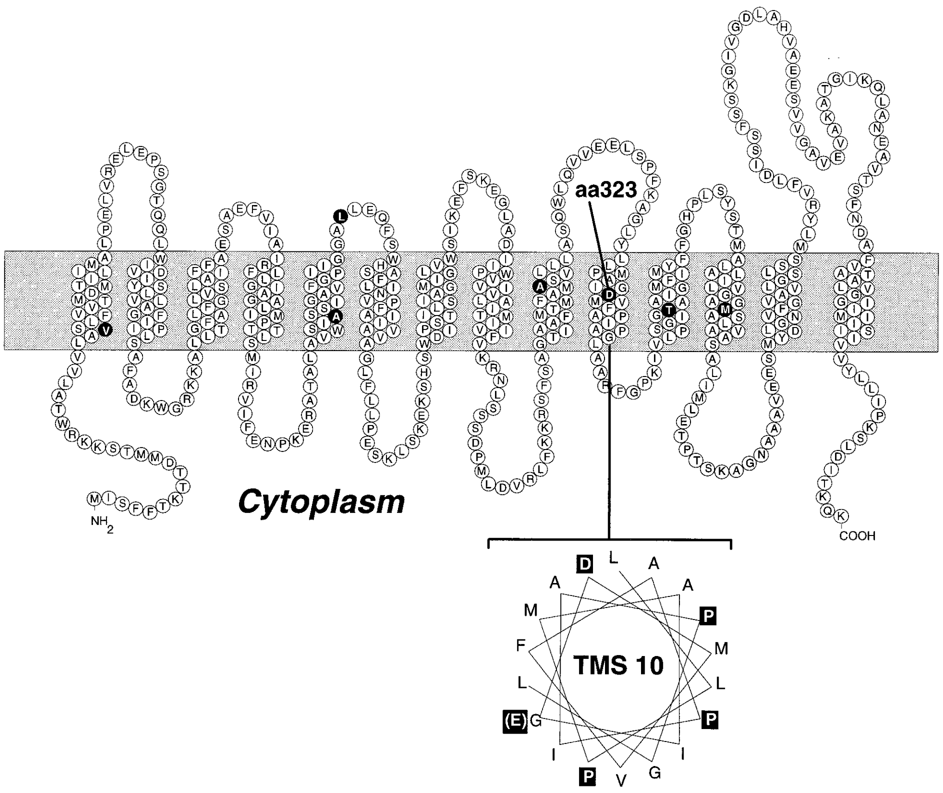


FIG. 5. Two-dimensional model of the 14-TMS *Staphylococcus aureus* multidrug resistance protein QacA in the cytoplasmic membrane (gray). The residues which differ between the closely related QacA (GenBank accession number X56628) and QacB (GenBank accession number U22531) proteins are highlighted in black. The α-helical structure of TMS 10 is displayed as a helical wheel, with each residue within the helical wheel offset from the preceding one by 100°. Asp-323 (highlighted D in the helical wheel) in QacA has been implicated in conferring resistance to divalent cations (205). Glu-322 in mutants of QacB (highlighted E residue in the helical wheel, in the equivalent position to Gly-322 in QacA) can apparently substitute for Asp-323 with respect to conferring resistance to divalent cations (205). Three proline residues (also highlighted) are located in this helix on the opposite face to Asp-323 and Glu-322 (see the text for a discussion).

between *qacA* and *qacB* are due solely to the presence of an acidic residue (Asp) at residue 323 in QacA (Fig. 5 and 6) instead of an uncharged residue (Ala) in QacB (205). Additionally, it was demonstrated that QacB mutants containing an acidic residue at amino acid 322 were able to mediate resistance to divalent cations (205).

The proposed 14-TMS membrane topology of the QacA protein (Fig. 6) has been confirmed by analysis of alkaline phosphatase and β-galactosidase fusions (205). A two-dimensional model of QacA/B based on these analyses is presented in Fig. 5. Residues 322 and 323 are located within TMS 10 of QacA/B, and Paulsen et al. (205) have suggested that the negative charge of an intramembranous acidic residue at either of these positions may be involved in substrate binding, possibly by interacting directly with one of the positively charged moieties of the divalent cation. However, other possibilities, e.g., that the acidic residue is involved in energization of transport of divalent cations or that the mutations indirectly affect a binding site located elsewhere in the protein via conformational alterations, cannot be ruled out at this stage. Unusually, TMS 10 contains three intramembranous proline residues, and these are located on the opposite face of this amphipathic helix compared with either the wild-type QacA or mutant QacB acidic residues implicated in substrate specificity (Fig. 5). It has been suggested that one or more of these proline residues may be involved in conformational changes in the protein associated with drug export, as a consequence of substrate interaction with the acidic residue (205).

EmrB 14-TMS Multidrug Efflux Protein

The *emr* locus was identified at 57.5 min on the *E. coli* chromosome and confers resistance to hydrophobic uncouplers, such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and tetrachlorosalicylanilide, to organomercurials, and to some hydrophobic antibiotics, such as nalidixic acid and thiolactomycin (75, 159). The locus consists of three cotranscribed genes (Fig. 8), *emrR* (formerly *mprA*), *emrA*, and *emrB* (159, 160). *emrB* codes for a member of the 14-TMS of the MFS (Fig. 3 and 6) (210), *emrA* codes for a member of the MFP family of proteins (54) (see below), and *emrR* codes for a regulator of the *emrRAB* operon (160). Indirect evidence has suggested that 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 (Fig. 1) (149, 159). EmrA and EmrB may also function cooperatively with a member of the OMF family of proteins (150) in an analogous manner to RND family proteins in gram-negative bacteria (Fig. 1; also see below).

Expression of the EmrAB efflux system is induced by its substrates (160), as has also been found with another uncoupler resistance multidrug efflux protein, EmrD (Table 2) (182). Transcriptional studies with *emr-lacZ* fusions have revealed that induction of the *emrRAB* operon is dependent on EmrR, which appears to act as a negative transcriptional regulator (160). Inducers of *emr* expression include EmrB substrates, such as CCCP and nalidixic acid, and also compounds which

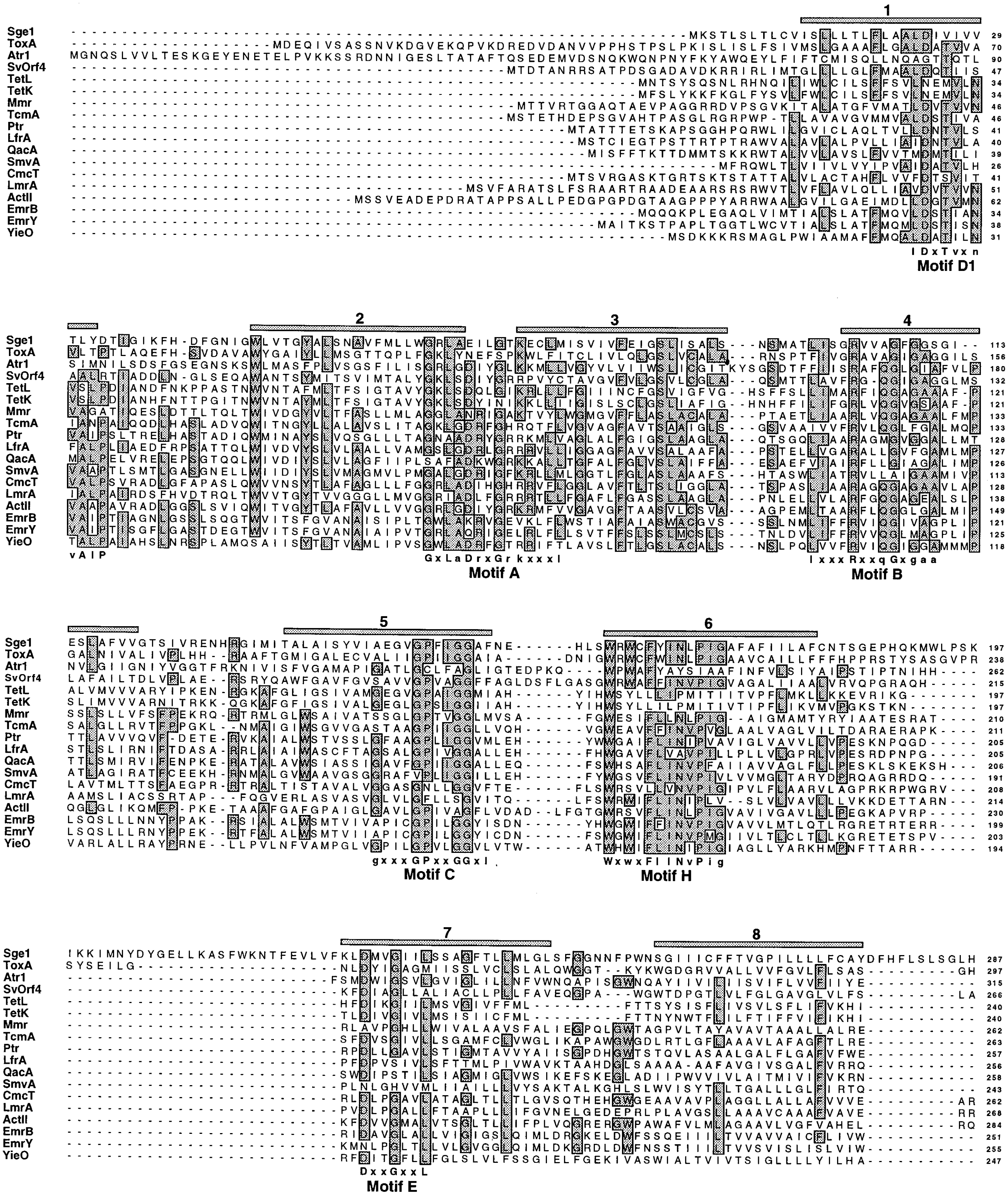


FIG. 6. Multiple-sequence alignment for representative members of the 14-TMS family of the MFS. This was prepared with PILEUP (53) and SEQVU (kindly provided by James Gardner, The Garvan Institute of Medical Research, Sydney, Australia). Sequence names are shown on the left. The shaded horizontal bars above the alignment correspond to the predicted positions of the TMS. The locations of the TMS were determined by analysis of protein hydropathy profiles and by comparison with the predictions from TOPPRED II (39) and PROFILEGRAPH (110). Sequence numbers on the right refer to the position of the rightmost residue on each line, and residues conserved in at least 40% of the sequences at any position are shaded. Highly conserved motifs are displayed below the alignment; the consensus sequences of the motifs are displayed as follows: x, any amino acid; capital letters, the frequency of occurrence of the amino acid in the displayed sequences is greater than 70%; lowercase letters, the frequency of occurrence is greater than 40%. For relevant accession numbers of and references to these proteins, see Table 1. Motifs A, B, C, D1, E, and F correspond to the motifs previously described by Paulsen and Skurray (210).

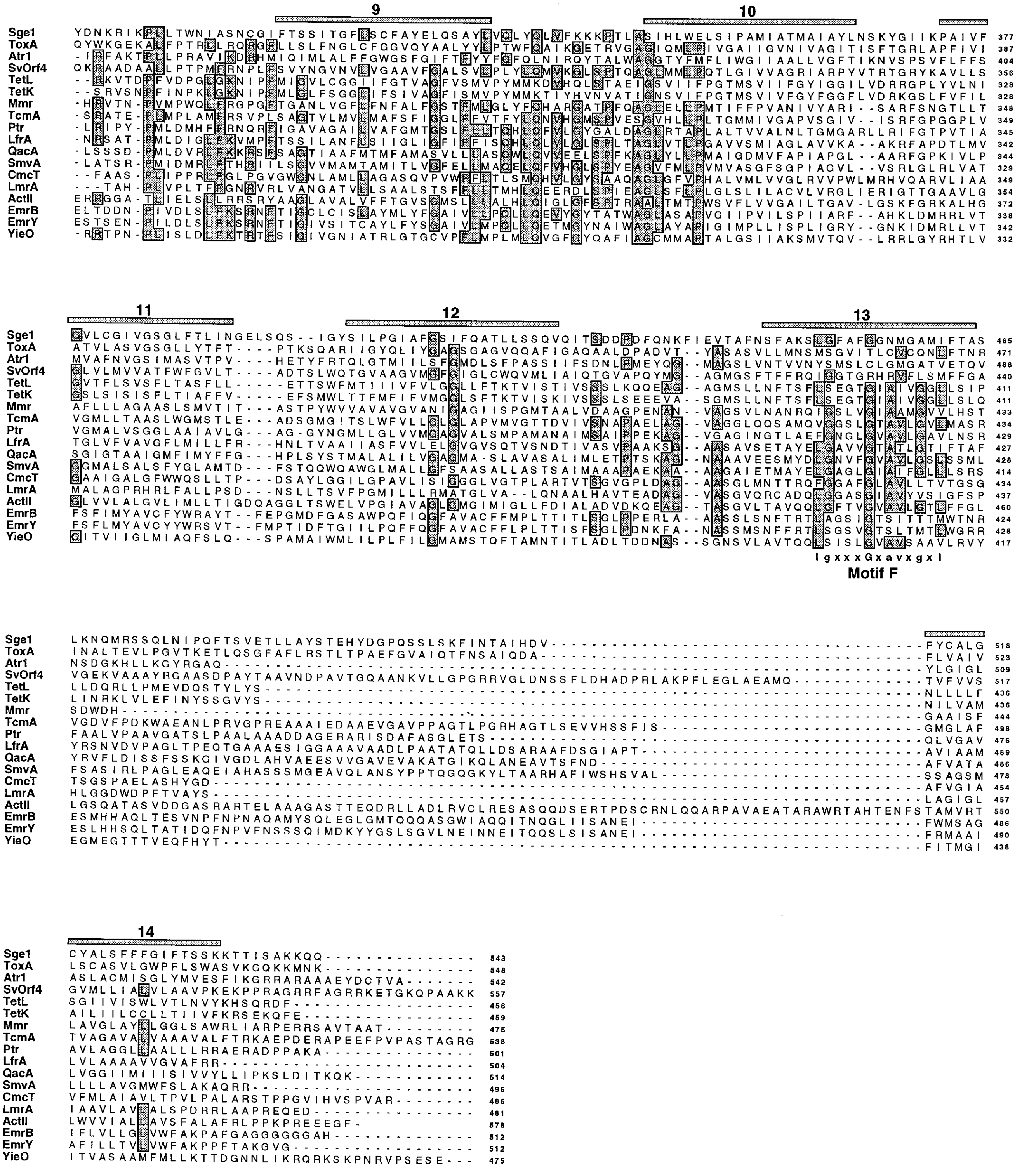


FIG. 6—*Continued.*

are not excreted by EmrB, such as ethidium bromide (160). EmrR (MprA) has previously been identified as a negative regulator of the microcin B-encoding gene *mcb* (50, 51). EmrR shares sequence similarity with a family of transcriptional regulators, including MarR, which regulates the pleiotropic multiple-antibiotic-resistance *mar* locus (40). It seems likely that EmrR binds to the promoter region of the *emr* operon and that expression of this locus is induced by EmrR binding directly with multiple drugs. Close homologs of EmrB, along with potential associated MFP family members, have been identified in *E. coli* and *H. influenzae* genome sequencing projects (EmrY and HI0897 respectively [Table 1; Fig. 3]), but the involvement of these putative proteins in multidrug efflux has not yet been investigated.

Other Putative 14-TMS Family Multidrug Resistance Proteins

Five other probable multidrug resistance proteins belonging to the 14-TMS family have so far been identified and presumably function as drug efflux proteins (Table 1; Fig. 3 and 6). Two of these, Sge1 and Atr1, are from the yeast *Saccharomyces cerevisiae*. Atr1 has been shown to confer resistance to the structurally unrelated compounds aminotriazole and 4-nitroquinolone-*N*-oxide, and expression of *atr1* is inducible by the former but not the latter (83, 131). Sge1 appears to convey resistance to crystal violet (61), ethidium bromide (12, 81), and probably other organic cations. The *Salmonella typhimurium smvA* gene also codes for a 14-TMS family member which confers resistance to methyl viologen, ethidium bromide, and probably other organic cations (111). The *Mycobacterium smegmatis lfrA* gene mediates resistance to hydrophilic fluoroquinolones and organic cations, such as ethidium, acridine, and some quaternary ammonium compounds (287), probably via PMF-dependent efflux (156a, 286). Hybridization analysis has indicated that genes homologous to *lfrA* are also found in pathogenic mycobacteria, such as *M. tuberculosis* and *M. avium* (287).

The fifth multidrug resistance protein in this family is encoded by the *Streptomyces pristinaespiralis ptr* gene and conveys resistance to two structurally unrelated antibiotics, pristinamycin I and II, produced by the organism, as well as to rifampin (24). The *ptr* promoter has been transcriptionally mapped (24), and expression from this promoter is induced by pristinamycin I or II in various *Streptomyces* species or by a wide range of toxic compounds in *Streptomyces lividans*. Induction of expression from the *ptr* promoter is growth phase dependent, with maximal expression occurring during a transition phase when expression of antibiotic biosynthesis genes begins (251). Gel shift and DNA footprinting assays indicated that a pristinamycin I-induced regulatory protein found in a range of *Streptomyces* species appears to bind three direct repeats located in the *ptr* promoter region (252).

Bmr, Blt, and NorA 12-TMS Multidrug Efflux Proteins

The three proteins Bmr, Blt, and NorA (Table 2) form a phylogenetically related cluster (Fig. 4) 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 (188). Bmr is encoded at 216 min on the *B. subtilis* chromosome, and overexpression of *bmr*, as a result of amplification of this locus, leads to high levels of resistance to these compounds. Disruption of the *bmr* gene leads to a corresponding increase in drug susceptibility (188). Bmr-mediated ethidium export is dependent on the PMF (188) and is probably driven by the ΔpH, suggesting an electroneutral drug/proton antiport mechanism (187).

Drug transport and resistance mediated by Bmr are sensitive to inhibitors of the mammalian P-glycoprotein pump, such as reserpine and verapamil (188). Alterations within the proposed TMS 9 of Bmr (Fig. 7) affect the degree of reserpine inhibition without affecting the substrate specificity of Bmr. Specifically, substitution for Val-286 in Bmr (Fig. 7) with Leu (larger side chain) decreased the binding of the transport inhibitor reserpine whereas replacement with Gly (smaller side chain) increased reserpine binding, with corresponding effects on the sensitivity of Bmr to reserpine (2). Since these mutations did not affect the sensitivity of Bmr to rescinnamine, a close structural analog of reserpine, it seems likely that Val-286 does not play a direct role in inhibitor recognition but may instead form part of a reserpine-binding "pocket" (2). Further mutational analysis of Bmr has indicated that mutations in TMS 4, 7, 9, 10, and 11 affect the spectrum of cross-resistance to various drugs (186).

Some transport substrates of the Bmr efflux system, such as rhodamine 6G and TPP, induce expression of *bmr*, and this regulation is dependent on BmrR, which is encoded downstream of *bmr* (Fig. 8) (3). BmrR shares sequence similarity with a family of transcriptional activator proteins, which includes the *E. coli* MerR and SoxR regulatory proteins. BmrR has been demonstrated, by using gel retardation and DNase I protection assays, to specifically bind, as a dimer, to the *bmr* promoter. Inducers, such as rhodamine 6G and TPP, increase the binding affinity between BmrR and its target site, and these compounds have been shown to bind BmrR in a ratio of one drug molecule for each BmrR dimer (3). The C-terminal domain of BmrR has been purified and shown to be capable of directly binding both rhodamine 6G and TPP, suggesting that this domain is responsible for drug binding (170).

Close functional homologs of Bmr have been identified in both *B. subtilis* (4) and *Staphylococcus aureus* (185, 189). The *B. subtilis blt* gene, encoded at 230 min on the chromosome, mediates resistance to a similar range of compounds to those for *bmr*, but unlike *bmr*, it appears not to be expressed in wild-type *B. subtilis* under standard conditions (4). Expression of *blt* is controlled by BltR, which is a close homolog of BmrR, but is encoded divergently from it (Fig. 8). BltR and BmrR share sequence similarity within their DNA-binding domains but share divergent drug-binding domains. Inducers of *bmr* expression, such as rhodamine 6G, do not induce expression of *blt*, indicating that BltR and BmrR apparently respond to different inducers (4). *blt* is cotranscribed and coregulated together with a downstream gene, *bltD* (Fig. 8), whose product shares homology with acetyltransferase enzymes. This operon structure suggests that Blt and BltD have some physiological role or function in common.

The *S. aureus norA* gene was initially identified as a chromosomal fluoroquinolone resistance gene (324) but was subsequently shown to also confer resistance to a similar range of substrates to that encoded by *bmr* (189). Drug transport studies have suggested that NorA-mediated export of ethidium (189) and the fluoroquinolone norfloxacin (126, 190) is dependent on the PMF and, in the case of norfloxacin, is driven by the ΔpH (190). NorA-mediated drug transport is also reserpine sensitive, but to a lesser extent than is Bmr-mediated transport (126), possibly because of the presence of Leu in an equivalent position to Val-286 in Bmr (see above). One NorA mutant with an altered resistance spectrum has been identified; alteration of Ala-362 to Asp within the putative TMS 12 of NorA (Fig. 7) leads to reduced resistance to norfloxacin (126, 201). *norA* is potentially regulated by a divergently encoded open reading frame, *norR* (Fig. 8), whose product shares sequence similarity with repressor proteins, such as the QacR and TetR repressors (see above) (126).

VMAT1 and VMAT2 12-TMS Multidrug Efflux Proteins

Synaptic transmission in higher eukaryotes requires the regulated release of neurotransmitters to the synaptic cleft. Neurotransmitters are stored in subcellular organelles to ensure their regulated release. Two broad-substrate-specificity transporters, VMAT1 and VMAT2 (Table 2; Fig. 4 and 7), which belong to the 12-TMS family of the MFS and which catalyze the accumulation of various monoamines, such as catecholamines (e.g., dopamine, epinephrine, and norepinephrine) and indoleamines (e.g., serotonin) within intracellular vesicles have been identified (for recent reviews, see references 261 and 264). Studies with chromaffin granules have indicated that VMATs mediate monoamine transport in exchange for two H^+ and are thus dependent on both the ΔpH and the $\Delta\Psi$ of the PMF (139, 198).

Reserpine and tetrabenazine (TBZ) are potent inhibitors of vesicular monoamine transport (136, 221). Reserpine competitively and almost irreversibly inhibits VMAT-mediated amine transport, probably by binding at the site of amine recognition (46, 258). Reserpine binding is accelerated by both the ΔpH and the $\Delta\Psi$ of the $\Delta\mu_{H^+}$ and is less sensitive than substrate transport to changes in pH (243). This has led to the development of a model for reserpine binding and monoamine transport (243, 261, 264). In this model, translocation of a single proton generates a high-affinity binding site for monoamines or reserpine. In the case of a monoamine, the substrate is released on the opposite side of the membrane following a conformational change and the translocation of an additional proton. However, in the case of reserpine, the drug prevents such a conformational change from taking place and the transporter becomes blocked at this point, with reserpine unable to dissociate and further proton translocation inhibited. TBZ also inhibits VMAT-mediated amine transport, probably by binding to a site on the protein different from the reserpine- and substrate-binding site, since TBZ binding is not inhibited by reserpine at concentrations which block transport and is not affected by the $\Delta\mu_{H^+}$, and substrates block TBZ binding only at high concentrations (46, 104, 259).

VMAT-encoding genes have been cloned and sequenced from several different species. VMAT1 has been cloned from rats (157) and humans (217), and VMAT2 has been cloned from rats (65), cows (113), and humans (64, 285) (only representative VMAT1 and VMAT2 proteins are included in Table 2 and Fig. 4 and 7). VMAT1 and VMAT2 share 62% identity at the amino acid level and differ mainly at their N and C termini and within the large hydrophilic loop between TMS 1 and TMS 2 (Fig. 7). Comparisons of the transport properties of rat VMAT1 and VMAT2 proteins has revealed that VMAT2 possesses a higher affinity for all monoamine substrates examined, particularly for histamine (218), and VMAT1 is less sensitive to the inhibitor TBZ (157, 218). VMAT1 and VMAT2 are also related to the VAChT proteins identified in *Caenorhabditis elegans* (Unc17 in Table 2 and Fig. 4), rats, humans, and the marine rays *Torpedo marmorata* and *T. ocellata*, which mediate the vesicular transport of the neurotransmitter acetylcholine (6, 66, 239, 300).

In addition to their ability to import neurotransmitter molecules into intracellular vesicles, both VMAT1 and VMAT2 have been shown to interact with a range of cytotoxic compounds, including isometamidium, ethidium, *N*-methyl-4-phenylpyridinium (MPP), rhodamine 6G, tacrine, TPP, and doxorubicin (261, 322). These compounds were shown to inhibit serotonin uptake and reserpine binding. VMAT1 was initially cloned on the basis of its ability to confer resistance to the neurotoxin MPP, and VMAT1 and VMAT2 have been shown to actively transport rhodamine 6G in an ATP-independent, reserpine-sensitive manner into intracellular storage vesicles (322). These findings led to the proposal that VMAT1 and VMAT2 may mediate a novel mechanism of drug resistance: accumulation of toxic compounds within intracellular storage vesicles (261, 264). It seems extremely likely that transport of such cytotoxic compounds is driven by the PMF across the membranes of intracellular vesicles. Thus, VMAT1 and VMAT2 appear to be mechanistically analogous to other PMF-dependent multidrug transporters, such as QacA and Bmr, by acting as multidrug/proton antiport systems.

Site-directed mutagenesis and residue-specific chemical reagents have been used to investigate the roles of specific residues in the function of VMAT1 and VMAT2 (for reviews, see references 263 and 264). The carboxyl-specific reagent *N*,*N*′-dicyclohexylcarbodiimide (DCCD) inhibits VMAT-mediated monoamine transport and inhibits reserpine and TBZ binding (76, 262, 283). Mutagenesis of Asp-33 in rat VMAT2 has indicated that a negative charge is essential at this position for transport. Substitutions at Asp-33 did not affect reserpine binding but did affect serotonin inhibition of reserpine binding, suggesting a role for this residue in substrate recognition (173). Substitutions have also been introduced for Asp-404 and Asp-431 in rat VMAT1 (Fig. 7); an Asp-404-to-Glu alteration changed the pH optimum of transport, and other changes to Asp-404 or Asp-431 abolished transport activity (176).

Mutations at His-419 in rat VMAT1 (Fig. 7) abolished monoamine transport but not reserpine or TBZ binding, although $\Delta\mu_{H^+}$ acceleration of reserpine binding was inhibited, suggesting a role for His-419 in either proton translocation or conformational changes that might occur in the transporter after substrate binding (272). Replacement of the serine residues Ser-180, Ser-181, and Ser-182 in TMS 3 of rat VMAT2 with alanine abolished serotonin transport but did not affect reserpine binding. However, reserpine binding was no longer inhibited by serotonin in these mutants, suggesting that Ser-180 to Ser-182 may play a role in substrate recognition (173). Mutagenesis targeting of the serine residues in TMS 4 of rat VMAT2 (Ser-197, Ser-198, Ser-200, and Ser-201) and other residues in rat VMAT2 (Gly-151, Thr-154, Asn-155, and Gly-158) and His-384 in rat VMAT1 indicated that these residues are not essential for monoamine transport or reserpine binding (173, 272).

Thus, VMAT1 and VMAT2 are multidrug antiport systems which display specificity for various monoamine neurotransmitters, which are presumably the natural substrates for these transporters. They are also capable of transporting various hydrophobic drugs, either fortuitously or as a novel mechanism of drug resistance, i.e., concentration of toxic compounds within intracellular storage vesicles. As these monoamine transporters have been well characterized at the biochemical level, they may serve as good model systems with which to study the phenomenon of multidrug transport.

Other Putative 12-TMS Multidrug Efflux Proteins

Lactococcus lactis expresses PMF- and ATP-driven multidrug efflux systems (25). A gene responsible for the former activity, *lmrP*, has been characterized and found to code for a member of the 12-TMS family (Table 2; Fig. 7) (26). However, as can be seen in the phylogenetic tree in Fig. 4, LmrP is one of the most divergent members of this family. Overexpression and construction of a chromosomal deletion mutant indicated that *lmrP* confers resistance to ethidium, daunomycin, and TPP ions. *lmrP*-encoded ethidium and daunomycin efflux is sensitive to ionophores and reserpine and insensitive to the ATPase inhibitor orthovanadate, supporting the notion that LmrP acts as a PMF-dependent multidrug efflux protein (26).

Two probable *E. coli* 12-TMS multidrug efflux proteins, encoded by the *bcr* and *emrD* genes, have been identified (Table 2; Fig. 7). *emrD*, in an analogous manner to *emrB*, confers resistance to various structurally unrelated hydrophobic un-

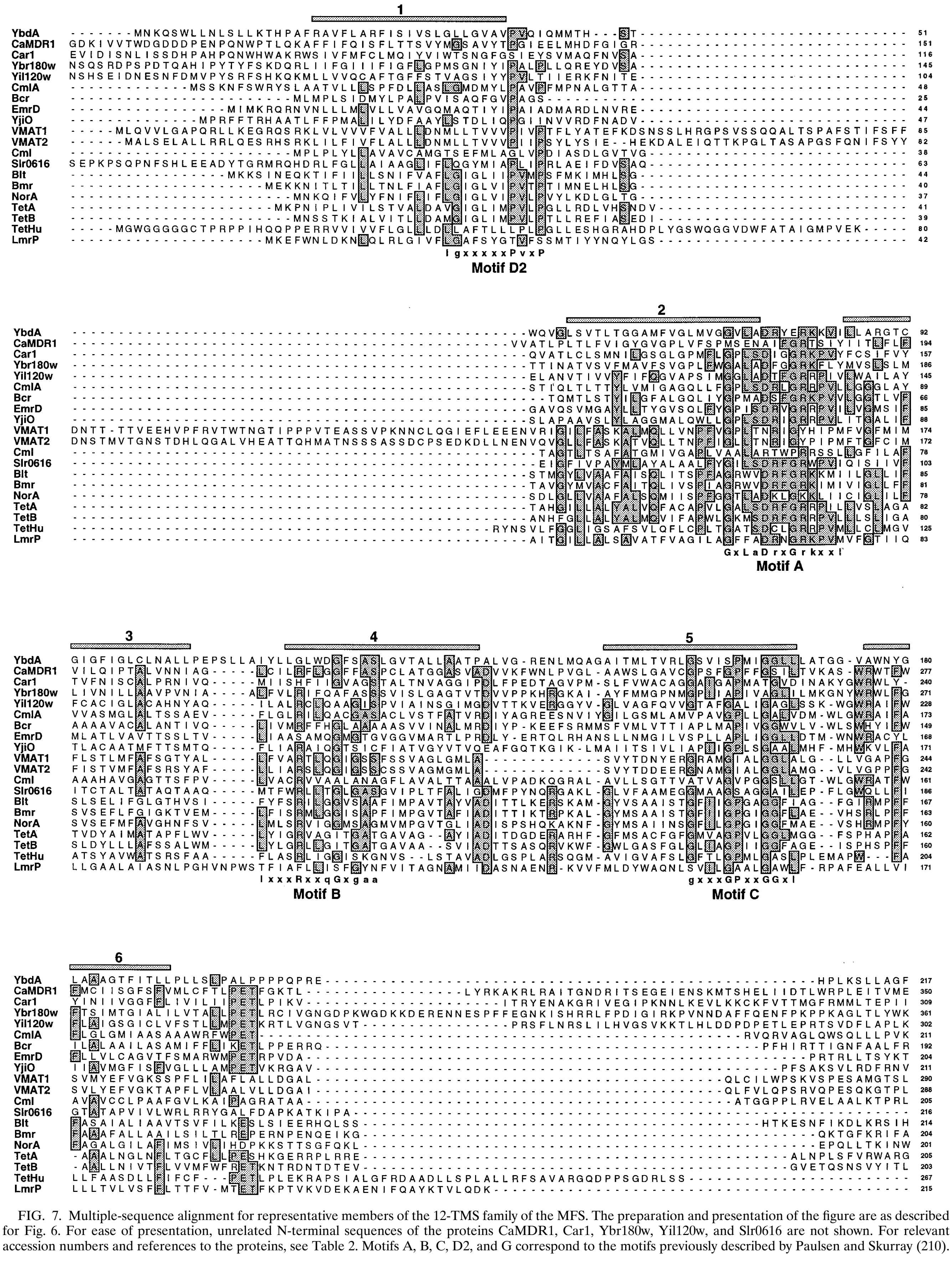


FIG. 7. Multiple-sequence alignment for representative members of the 12-TMS family of the MFS. The preparation and presentation of the figure are as described for Fig. 6. For ease of presentation, unrelated N-terminal sequences of the proteins CaMDR1, Car1, Ybr180w, Yil120w, and Slr0616 are not shown. For relevant accession numbers and references to the proteins, see Table 2. Motifs A, B, C, D2, and G correspond to the motifs previously described by Paulsen and Skurray (210).

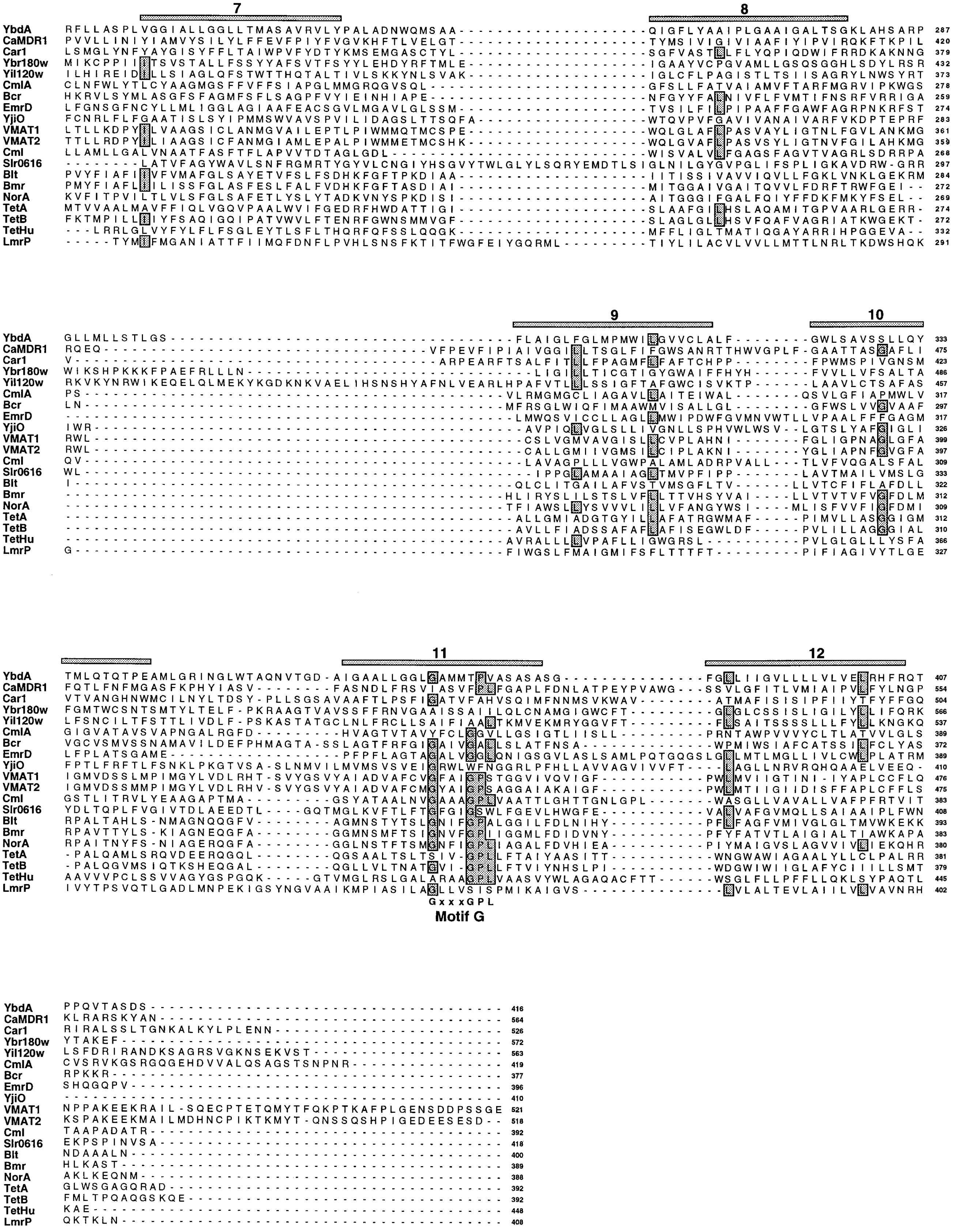


FIG. 7—*Continued.*

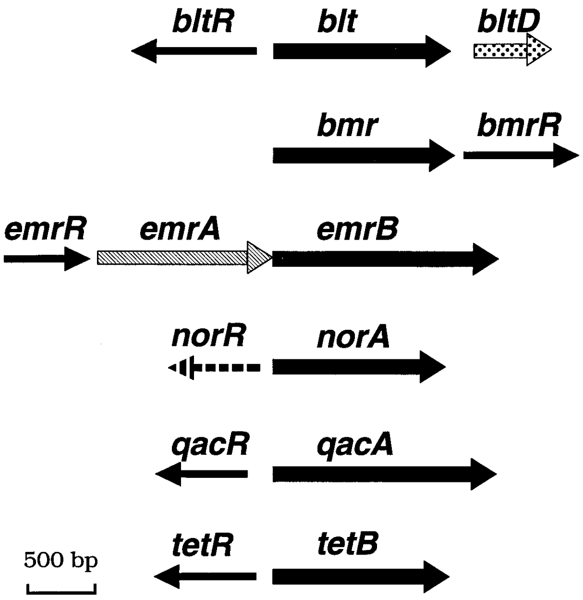


FIG. 8. Comparative genetic maps of the regions encoding the multidrug efflux loci *blt*, *bmr*, *emrAB*, *norA*, and *qacA* and the well-characterized tetracycline resistance locus *tetB*. Genes are denoted by arrowed lines: (i) MFS, thick black; (ii) MFP, thick striped; (iii) others, stippled; (iv) regulatory, thin black; (v) putative regulatory, broken thin black. It should be noted that TetR, QacR, and the putative protein NorR are homologous, BltR and BmrR are members of the MerR family of transcriptional repressors (3), and EmrR is a member of the MarR family of regulatory proteins (160).

couplers, although it apparently has a narrower range of specificity than *emrB* (Table 1) (182). Unlike *emrB*, *emrD* is not encoded in an operon with an MFP-encoding gene, and it is not known whether it requires the action of any auxiliary proteins. The *bcr* gene (also known as *bic*) confers resistance to bicyclomycin (20) and to sulfathiazole (192). The *Candida albicans CaMDR1* gene, formerly known as *BEN* or *bmrP*, was originally identified as conferring resistance to benomyl and methotrexate (70). Subsequently, overexpression and gene knockout studies have indicated that *CaMDR1* also encodes resistance to cycloheximide, benztriazoles, 4-nitroquinolone-*N*-oxide, and sulfometuron methyl (21, 82). Disruption of the *CaMDR1* gene reduces the virulence of *C. albicans* (18).

Other PMF-Dependent Multidrug Transport Systems within the MFS?

As discussed above, the MFS includes several separate families of proteins, with two of these families (the 12- and 14-TMS families) consisting primarily of drug resistance efflux proteins, including a number of multidrug efflux proteins (Tables 1 and 2). The remaining families consist primarily of symporters and uniporters involved in the uptake of essential nutrients and of proteins of unknown function (Fig. 2).

Recently, Grundemann et al. (93) identified and sequenced a gene from rat kidney, which encoded a novel multidrug antiporter, designated Oct1 (Fig. 9). In mammals, various structurally distinct cationic drugs, e.g., antihistamines, sedatives, opiates, and antibiotics, are excreted by epithelial cells of the renal proximal tubes, and two functionally distinct transport systems are localized in the basolateral and luminal plasma membranes of these cells (for a review, see reference 294). Expression of Oct1 in *X. laevis* oocytes conferred high levels of tetraethylammonium transport, which was dependent on the membrane potential and was inhibited by both hydrophobic and hydrophilic organic cations (93). Oct1 was also demonstrated to confer MPP transport in oocytes. The *oct1*-encoded cation antiport system displayed similar transport parameters to those determined for the basolateral membrane cation uptake system, suggesting that Oct1 is responsible for the observed multidrug transport of organic cations in the basolateral membranes of cells in the renal proximal tubes. Consistent with this notion, Northern (RNA) blot analysis indicated that *oct1* is expressed in the liver, kidney, and intestine of rats (93).

Grundemann et al. reported that the Oct1 protein did not share sequence similarity with any known proteins (93). However, our analyses indicate that the Oct1 protein shares significant sequence similarity with the proteins in family 3 of the MFS (Fig. 2 and 9), which includes many sugar uniport proteins, e.g., the human glucose facilitator GLUT proteins, and sugar/H^+ symport proteins, e.g., the *E. coli* transporters AraE, GalP, and XylE, specific for arabinose, galactose, and xylose, respectively (100, 101). Within this family, the Oct1 protein is most closely related to the rat SV2 synaptic vesicle protein and to various *Caenorhabditis elegans* proteins of unknown function identified by genome sequencing, e.g., Zk637.1 (Fig. 9). As can be seen in the sequence alignment presented in Fig. 9, Oct1 is clearly homologous to representative members of this family.

Comparison of the Oct1 protein with members of the 12- and 14-TMS efflux protein families analyzed above indicated that Oct1 did not contain any of the motifs specific for either or both of these two families. Oct1 does contain motif A and motif B common to most MFS proteins (90, 210) and also contains motifs which are specific for family 3 of the MFS (Fig. 9) (103), confirming the notion that Oct1 belongs to family 3 within this superfamily and is distinct from the other families containing multidrug efflux proteins. Furthermore, our hydropathy analyses suggest that Oct1 contains 12 TMS (Fig. 9), as is consistently found in other members of family 3 of MFS.

The novel finding that the Oct1 multidrug efflux protein is a member of a family of the MFS hitherto thought to contain only symporters and uniporters presents the exciting possibility that other members of this family, such as the SV2 rat synaptic vesicle protein, also mediate multidrug antiport. It also reveals that the families within the MFS are more functionally diverse than was previously thought (90, 169, 210), since this single family includes known uniporters, symporters, and antiporters. Thus, for transporters in the MFS, vectorial movement of the substrate appears to be governed by subtle factors which are not obvious from sequence gazing at the primary amino acid sequences of the proteins.

Structure and Function of the MFS Transporters

The 12- and 14-TMS families within the MFS contain a variety of drug resistance proteins, including multidrug efflux proteins, and proteins of unknown function. The best characterized protein within one of these families is the *E. coli* TetB protein, which has been purified, reconstituted as a tetracycline transporter, and shown to function as an electroneutral antiport system which catalyzes the exchange of a tetracycline–divalent-metal-cation complex for a proton (5, 106, 132, 277, 320). A similar mechanism operates for the TetK tetracycline transporter from gram-positive bacteria (318). Other families within the MFS include symporters and uniporters which have been characterized in detail. For example, *E. coli* LacY (172, 184), PgtP (298), and UhpT (13) have been purified, reconstituted, and shown to mediate H^+/lactose, H^+/phosphoglycerate, and H^+/sugar phosphate symport, respectively (Fig. 2).

As discussed above, sequence analyses have suggested that the proteins within the various families of the MFS share greater sequence similarity between their N-terminal halves than between their C-terminal halves, which has led to the proposal that the C-terminal regions of the MFS proteins are involved primarily in substrate recognition and the N-terminal halves are involved primarily in proton translocation. Extensive mutagenesis of members of the MFS, particularly of the LacY and TetB proteins (for details, see below), has shed some light on the important conserved structural and functional features of the MFS proteins (for extensive reviews on the mutagenesis of the LacY protein, see references 128 to 130).

Schematic models of a typical 12-TMS family protein and a typical 14-TMS family protein of the MFS are displayed in Fig. 10, with the conserved motifs highlighted. The conservation of such motifs (see also Fig. 6 and 7) among transporters specific for various substrates and among multidrug transporters suggests that they play essential structural or functional roles common to these proteins and are probably not involved in substrate discrimination (for reviews, see references 90, 169, and 210).

Motif A, located in the cytoplasmic loop between TMS 2 and TMS 3, is conserved not only in the 12- and 14-TMS families but also in the other four well-characterized families of the MFS (families 3 to 6 in Fig. 2) (90, 169, 210). Mutagenesis of TetB has suggested that Gly-62 and Gly-69 (corresponding to positions 1 and 8, respectively, within this motif) play an essential structural role in forming a β-turn, and Asp-66 and Arg-70 are also essential (Fig. 7) (316, 317). Similarly, in LacY, Gly-64 and Asp-68 (corresponding to positions 1 and 5, respectively within this motif) are essential, with the former probably playing a structural role (119). In TetB, following substitution of cysteine for various residues in this motif, only a Ser-65-to-Cys mutant was sensitive to *N*-ethylmaleimide (NEM) inhibition; inhibition by sulfhydral reagents depended on the size of the reagent, and NEM inhibition was accelerated by tetracycline (135, 315), suggesting that this motif may be involved in initial contact with the substrate in TetB. The available data suggest that this motif acts as a cytoplasmic gate which controls passage of the substrate to and from the cytoplasm (317, 319). Alternatively, it may be involved in promoting global conformational changes in the protein that enable the substrate to translocate across the membrane (119). Possibly supporting the latter contention, second-site suppressor mutations which restore function to TetB Asp-66 or LacY Asp-68 mutants have been identified; these mutations occur in various locations throughout the proteins, i.e., in the external loop between TMS 1 and 2 in TetB and LacY (118, 314), within TMS 7 or 11 in LacY (118), or within the loops between TMS 7 and 8 or TMS 11 and 12 in LacY (118).

Motif B is conserved in the 12- and 14-TMS families and in family 3 of the MFS and is located within TMS 4 of these proteins (Fig. 6, 7, and 9). The role of this motif has not been investigated by mutagenesis, but it has been proposed to be involved in energy coupling (210). Motif C is located in TMS 5 of the drug/proton antiporters of the 12- and 14-TMS families but not in symporters from other MFS families, suggesting that it may be required for linking proton translocation to antiport but not to symport of a substrate (90, 210). Mutagenesis of Gly-147 (corresponding to position 4 within this motif) in TetC has implicated this residue in tetracycline/H^+ antiport, and on the basis of molecular modelling, Varela et al. (299) have proposed that motif C forms a kink in the helix of TMS 5. This has led to the speculation that motif C may determine the orientation of the unoccupied substrate-binding site and hence dictate the direction of transport (299).

Like motif C, motif D is found only in members of the 12- and 14-TMS families, with some variation between the two families, and is located within TMS 1 (210). However, the role of this motif has not yet been investigated. Motif H is conserved in the 14-TMS family proteins but can also be recognized in a divergent form in some 12-TMS family proteins. Motifs E and F are conserved only in the 14-TMS family proteins, and no experimental evidence regarding the potential roles of these motifs is available, although, interestingly, motif E contains a highly conserved, intramembranous charged residue, Asp.

Motif G is conserved only in the 12-TMS family proteins, and it probably corresponds to a C-terminal duplication of motif C (210). Whether this motif plays a similar role to motif C (see above) has not yet been investigated. A C-terminal duplication of motif A located at the end of TMS 8 is also recognizable in some 12-TMS family proteins (Fig. 7) and in proteins belonging to other families in the MFS.

Mutations resulting in altered substrate specificities in the multidrug efflux proteins QacA/B, Bmr, and NorA have been found mainly in the C-terminal regions of these proteins (see above for details), lending some credence to the proposal that the C-terminal regions of the MFS proteins are primarily involved in substrate recognition and the N-terminal regions of the transporters are involved in energy coupling (90, 240). However, in other MFS proteins, residues in various regions of the transporters have been implicated in substrate binding, namely, Ser-180 to Ser-182 (TMS 3) in VMAT2 (173) (see above), Cys-148 and Cys-154 (TMS 5) in LacY (124, 297), and Gln-54 (TMS 2), Asp-84 (TMS 3), and Gln-261 (TMS 8) in TetB (312, 313), or in energy coupling, namely, His-322, Glu-325 (TMS 10), and Arg-302 (TMS 9) in LacY (34, 125, 143, 230) and His-257 (TMS 9) in TetB (311). The construction of GalP-AraE fusions has indicated that TMS 1, 11, and 12 are not involved in discrimination between pentose and hexose sugars (102). Thus, it is difficult to draw any generalized conclusions regarding the roles of specific regions in the MFS transporters. Conclusions which can be safely drawn, although they are neither novel nor specific to MFS-type transporters, are that essential functional residues, particularly those associated with substrate recognition, are frequently located within TMS and that intramembranous charged residues are frequently important.

The LacY transporter is the most extensively studied of any member of the MFS; the majority of the residues in the protein have now been analyzed by cysteine-scanning mutagenesis, and only a few residues have been shown to be essential for activity (58, 73, 246, 247, 304, 305). Studies involving site-directed fluorescence labelling and inactivation by sulfhydryl reagents have indicated that the reactivity of various introduced cysteine residues in LacY is influenced by sugar binding or by imposition of a proton electrochemical gradient (124, 246, 304, 309, 310). This suggests a model whereby the interaction between the substrate and the protein involves only a few essential residues but transport of the substrate involves widespread conformational changes in the protein.

Because of the difficulties in crystallization of hydrophobic membrane proteins (141), the three-dimensional structure of any MFS proteins, or indeed any other secondary transporter, has not been solved at high resolution. Some details regarding the arrangement of the transmembrane helices of the LacY protein have been uncovered by second-site suppressor analysis and site-directed excimer fluorescence (125). Goswitz and Brooker (84) have proposed a speculative model of the three-dimensional arrangement of the helices in the members of the MFS with 12 TMS on the basis of hydropathy, amphipathicity, loop lengths, rotational symmetry, and available experimental evidence, where TMS 1, 2, 4, 5, 7, 8, 10, and 11 potentially form a transmembrane pathway, and the other four TMS do not line the pathway. Yan and Maloney (321) have suggested that Cys-265 in UhpT may be part of a transmembrane pathway in this transporter, since this residue is accessible to membrane-impermeable sulfhydryl reagents from both sides of the membrane.

Some of the available mutagenesis data seem at odds with this proposed three-dimensional model, since residues in TMS 3, 6, 9, or 12 in some MFS transporters have been implicated in substrate binding or other essential functions. However, since it is possible that there is some access for side chains from these helices to the transmembrane pathway or, alternatively, in the case of multidrug efflux proteins, hydrophobic substrates may gain access to the transporter via the lipid bilayer rather than from outside the membrane, these data do not serve to confirm or refute this model.

SMALL MULTIDRUG RESISTANCE FAMILY

The smallest known secondary transporters belong to the SMR family (Table 3) (for a review, see reference 213). These proteins are typically around 110 amino acid residues in length with 4 predicted TMS (Fig. 11), and they do not exhibit sequence homology with the 12- or 14-TMS family previously discussed. Since these proteins are so small, it has been proposed that they may function as oligomeric complexes (208, 213). The best-characterized member of this family is a staphylococcal multidrug efflux protein known variously as Smr, QacC, QacD, or Ebr (91, 155, 162, 256), which we refer to hereafter as Smr. Other members of this family which mediate multidrug efflux include the chromosomally encoded *E. coli* resistance protein EmrE, previously known as MvrC and Ebr (149, 177, 228), and the QacE protein encoded on an integron from the *Klebsiella aerogenes* plasmid R751 (208).

The SMR family also includes the product of the *E. coli* chromosomal *sugE* locus (previously thought to contain two open reading frames, *sugES* and *sugEL* because of a sequencing error) (213), which is apparently capable of phenotypically suppressing mutations in the molecular chaperone gene *groE* (89). The actual function of SugE remains unclear, although it has been suggested to potentially be involved in peptide efflux (213). Homologs of SugE have been identified in *Proteus vulgaris* (42), *Citrobacter freundii* (22), *Myxococcus xanthus* (213), and *B. subtilis* (Table 3; Fig. 11). These SugE-like proteins have not been functionally characterized, with the exception that the *C. freundii sugE* gene apparently does not confer multidrug resistance or catalyze efflux (22).

Despite being substantially larger than other SMR family proteins, the *E. coli* tellurite resistance protein TehA (288, 301) may be distantly related to the SMR protein family (213) based on limited sequence similarity and an apparent functional similarity, since TehA can confer resistance to various organic cations (292). Determination of the complete sequence of the *Haemophilus influenzae* genome has identified a close homolog (HI0511) of TehA (69), which may also be involved in multidrug efflux.

Recent experiments have confirmed that two members of the SMR family function as independent transporters (92, 323). Grinius and Goldberg (92), using purified Smr protein reconstituted into liposomes, have shown that it transports substrates such as ethidium and MPP. Similarly, EmrE has been purified by extraction with a chloroform-methanol mixture and reconstituted in proteoliposomes as a multidrug efflux system (323). In both cases, drug efflux was driven by the PMF.

Phylogenetic analysis indicates that this family contains two distinct clusters of proteins (Fig. 12) (213). The first cluster consists of the multidrug resistance proteins Smr, QacE, and EmrE. The proteins that make up the second cluster include the *E. coli* SugE protein, as well as SugE homologs from other bacteria. These two clusters may define functionally separate groups of proteins within this family (213). Because of their limited sequence and structural similarities with the SMR proteins, the tellurite resistance TehA protein from *E. coli* and its homolog *H. influenzae* were not included in this analysis.

Multiple-sequence alignment of SMR family proteins (Fig. 11) reveals a number of residues which are absolutely conserved, implying that they may play essential structural or functional roles (see below). Three signature sequences (motifs A, B, and C in Fig. 11 and 13) specific to the SMR family have been previously defined (213).

The following sections consider in detail each of the SMR multidrug efflux systems which have been characterized.

Smr Multidrug Efflux Protein

This multidrug resistance determinant, first described as *qacC* (155, 162) and also known as *qacD* (155) or *ebr* (256), has now been renamed as *smr* (91, 92, 213). The *smr* gene is typically located on both conjugative and nonconjugative plasmids in clinical isolates of *Staphylococcus aureus* and other staphylococci (145, 146, 155, 156) and encodes resistance to a variety of organic cations, including quaternary ammonium compounds, dyes, such as ethidium, and other compounds, such as TPP (91, 156).

Studies with whole cells have suggested that *smr* mediates PMF-dependent ethidium and TPP efflux (91, 123, 156). The Smr protein has been purified and reconstituted into proteoliposomes where it has been shown to mediate multidrug transport (92). Smr-mediated ethidium and MTP ion transport in liposomes could be driven by the ΔpH but not the $\Delta\Psi$. However, the $\Delta\Psi$ was shown to accelerate the rate of ΔpH-dependent drug transport, leading to the proposal that Smr functions as an electrogenic drug/proton antiport system (92).

The membrane topology of the Smr protein has been investigated by using alkaline phosphatase and β-galactosidase fusions (204). These studies generally supported a four-TMS model of this protein with the N terminus located cytoplasmically, although the localization of the C terminus of the protein remains to be clarified (204).

As noted above, the SMR proteins contain a number of conserved residues (see reference 213 for a detailed analysis of the conserved residues in the SMR proteins); site-directed mutagenesis has been used to investigate the role of a number of these residues in the staphylococcal Smr protein (92, 204). A structural model for the Smr protein is presented in Fig. 13, with conserved and mutagenized residues indicated.

The conserved charged Glu-13 residue in TMS 1 of Smr (Fig. 11 and 13, motif A) appears to be essential for activity of the efflux system, since even conservative substitutions, such as substitution with asparagine, effectively abolished transport activity (92). It has been postulated that this acidic residue may potentially be involved in substrate binding and/or the exchange of drug molecules for protons (92).

The role of the sole cysteine residue in Smr, Cys-42, located in TMS 2 (Fig. 13), has been investigated by NEM inhibition studies and by site-directed mutagenesis (204). Smr-encoded ethidium export is sensitive to the effects of NEM, and the presence of excess substrate appears to partially protect against NEM inhibition (204). Analysis of Cys-42 site-directed mutants revealed that this residue is not absolutely essential

FIG. 9. Multiple-sequence alignment of the multidrug transporter Oct1 (EMBL accession number X78855) and representative members of family 3 of the MFS. Sequences of other members of the family shown are the *E. coli* galactose/H$^+$ symporter GalP (SwissProt accession number P37021) and arabinose/H$^+$ symporter AraE (SwissProt accession number P09830), the human glucose facilitator GLUT1 (PIR accession number A27217) and synaptic vesicle protein SV2 (SwissProt accession number Q0256), and the *Caenorhabditis elegans* hypothetical protein Zk637.1 (SwissProt accession number P30638). Presentation of the figure is as described for Fig. 6. For ease of presentation, unrelated N-terminal sequences of SV2 and Zk637.1 are now shown. Motifs A and B of the MFS proteins (Fig. 6 and 7) are highlighted; see the text for details.

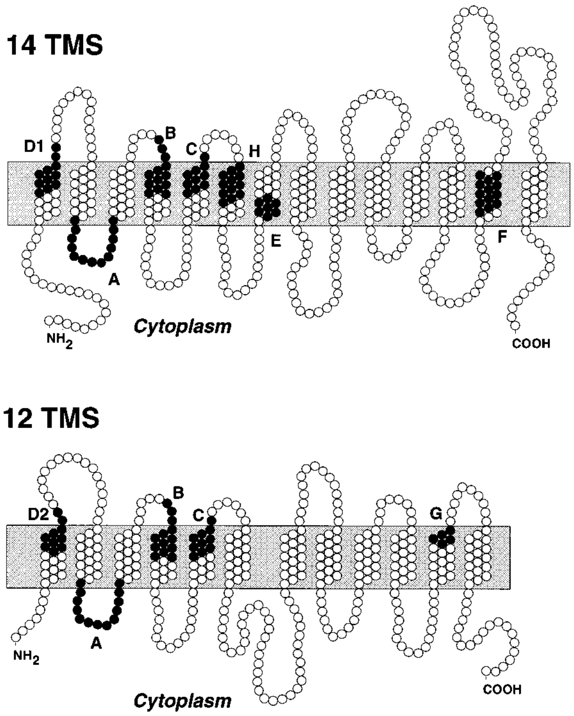


FIG. 10. Schematic two-dimensional representations of a typical member of the 14- and 12-TMS families in the cytoplasmic membrane (gray). The locations of the conserved motifs shown in Fig. 6 and 7 are highlighted in black and labelled with the letter representing the particular motif.

for activity, since a conservative substitution to threonine retained full activity. However, other, more radical mutations at this site altered the substrate specificity of the export system. Together, these studies indicate that Cys-42 may be located near the substrate-binding site of Smr (204).

The conserved aromatic residues Tyr-59 and Trp-62 in the Smr protein (Fig. 11 and 13, motif C) have also been targeted by site-directed mutagenesis (204). These residues appear to be essential, since substitutions at these positions, even with other aromatic residues, abolished the ability of Smr to catalyze drug efflux. These two residues are located on the same polar face of TMS 3, leading to the proposition that their side chains may directly interact with the hydrophobic regions of substrates of the Smr efflux system (204) (see below).

The introduction of separate substitutions for the residues Glu-24, Pro-31, Cys-42, and Glu-80 in Smr (92, 204) affected the substrate specificity of this efflux system; i.e., they reduced or abolished the ability of the protein to confer resistance to ethidium bromide, but not to other compounds, such as benzalkonium or cetrimide (92, 204). The basis of the common phenotypic effects resulting from these different mutations remains unclear, but it seems unlikely that all of these residues are directly involved in substrate recognition, given their disparate locations (Fig. 13).

EmrE Multidrug Efflux Protein

Two groups have independently cloned and sequenced an *E. coli* chromosomal resistance gene which conferred PMF-dependent efflux of organic cations (177, 228, 229) and was designated *ebr* or *mvrC* but has since become known as *emrE* (323). *emrE* confers resistance to monovalent cations, such as ethidium, proflavine, pyronin Y, safranin O, and methyl viologen (177, 229), as well as to erythromycin, sulfadiazine, TPP, and tetracycline (323). The EmrE efflux system may correspond to the *E. coli* chromosomal ethidium and phosphonium efflux system previously identified by Midgley (174, 175).

The EmrE protein is soluble in a chloroform-methanol mixture and has been purified via extraction with these organic solvents (323). The purified EmrE protein has been reconstituted into proteoliposomes and shown to mediate ΔpH-dependent ethidium and methyl viologen transport (323), suggesting a drug/proton antiport mechanism. Transport of these substrates could be competitively inhibited by each other or by various other compounds to which *emrE* confers resistance, e.g., TPP, acriflavine, and tetracycline, as well as by the P-glycoprotein inhibitor reserpine (323).

QacE/QacEΔ1 Multidrug Efflux Proteins

The multidrug resistance *qacE* gene was initially identified on the *Klebsiella aerogenes* plasmid R751 (208), where it is located on an integron, a potentially mobile element found in gram-negative bacteria (281). Drug susceptibility studies have indicated that *qacE* confers a similar drug resistance phenotype to that encoded by the staphylococcal *smr* gene. Ethidium transport experiments have suggested that *qacE* confers resistance via PMF-dependent efflux (208). A semifunctional derivative of the *qacE* gene, known as *qacEΔ1*, is widely distributed throughout gram-negative bacteria because of its location on the 3′ conserved segment of most integrons (208, 281). *qacEΔ1* probably represents a disrupted form of *qacE* which evolved by the insertion of a DNA segment near the 3′ end of the *qacE* gene (208, 232).

Structure and Function of the SMR Transporters

Experiments with purified, reconstituted Smr and EmrE proteins have demonstrated that these proteins function as PMF-dependent efflux pumps probably via a multidrug/proton antiport mechanism (92, 323). The apparently electrogenic nature of Smr-catalyzed efflux (92) has suggested a stoichiometry of 2 or 3 H^+ per drug cation, and Grinius and colleagues have suggested that the essential intramembranous Glu-13 residue in Smr (92, 213) may be involved in the H^+/drug exchange reaction. Paulsen et al. (213) have proposed a model for multidrug efflux catalyzed by the SMR proteins based on the available experimental data and the observation that the first three TMS in the SMR proteins are amphipathic, with a number of conserved glutamate, serine, tyrosine, and tryptophan residues located on the polar faces of these helices (Fig. 13). These residues may form part of a transmembrane pathway through which protons and drugs pass, with the possibility that the side chains of conserved residues, such as Tyr-59 and Trp-62 in Smr, directly interact with the hydrophobic regions of substrates, facilitating their transport through the transmembrane pathway in a similar fashion to that proposed for the mammalian multidrug pump, P-glycoprotein (204, 213, 214).

The small size (4 TMS; ~110 amino acids) of the SMR family of multidrug efflux proteins makes them unique among secondary transporters, which typically consist of 10 to 14 TMS (226). Thus, the SMR proteins may serve as an excellent model for the study of membrane transport, well suited to three-dimensional structural determination via nuclear magnetic resonance (NMR) spectroscopy, NMR or fluorescence spectroscopic investigations of substrate interactions, and saturation mutagenesis. However, such analyses may well be complicated by the potential oligomeric structure of the SMR proteins.

TABLE 3. SMR family proteins

Protein	Organism	Representative substrates[a]	Accession no.[b]	Reference(s)
Multidrug resistance				
EmrE	*Escherichia coli*	Monovalent cations, e.g., CT, CV, EB, MV, TET, TPP	SW P23895	149, 177, 228
Smr	*Staphylococcus aureus*	Monovalent cations, e.g., CT, CV, EB	SW P14319	91, 155, 256
QacE	*Klebsiella pneumonia*	Similar range of substrates to Smr	PR S25583	208
QacEΔ1	Gram-negative bacteria	Similar range of substrates to Smr	GB L06418	33, 208, 284
Other function				
CfSugE	*Citrobacter freundii*	Unknown[c]	NA[d]	22
EcSugE	*Escherichia coli*	Unknown[c]	GB X69949	89, 213
Hypothetical or uncharacterized				
BaOrf6	*Bacillus subtilis*	Unknown	GB D78189	181
PvSugE	*Proteus vulgaris*	Unknown[c]	SW P20928	42
SocA2	*Myxococcus xanthus*	Unknown	PR B55208	144

[a] Abbreviations as for Tables 1 and 2.
[b] Accession numbers as for Table 2.
[c] These proteins have been hypothesized to suppress chaperone defects (89, 213).
[d] NA, not available.

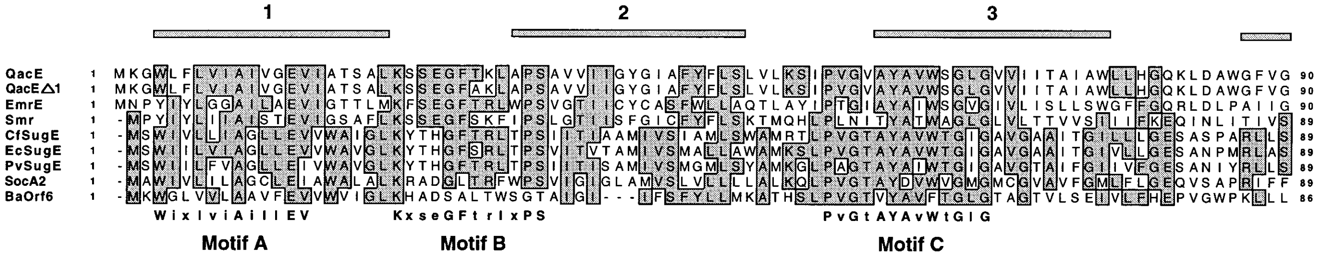
RESISTANCE/NODULATION/CELL DIVISION FAMILY

A third family of PMF-dependent drug efflux proteins, known as the RND family, has been identified (Table 4) (54, 249). 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 (see Fig. 17) (249). The RND family includes a number of multidrug resistance proteins: AcrB (formerly AcrE) and AcrF (formerly EnvD) from *E. coli* (165–167), MexB from *Pseudomonas aeruginosa* (224, 225), and MtrD from *Neisseria gonorrhoeae* (95, 203). These probable multidrug drug efflux proteins share an extremely broad substrate specificity. Two other putative *E. coli* proteins, AcrD and YhiV (OrfB), may also be multidrug efflux proteins (167). The existence of a further multidrug efflux protein, MexD from *P. aeruginosa*, has been hypothesized (151) and recently confirmed (223). Other members of this family include the *Alcaligenes* heavy-metal ion export proteins CzcA (194), CnrA (154), and NccA (260); the NolGHI system from *Rhizobium meliloti*, which may export oligosaccharides involved in nodulation signalling (14); and the products of hypothetical open reading frames from a number of organisms. Although none of the members of this family have been unequivocally demonstrated to be membrane transport proteins, there is accumulating indirect evidence for several members of this family, suggesting that they confer PMF-dependent transport (165, 167, 193, 194, 225).

Comparative sequence analyses have indicated that the N- and C-terminal halves of RND proteins share sequence similarity, implying that they may have evolved via tandem intragenic duplication in an analogous manner to that proposed for the MFS (249). Thus, the RND proteins appear also to have evolved from an ancestral protein containing six TMS. Phylogenetic analysis has revealed that the majority of multidrug efflux proteins within this family fall within a single closely related cluster, with only MtrD being somewhat divergent (Fig. 14). Hypothetical proteins within this cluster (AcrD, BuOrf2, Slr0369 and HI0895 in Fig. 14) may also be multidrug exporters, in which case this entire cluster will be composed of multidrug efflux proteins. There are two other functional groupings within the RND family tree: a cluster which includes three metal ion efflux proteins, CzcA, CnrA, and NccA; and a single branch which contains the NolGHI system, which may export oligosaccharides. Thus, the clustering pattern of the RND phylogenetic tree appears to reflect functional differences between the proteins, and there may be only a single distinct cluster of multidrug efflux proteins, contrasting the situation with the phylogeny of the 12- and 14-TMS families of the MFS (Fig. 3 and 4). Sequence alignment has previously identified three highly conserved motifs shared by RND proteins (249) (see motifs A, B, and C in Fig. 1, 15, and 17). The multiple-sequence alignment presented in Fig. 15 reveals that these conserved motifs are also found in recently discovered family members. The potential roles of these motifs have not yet been clarified, but their conservation suggests that they may play an essential structural or functional role in these proteins. We have identified an additional highly conserved motif in the RND proteins (motif D in Fig. 15 and 17).

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 (Fig. 16) (54, 249). Genetic evidence has suggested that RND and MFP proteins interact cooperatively to enable drug transport across both the inner and outer membranes of gram-negative bacterial cells (Fig. 1) (for reviews, see references 149, 167, and 196a). Dinh et al. (54) 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 (54). Examples include the *E. coli* EmrA protein, which cooperates with the MFS multidrug efflux protein, EmrB (159), and the *E. coli* HlyD protein, which interacts with the ABC hemolysin transporter, HlyB (140).

The MFP proteins are apparently tethered to the inner membrane (54) either by a single N-terminal TMS-spanning segment, e.g., HlyD (266), or by a lipid moiety (i.e., some MFP members are lipoproteins), e.g., AcrE (267). Dinh et al. (54) have proposed, on the basis of secondary-structure analysis, that the MFP proteins span the periplasmic space and interact with constituents in both membranes (Fig. 1). Multiple-sequence analysis has revealed that the MFP family is quite



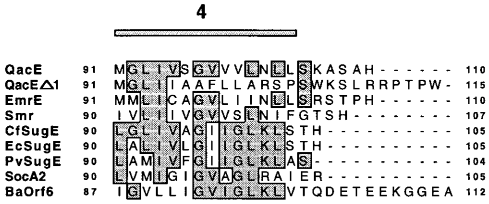


FIG. 11. Multiple-sequence alignment of members of the SMR family. The presentation of the figure is as described in the legend to Fig. 6, and the motifs shown are equivalent to the signature sequences defined by Paulsen et al. (213). For relevant accession numbers and references to these proteins, see Table 3.

divergent and that its members share no globally conserved residues (206).

In a manner similar to that for the RND proteins, the phylogeny of the MFP proteins correlates with their substrate specificities and also with the types of transport system with which they interact (54). This observation suggests that the MFP proteins may interact directly with both the transported substrates and the transport proteins with which they are associated (54).

In some cases, MFP proteins and their respective transport proteins have been proposed to interact with members of a third protein family, namely, the OMF family (56). For example, the OMF protein TolC is required for hemolysin export by HlyB and HlyD (302), and OprM is involved in multidrug efflux mediated by the *P. aeruginosa* MexA and MexB proteins (225). OMF family members are outer membrane proteins, and Ma et al. (167) have suggested that they act as outer membrane channels and function cooperatively with RND and MFP proteins, as shown schematically in Fig. 1.

Thus, in some cases, the RND efflux proteins appear to utilize two further components, the MFP and OMF proteins, to enable substrate transport across the outer membranes of gram-negative bacteria (Table 4; Fig. 1). Some efflux proteins from other families, such as the MFS multidrug efflux protein EmrB (Fig. 1 and see above), also appear to utilize such components, although a definitive identification of the OMF protein involved with EmrB has not yet been obtained. This generalization has not yet been shown to be applicable to all RND proteins; e.g., OMF proteins have not been identified for some of these systems, and in the case of AcrD (167), neither an MFP nor an OMF protein associated with this system has been identified (Table 4). Consistent with the hypothesis that the MFP and OMF constituents enable transport across the outer membrane of gram-negative cells, the currently identified RND proteins from gram-positive bacteria do not have corresponding MFP or OMF proteins, nor have any members of the MFP or OMF families been identified in gram-positive bacteria.

The following sections consider in detail each of the RND multidrug efflux systems which have been characterized.

AcrAB Multidrug Efflux System

The *E. coli* chromosomal *acrA* locus has long been known to be involved in determining resistance to acriflavine and other cationic dyes, as well as to detergents and antibiotics (179, 180). Cloning, sequencing, and characterization of this locus (165) identified an operon with two genes, *acrA* and *acrB*, encoding members of the MFP and RND families, respectively (Fig. 16). Deletions within each of these genes confirmed that they are both required for drug resistance (166). Drug susceptibility studies have indicated that AcrAB mediate resistance to a very wide range of antibiotics and toxic compounds, and acriflavine accumulation experiments have supported the notion that these proteins constitute a PMF-dependent drug efflux system (165, 166). An OMF protein associated with the AcrAB system has not yet been firmly identified, although it is possible that the TolC channel acts in this capacity (167), since *tolC* mutants show increased susceptibility to various substrates of the *acrAB* system (49). Additionally, a *tolC* mutation does

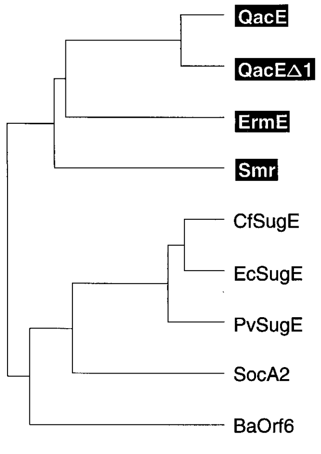


FIG. 12. Phylogenetic tree displaying the relationships among proteins of the SMR family. The tree was constructed as described in the legend to Fig. 2. Known multidrug efflux proteins are highlighted in reverse type. See Table 3 and the text for further details about specific proteins in the family.

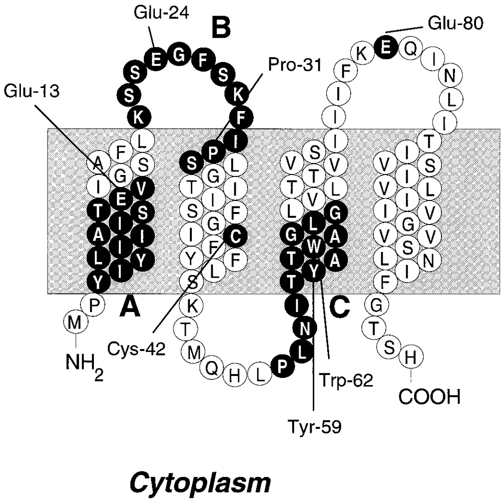


FIG. 13. Schematic two-dimensional representation of the *Staphylococcus aureus* Smr protein in the cytoplasmic membrane (gray). The locations of the signature sequences A, B, and C, shown in Fig. 11, are highlighted in black. Residues in Smr which have been mutagenized (92, 204) are highlighted in black and indicated (see text for details).

not further increase the sensitivity of *acrAB* mutant strains to these substrates, inferring that TolC and AcrAB function together (71a),

Deletion of the *acrAB* operon also leads to increased susceptibility to bile salts and fatty acids, such as decanoate (166). Bile salts and fatty acids are present in high concentrations in the natural environment of an enteric bacterium, such as *E. coli*, suggesting that efflux of these compounds may be one of the physiological roles of the AcrAB efflux system in *E. coli*. Consistent with this hypothesis, *acrAB* expression has been demonstrated to be induced by decanoate (166).

In addition to decanoate, *acrAB* expression is induced by other stress conditions, e.g., 4% ethanol, 0.5 M NaCl, and growth of the cell to the stationary phase (166). Upstream of the *acrAB* operon is a divergently transcribed gene, *acrR* (Fig. 16) (167), whose product shares sequence similarity with the regulatory proteins TetR and QacR (Fig. 8 and see above). Analysis of *acrAB-lacZ* fusions has suggested that expression of this operon is subject to regulation by the *E. coli mar* regulon (166) and also by AcrR (163). Ma et al. (163) have proposed that regulation of *acrAB* expression is mediated primarily by global regulatory pathways, and AcrR acts as a secondary modulator to prevent excessive expression of *acrAB* (163).

At least three genes encoding close homologs of AcrB are present on the *E. coli* chromosome (Table 4): *acrF* (formerly *envD*) (165, 167), *yhiV* (*orfB*) (167), and *acrD* (167). *acrF* and *yhiV* are located in operons together with *acrE* and *yhiU* (*orfA*) (Fig. 16), which encode MFP constituents, and the *acrEF* operon is probably regulated by the upstream *acrS* gene, whose product is similar to AcrR (Fig. 16). Mutations in either *acrEF* (165) or *yhiVU* (282) lead to increased susceptibility to multiple drugs, strongly suggesting that these are also PMF-dependent multidrug efflux systems.

MexAB/OprM Multidrug Efflux System

Pseudomonas aeruginosa exhibits a high level of intrinsic resistance to a range of antimicrobial agents, partly because of its outer membrane composition (195). Additionally, drug accumulation and efflux studies have suggested the presence of at least two distinct PMF-dependent multidrug efflux systems (152, 153). Poole and colleagues (86, 224, 225) identified a chromosomal operon, involved in conferring resistance to a range of antimicrobial agents, which encodes three genes, *mexA*, *mexB*, and *oprM* (Fig. 16). The *mexA* and *mexB* genes code for members of the MFP and RND families, respectively. The *oprM* gene codes for a member of the OMF family, which was initially identified as the outer membrane protein OprK (225). However, the *oprM* gene has recently been demonstrated to code for a different outer membrane protein, OprM (Fig. 1) (86, 98), which had previously been shown to be involved in conferring resistance to multiple drugs (85, 171, 235).

The *mexAB*/*oprM* operon was originally identified on the basis of its ability to complement an iron metabolism defect (224). Expression of the *mexAB*/*oprM* operon was inducible under iron-limited conditions and appeared to be coregulated with components of the pyoverdine-mediated iron transport system (224). Additionally, mutants lacking *mexA* or *mexB* were found to be unable to grow on iron-deficient medium. This led Poole et al. to suggest that this operon may be involved in the secretion of the iron-chelating molecule pyoverdine (224) or, more generally, that it may be involved in the general secretion of secondary metabolites such as pyoverdine, which may explain its ability to confer resistance to antibiotics (222).

Additional to their proposed role in pyoverdine secretion, MexAB and OprM appear to function cooperatively as a multidrug efflux system (Fig. 1), providing a significant contribution to the intrinsic resistance of *P. aeruginosa*. Mutations in *mexA*, *mexB*, or *oprM* result in enhanced sensitivity to tetracycline, chloramphenicol, ciprofloxacin, and iron-binding compounds (225), as well as to other quinolones and a range of β-lactam compounds (86). Mutations in *mexA* or *oprM* lead to increased cellular 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 (153). Recently, a divergently encoded open reading frame upstream of the *mexAB*/*oprM* operon has been identified and named *mexR* (Fig. 16) (225a). This encodes a protein which exhibits some similarity to MarR and appears to function both as a repressor and as an activator. Preliminary experiments have raised the possibility of the participation of a second gene product in the regulation of the *mexAB*/*oprM* operon (225a).

In addition to the MexAB/OprM system, other studies have identified a similar multidrug resistance system in *P. aeruginosa* that apparently consists of the components MexC, MexD, and OprJ (previously thought to be OprK) (98, 152, 153, 223). Analysis of *oprJ* (*oprK*) mutants and MexCD/OprJ (OprK)-overproducing strains has suggested that this system shares a similar substrate specificity to MexAB/OprM, with the exception of some compounds; e.g., only MexAB/OprM confers resistance to carbenicillin (98). This operon has recently been cloned and found to contain the *mexC*, *mexD*, and *oprJ* genes; overexpression of the operon confers resistance to quinolones, tetracycline, chloramphenicol, and newer cephems (223).

MtrCDE Multidrug Efflux System

Mutations in the *mtr* locus of *Neisseria gonorrhoeae* confer resistance to hydrophobic antibiotics, detergents, and dyes, as well as to bile salts and fatty acids typically found on mucosal surfaces (168, 278). Such resistant strains have been commonly reported in clinical *N. gonorrhoeae* isolates (178), particularly in isolates from rectal infections, suggesting a role for the *mtr* locus in providing resistance to toxic fecal lipids. The *mtr* locus

TABLE 4. RND family export proteins[a]

RND protein	MFP protein	OMF protein	Organism	Representative substrates[b]	Accession no.[c]	Reference(s)
Multidrug resistance						
AcrB	AcrA	TolC (?)	*Escherichia coli*	AC, CV, detergents, decanoate, EB, erythromycin	EM U00734	165, 166
AcrF	AcrE		*Escherichia coli*	AC, actinomycin D, vancomycin	EM X57948	137, 138
MexB	MexA	OprM	*Pseudomonas aeruginosa*	CML, β-lactams, fluoroquinolones, TET[d]	GB L11616	224
MexD	MexC	OprJ	*Pseudomonas aeruginosa*	CML, quinolones, TET	GB U57969	223
MtrD[e]	MtrC	MtrE	*Neisseria gonorrhoeae*	Detergents, hydrophobic antibiotics, dyes	SW P43505	95, 203
YhiV	YhiU		*Escherichia coli*	Multidrugs[g]	EM U00039	167, 276
Other resistance						
CnrA	CnrB		*Alcaligenes eutrophus*	Cobalt, nickel	EM M91650	154
CzcA	CzcB		*Alcaligenes eutrophus*	Cobalt, cadmium, zinc	EM M26073	193, 194
NccA	NccB		*Alcaligenes xylosoxidans*	Cadmium, cobalt, nickel	GB L31363	260
NolGHI	NolF		*Rhizobium meliloti*	Unknown[g]	EM X58632	14
Hypothetical or uncharacterized						
AcrD			*Escherichia coli*	Unknown	GB U10436	164, 167
BuOrf2[e]			*Bacteroides uniformis*	Unknown	GB L08472	275
HI0895	HI0894		*Haemophilus influenzae*	Unknown	GB L45533/L45532	69
Slr0369	Slr0628		*Synechocystis* sp.	Unknown	GB D63999/D64002	133
Slr0794			*Synechocystis* sp.	Unknown	GB D64005	133
YbdE			*Escherichia coli*	Unknown	SW P38054	27, 219

[a] Along with the RND family export protein are shown the associated MFP and OMF proteins, see the text for details.
[b] Abbreviations as for Tables 1 and 2.
[c] Accession numbers as for Table 1.
[d] Also involved in transporting pyoveridine.
[e] From our analyses, we predict that the sequence of these proteins may be incomplete and/or contain sequencing errors.
[f] Substrates not described (282).
[g] NolGHI and NolF are involved in secreting oligosaccharides that act as nodulation signals (14).

consists of *mtrR*, encoding a transcriptional repressor protein related to AcrR and AcrS (203), and an operon containing the *mtrC*, *mtrD*, and *mtrE* genes (95), which encode members of the MFP, RND, and OMF families, respectively (Table 4; Fig. 16).

The *mtrC* gene encodes a lipoprotein (95), and disruption of *mtrC* increased susceptibility to a range of hydrophobic drugs (95). Consistent with the notion that MtrCDE acts as a multidrug efflux system, accumulation experiments with the hydrophobic detergent Triton X-100 revealed that disruption of *mtrC* resulted in increased accumulation of Triton X-100, as did treatment with the protonophore CCCP (161). Overexpression of the *mtrCDE* operon as a result of a mutation in *mtrR* (see below) led to increased levels of multidrug resistance (95) and decreased Triton X-100 accumulation (161).

Deletion of the *mtrR* gene resulted in increased multidrug resistance (intermediate-level resistance), increased production of the MtrC protein (203), and increased transcription of *mtrC* and presumably of *mtrD* and *mtrE* (96). Similarly, mutations in *mtrC*, resulting in amino acid substitutions at residue 40 (269), 45 (95), or 105 (203) in MtrC, gave increased multidrug resistance (intermediate-level resistance). 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 site (97). 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 *N. gonorrhoeae* isolates (269).

Structure and Function of the RND Transporters

Although no RND protein has been purified, reconstituted, and shown to be a PMF-dependent transporter, genetic and biochemical evidence supports the notion that these proteins do function as PMF-dependent efflux systems. The RND multidrug efflux systems identified display a much wider substrate specificity than the MFS or SMR multidrug efflux proteins. Currently, no data regarding the molecular basis of substrate recognition by these transporters are available.

On the basis of hydropathy analyses and the multiple-sequence alignment partly presented in Fig. 15, a schematic model of a typical RND family protein is presented in Fig. 17. There are two large external loops, situated between TMS 1 and 2 and between TMS 7 and 8, and the duplication of the N- and C-terminal halves is evident in the arrangement of the TMS. The role of the four conserved regions identified has not yet been investigated.

For gram-negative bacteria, genetic evidence is consistent with the proposal that RND proteins typically function in conjunction with MFP and OMF proteins to mediate transport across both membranes of the cell envelope (Fig. 1). MFP and in some cases OMF proteins which function together with MFS (e.g., EmrB; see above) or ABC (e.g., HlyB) transporters have also been identified. RND proteins have also been iden-

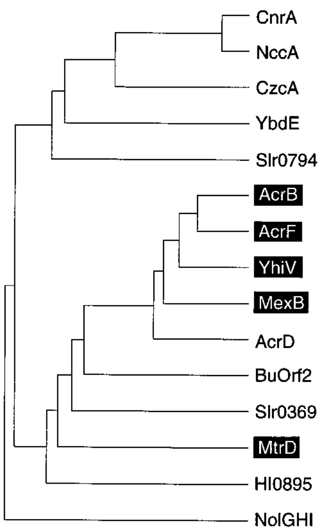


FIG. 14. Phylogenetic tree displaying the relationships among proteins within the RND family. Preparation and presentation are as for Fig. 2. Known multidrug efflux proteins are highlighted in reverse type. See Table 4 and the text for further details about specific proteins in the family.

tified in gram-positive bacteria, where they do not seem to be associated with MFP or OMF proteins.

OTHER PMF-DEPENDENT MULTIDRUG EFFLUX SYSTEMS

All of the sequenced PMF-dependent multidrug efflux systems characterized to date belong to one of the three families considered in this review. It is quite likely that other PMF-dependent multidrug proteins, not homologous with members of any of these three families, exist. Certainly, other unrelated families of PMF-dependent transporters are known, e.g., the MIT family of metal ion transporters (209), the APC family of amino acid and other transporters (233), and the POT or PTR family of peptide transporters (211, 280).

Biochemical and physiological studies have identified other PMF-dependent multidrug efflux systems whose genes have not been characterized. Whether these will prove to be members of the three families described above remains to be seen. For example, Charvalos et al. (37) have obtained mutants of *Campylobacter jejuni* which are resistant to multiple drugs, such as pefloxacin, erythromycin, chloramphenicol, tetracycline, and β-lactams. Accumulation assays have supported the notion that these strains extrude pefloxacin, ciprofloxacin, and minocycline in a protonophore-sensitive manner.

It must be cautioned that a multidrug resistance phenotype encoded by a single locus may not necessarily be due to a multidrug export system, even when one or more of the resistances appears to result from active export. For instance, the *E. coli* chromosomal *marRAB* locus confers resistance to tetracycline, chloramphenicol, fluoroquinolones, nalidixic acid, rifampin, penicillin, and other compounds (41). *marRAB* does not encode a multidrug efflux system; instead, it is a global regulatory locus which controls the expression of multiple genetic loci, such as the porin gene *ompF* and the *acrAB* multidrug efflux genes (40, 278). The mechanisms encoded by these multiply regulated loci, which possibly include additional drug efflux systems, cumulatively account for the Mar phenotype. Similarly, the *Klebsiella pneumoniae* multidrug resistance *ramA* gene is another regulatory locus which codes for a transcriptional activator homologous to MarA (78).

MOLECULAR BASIS OF BROAD SUBSTRATE SPECIFICITY

The proteins within the three distinct families or superfamilies of PMF-dependent multidrug efflux proteins described in this review possess the common feature of broad substrate specificity, despite their disparate evolutionary origins. However, the molecular basis of the broad substrate specificities of these multidrug efflux systems remains unclear.

One deduction that can be drawn from the current data is that the physical characteristics of the compounds, such as their charge, hydrophobicity, or amphipathicity, rather than their structures, appear to be a key determinant in the specificities of these PMF-dependent multidrug efflux systems. For instance, *emrAB* conveys resistance to hydrophobic quinolones but not to structurally related hydrophilic analogs of these drugs (159). In contrast, *bmr* and *norA* convey resistance to hydrophilic fluoroquinolones but not to more hydrophobic analogs (185, 189). All of the substrates of the *qacA*- and *qacB*-encoded export systems contain a positively charged moiety and in most cases one or more aromatic rings, and the key difference in their relative specificities appears to be the number of positively charged moieties present in the substrate (156, 205) (see above). The only common features observed in substrates and inhibitors of the VMAT1 and VMAT2 transporters are the presence of an aromatic ring and a positively charged moiety (261, 264). Introduction of a negative charge in particular VMAT substrates or inhibitors greatly reduces their binding affinity with the transporter (36, 242), whereas the introduction of hydroxyl, methoxy, or amino substituents in the aromatic ring increases their binding affinity for the transporter (198, 265).

In particular exporters, mutagenesis has identified specific residues implicated in substrate specificity. In most cases, the residues potentially involved in substrate binding are located within predicted transmembrane regions; for instance, in the MFS proteins, substitutions at Asp-323 (TMS 10) in QacA (205), Val-286 (TMS 9) (2) and within TMS 4, 7, and 9 to 11 (186) in Bmr, and Ala-362 (TMS 12) in NorA (126, 201) all alter substrate specificity (see above for details and discussion of roles of specific residues). Such studies have yet to provide a clear picture of the molecular basis of the broad substrate specificity of such multidrug efflux proteins.

The basis of their broad substrate specificity will probably be definitively answered only by the determination of high-resolution structures of one or more multidrug efflux proteins, together with biochemical analyses of the interactions between the substrates and the transporter. However, the ability of regulatory proteins, such as BmrR, EmrR, and QacR, to bind structurally diverse drugs in a manner akin to their corresponding efflux proteins provides a complementary approach to gain insights into the phenomenon of multidrug recognition. Such hydrophilic regulatory proteins are likely to prove more amenable to structural and functional studies than are their corresponding hydrophobic membrane proteins.

Comparison of the above characteristics of the PMF-dependent multidrug efflux systems with those of the well-studied ATP-dependent multidrug efflux pump P-glycoprotein reveals a number of similarities; i.e., its substrates are generally hydrophobic, the physical rather than structural characteristics of the substrates appear to be key determinants in the substrate specificity of the proteins, and specific mutations, typically within membrane-spanning regions, alter the substrate speci-

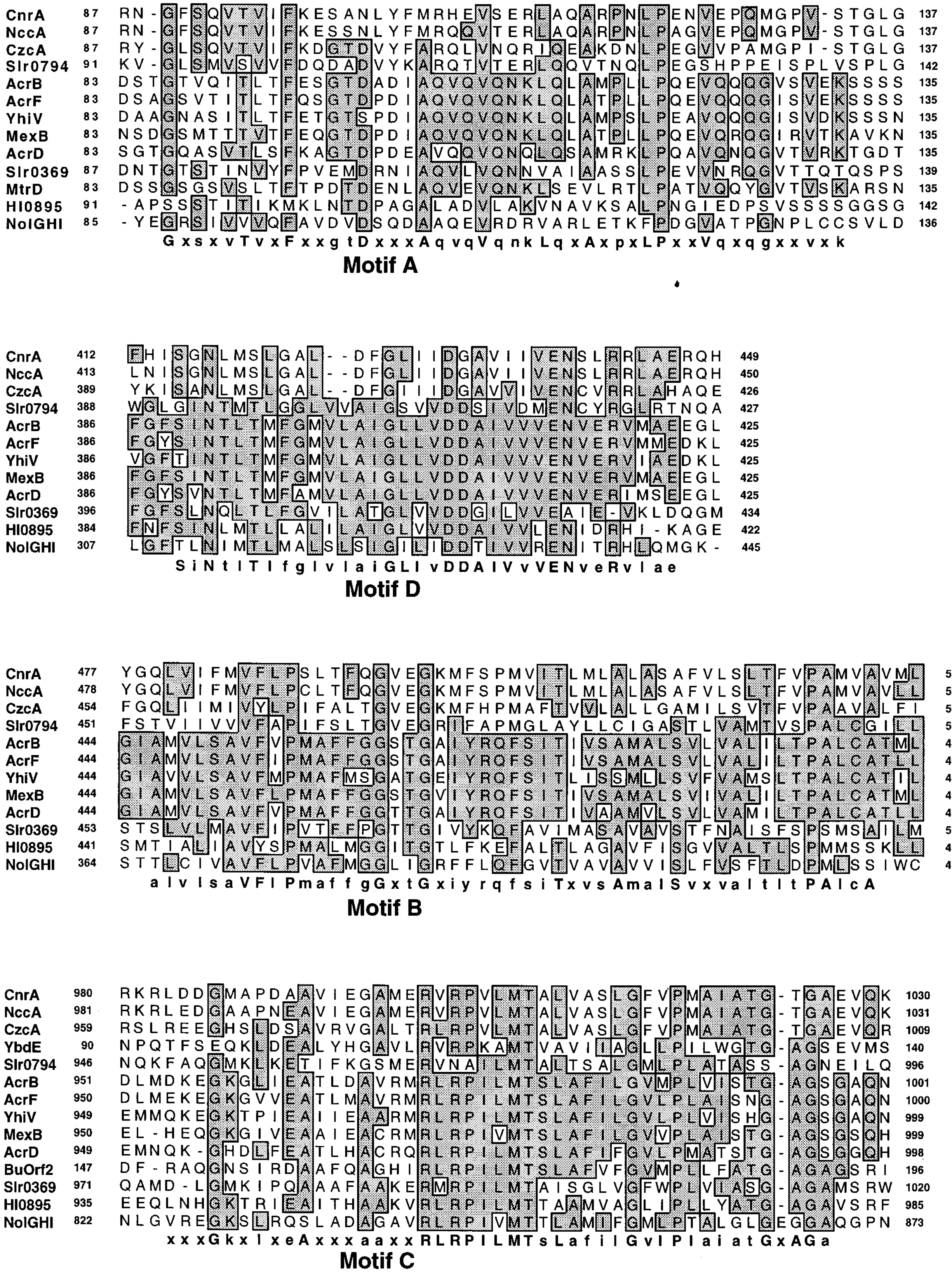


FIG. 15. Multiple-sequence alignment of the conserved regions A, B, C, and D for the members of the RND family. The presentation of the figure is as described in the legend to Fig. 6. Motifs A, B, and C correspond to the conserved regions previously identified by Saier et al. (249). For relevant accession numbers and references to these proteins, see Table 4.

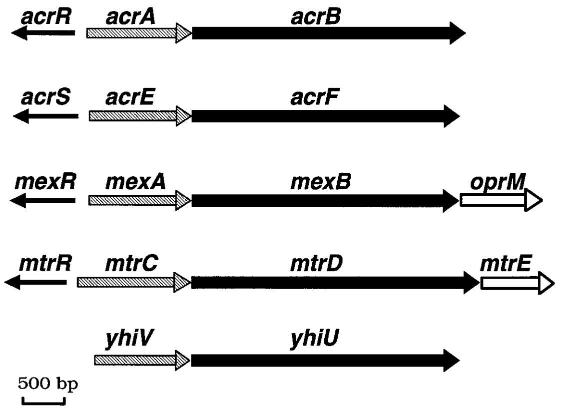


FIG. 16. Comparative genetic maps of the RND multidrug efflux loci *acrAB*, *acrEF*, *mexAB/oprM*, *mtrCDE*, and *yhiVU*. Genes are denoted by arrowed lines: (i) RND, thick black; (ii) MFP, thick striped; (iii) OMF, white; (iv) regulatory, thin black.

ficity of the efflux proteins (for a review of P-glycoprotein, see reference 87). These broad similarities suggest that P-glycoprotein and the PMF-dependent efflux systems examined in this review may share a similar mechanistic basis for recognizing structurally dissimilar drugs.

A number of models have been proposed to explain the capability of P-glycoprotein to recognize and transport multiple drugs. Recent studies with purified, reconstituted protein have confirmed the long-held supposition that P-glycoprotein is an active transporter which can transport drug molecules against a significant substrate concentration and have indicated that P-glycoprotein can transport drugs either from the cytoplasm or directly from the lipid membrane (for reviews, see references 244 and 270). These findings argue against alternative indirect mechanisms proposed for P-glycoprotein-mediated drug resistance, such as (i) P-glycoprotein acting as a proton pump, such that ATP hydrolysis drives proton transport and hydrophobic drugs follow passively (279), (ii) P-glycoprotein acting as a membrane channel for ATP (1), and (iii) P-glycoprotein being involved in intracellular pH regulation (237, 238). However, a number of other possible mechanistic models have also been proposed.

In a "conventional" model of membrane transport, P-glycoprotein would form a pore in the membrane, with drugs being bound at a substrate binding site on P-glycoprotein capable of recognizing a wide range of substrates and subsequently being released on the opposite side of the pore in an ATP-dependent process.

Gottesman and Pastan (87) have proposed that P-glycoprotein acts as a hydrophobic vacuum cleaner, whereby the protein recognizes its lipophilic substrates directly from the cell membrane or from the cell cytosol and pumps them through a single-membrane barrel in P-glycoprotein.

Higgins and Gottesman (108) suggested that P-glycoprotein may be a flippase, i.e., a protein involved in flipping drugs from the inner leaflet of the lipid bilayer to either the outer leaflet of the lipid bilayer or the external environment, and may form a cleft, whereby the substrate-binding site would be accessible from the lipid membrane, rather than a pore. In this situation, P-glycoprotein would be capable of exporting any hydrophobic substrates capable of intercalating appropriately in the lipid bilayer. Interestingly, construction of a null mutation in the *mdr2* gene, whose product is closely related to P-glycoprotein but does not confer multidrug resistance, has indicated that Mdr2 plays an essential role in the transport of phosphatidylcholine into bile and probably functions as a lipid flippase or as a phosphatidylcholine transporter (245, 274).

Pawagi et al. (214) have noted that P-glycoprotein contains a high concentration of aromatic amino acid residues within its putative TMS and, using computer modelling, suggested that typical P-glycoprotein substrates may be capable of intercalating between the aromatic side chains of these residues. This led to the suggestion that rather than containing a single substrate-binding site capable of recognizing diverse substrates, P-glycoprotein may be able to undergo wide-ranging drug-dependent dynamic reorganization; i.e., P-glycoprotein may adapt its structure to cope with the requirements of particular substrates (214).

It should be noted that particular features of some of these models are not exclusive and may be complementary. Although an understanding of the phenomenon of multidrug efflux at the molecular level remains elusive, it is hoped that the study of both P-glycoprotein and of the multidrug/proton antiport systems discussed in this review may clarify this matter.

WIDESPREAD DISTRIBUTION OF PROTON-DEPENDENT MULTIDRUG EXPORT SYSTEMS

PMF-dependent multidrug pumps appear to be widespread, since they are found in organisms of diverse origins, both eukaryotic and prokaryotic (Tables 1 to 4). In most instances, they are chromosomally encoded, but particularly in clinical isolates of some pathogenic bacteria, they are encoded by resistance plasmids. A variety of multidrug systems with overlapping specificities appear to be located in single organisms. For example, to date, nine definite (EmrA/B, EmrD, EmrE, Bcr, QacEΔ1, AcrA/B AcrE/F, YhiV/U and TehA) and a further two probable (AcrD and EmrX) proton-dependent multidrug extrusion systems have been identified in *E. coli* (Tables 1 to 4). As an example of their overlapping specificities, at least six of these systems can transport ethidium cations. In particular, 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.

In addition to these systems in *E. coli*, there are other hypothetical open reading frames, whose products belong to either the MFS, SMR, or RND family which may prove to be multidrug efflux proteins; there may be ATP-driven multidrug efflux pumps (e.g., the product of the *mdl* gene is a close homolog of P-glycoprotein and may function as a drug efflux pump) (10), and there are also other loci, such as the *marRAB* regulatory system (see above) involved in controlling resistance to multiple drugs. This apparent redundancy in the number of systems protecting a cell from the effects of toxic compounds remains to be explained, although it is possible that such an array of multiple efflux systems with overlapping specificities affords a high level of protection, while allowing a cell to fine tune the excretion of particular compounds. Alternatively, these multidrug exporters may play other physiological roles, and their excretion of toxic compounds is due to fortuitous recognition of these substrates. The potential physiological roles of the characterized multidrug efflux systems are discussed in the next section.

It is anticipated that genome-sequencing projects will soon allow the identification of a large number of putative multidrug proteins, homologous to known multidrug pumps. For instance, analysis of the complete sequences of 10 *Saccharomyces cerevisiae* chromosomes (corresponding to approximately 80% of the yeast genome) has identified 19 novel open reading

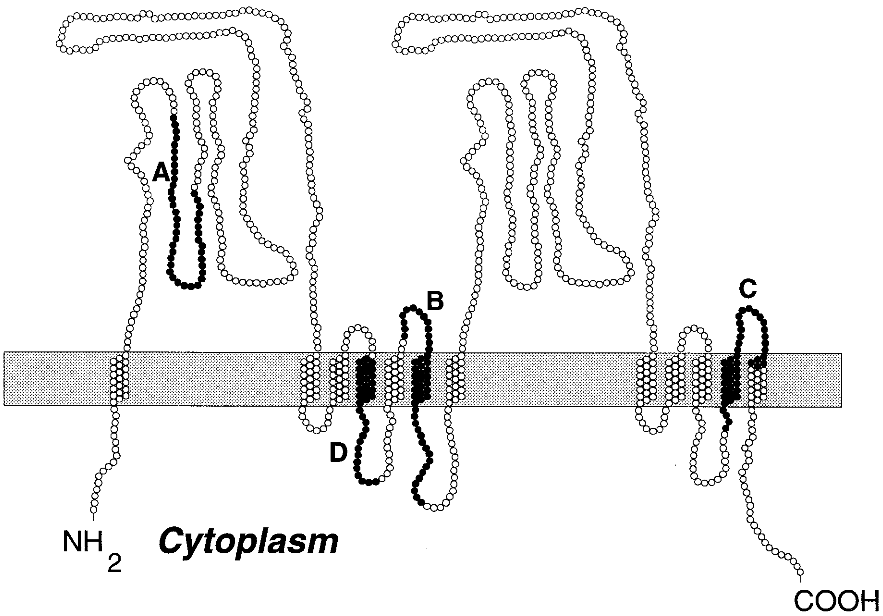


FIG. 17. Schematic two-dimensional representation of a typical member of the RND family in the cytoplasmic membrane (gray). The locations of the conserved motifs A, B, C, and D, as indicated in Fig. 15, are highlighted in black.

frames, which encode members belonging to the 12- and 14-TMS families of the MFS, all representing potential multidrug efflux proteins (81).

Recently, the complete genomic sequences from two free-living organisms, *Haemophilus influenzae* (69) and *Mycoplasma genitalium* (72), have been reported. In the case of *H. influenzae*, Fleischman et al. (69) identified close homologs of four multidrug efflux systems encoded on its chromosome: two operons consisting of *acrR-acrA-acrB* (HI0893-HI0894-HI0895) and *emrA-emrB* (HI0898-HI0897) homologs are located almost adjacent to each other, and homologs of *bcr* (HI1242) and *tehA* (HI0511) are located elsewhere on the chromosome. There are also two OMF proteins encoded on the *H. influenzae* chromosome which may act in conjunction with the MFP proteins encoded by the *emrA* and *acrA* homologs. Thus, *H. influenzae* probably contains two MFS efflux proteins (one from the 12-TMS family and one from the 14-TMS family) and one RND/MFP efflux system but no 4-TMS SMR family member (other than the distantly related TehA homolog).

In contrast, the *M. genitalium* genome does not contain close homologs of known multidrug efflux systems (72). *M. genitalium* is thought to have the smallest genome of any self-replicating organism, thus providing a model for the minimal set of genes required for cell survival. The apparent lack of multidrug efflux systems in this organism suggests that although multidrug efflux systems provide selective advantages under some environmental conditions or for some organisms, they are not obligatory for cell survival.

PHYSIOLOGICAL ROLES OF PMF-DEPENDENT MULTIDRUG EFFLUX SYSTEMS

The prevalence of PMF-dependent multidrug systems in a diversity of organisms raises several questions. What is the normal physiological role of such multidrug export systems? Is their primary role to protect the cell by removing environmental toxins? Or do they play roles other than detoxification, such as the transport of a particular substrate? In which cases, are their abilities to transport multiple drugs only fortuitous?

It is not yet possible to provide definitive answers to these questions, and the physiological roles of most multidrug efflux systems remain uncertain. However, some insights into these issues can be gained by an examination of the genetic organization, regulation, and occurrence of the genes encoding the multidrug efflux proteins, in addition to biochemical characterization of the proteins themselves and examination of the physiologies of the organisms in question. Such indirect evidence suggests that the native cellular roles of some efflux systems are to defend the cell from exogenous toxic compounds; however, in other instances, multidrug efflux systems appear to fulfil primary functions unrelated to drug resistance and transport multiple drugs only fortuitously or opportunistically. Both "natural" and "opportunistic" multidrug efflux systems appear to have been recruited by cells to protect themselves from chemotherapeutic drug treatments in clinical situations.

The *Pseudomonas aeruginosa* multidrug resistance *mexAB/oprM* operon has been proposed to be involved in the secretion of the iron chelator molecule pyoverdine under conditions of iron starvation and is regulated by iron concentration and coregulated with other elements involved in pyoverdine secretion and uptake (224, 225) (see above). The mammalian multidrug transporter VMAT1 catalyzes the accumulation of neurotransmitter amine molecules in intracellular vesicles, enabling the cell to regulate the concentration of such biogenic amines (261, 264) (see above). In both of these cases, physiological evidence suggests that resistance to toxic inhibitors is not the primary role of these transporters but that, instead, they are involved in iron metabolism and neurotransmission, respectively. Other substrates of the *mexAB/oprM* efflux system do share some structural similarities with the catechol-containing chromophore of pyoverdine, suggesting that they may only be excreted fortuitously. Similarly, other substrates of VMAT1 share structural features with biogenic amines (261).

Further insight into a possible native physiological role for some tetracycline transport proteins is provided by the observations that TetL (and TetK) may also act in the cell as sodium/proton antiport systems (38a). This finding also emphasizes the liklihood that other hitherto considered single-substrate transporters may indeed recognize multiple substrates, some proving to be multidrug export proteins on further examination.

The *Streptomyces pristinaespiralis ptr* gene confers resistance to the structurally unrelated antibiotics pristinamycin I and II, which are synthesized by the organism, as well as to rifampin (24). It seems likely that the normal physiological role of the Ptr transporter is to transport endogenously produced pristinamycin I and II. It also makes physiological sense that an organism which produces multiple antibiotics may contain a multidrug efflux system capable of excreting such toxic secondary metabolites. This suggests that at least some multidrug efflux systems may have originated as excretion systems for secondary metabolites in antibiotic-producing organisms, as has been proposed on many occasions for resistance genes in general (48, 148).

The native physiological roles of the closely related *Bacillus subtilis bmr* and *blt* and the *Staphylococcus aureus norA* genes remains unclear. However, the *bmr* and *blt* genes have distinct operon organizations and are regulated independently, suggesting that they may perform separate physiological functions (4). This may indicate that their native roles are not related to the efflux of exogenous toxins.

The *E. coli* AcrAB efflux system confers resistance to bile salts and fatty acids, such as decanoate, and expression of *acrAB* is induced by decanoate and various stress conditions (166). Since the natural environment of enteric bacteria such as *E. coli* is rich in bile salts and fatty acids, these data support the hypothesis that the primary function of the AcrAB efflux system is protection against such natural hydrophobic inhibitors. Similarly, it has been proposed that the *Neisseria gonorrhoeae* MtrCDE efflux system, which is induced by and confers resistance to hydrophobic compounds, may serve to regulate the permeability of the *N. gonorrhoeae* cell envelope such that it can grow in the presence of toxic fecal lipids and bile salts in the rectum (95, 96, 203). Supporting this notion, increased expression of the *mtr* locus has been observed in various *N. gonorrhoeae* isolates from rectal infections (178, 269). Disruption of the *Candida albicans* multidrug resistance gene *CaMDR1* reduces the virulence of this fungal pathogen, suggesting a role in pathogenesis for this multidrug efflux system (18). These examples suggest that multidrug efflux systems may also play an important role in pathogens and other organisms by enabling them to survive in hostile environments, rich in toxic compounds.

The *E. coli emrAB* and *emrD* genes confer resistance to hydrophobic uncouplers, and expression of *emrD* gene is induced by a reduction in the PMF (182), whereas expression of *emrAB* is induced by various uncouplers (160). Lewis (149) has inferred that the primary physiological roles of these genes may be to protect *E. coli* from natural uncoupling compounds which dissipate the cellular PMF. Interestingly, the close genetic localization of the *acrRAB* and *emrAB* operons in *H. influenzae* (see above) suggests that they are involved in similar or common functions, supporting the hypothesis that both of these systems are involved in protecting the cell from toxic compounds in the environment.

The original physiological function of the staphylococcal *qacA*, *qacB*, and *smr* genes remains unclear. However, their widespread distribution in conjunction with various antibiotic resistance determinants on multiresistance plasmids in clinical isolates of *Staphylococcus aureus* (146, 156, 162, 273), the ability of antiseptics such as benzalkonium chloride to induce the expression of *qacA* and *qacB* (28), and the observation that the chronological emergence of these genes in clinical *S. aureus* isolates mirrors the introduction and usage of various organic cationic compounds such as clinical antiseptic and disinfectants (206) support the notion that the dissemination of these genes among pathogenic staphylococci has been due to the selective pressure imposed by the clinical use of agents such as acriflavine, benzalkonium chloride, chlorhexidine, and cetrimide as the active ingredients in antiseptic and disinfectant formulations. Similarly, the enterobacterial *qacE* and *qacE*Δ*1* determinants are encoded on potentially mobile elements, known as integrons, and are typically located in association with a variety of antibiotic resistance determinants in clinical isolates (208, 281). Thus, although the *qac* genes may once have played other physiological roles in their original host organism, they appear to have been acquired by clinical pathogens for the primary purpose of protection against hydrophobic organic antimicrobial agents.

OVERVIEW

The multidrug efflux systems which have been identified appear to have diverse origins and/or physiological functions. Some are involved in the excretion of exogenous or endogenous toxins, whereas others are involved in unrelated metabolic functions, such as iron metabolism. This is consistent with the notion that multidrug and specific or single-drug transporters can evolve from each other through either an increase or decrease in substrate specificity (149). In the case of the multidrug efflux proteins in the 12- and 14-TMS families in the MFS, this proposition is supported by phylogenetic analyses (Fig. 3 and 4) which reveal that multidrug efflux proteins are not more closely related to each other than they are to other, more specific transporters. However, in contrast, analyses of the SMR and RND families (Fig. 12 and 14) indicate that the multidrug efflux systems belong to distinct phylogenetic clusters consisting only of multidrug transporters or, in the case of the RND family, also including hypothetical proteins of unknown function, implying that the multidrug efflux systems in these families may have derived from a single ancestral multidrug transporter within the particular family.

Despite the apparent different native physiological functions of various multidrug efflux systems, both prokaryotic and eukaryotic cells appear to have recruited multidrug resistance proteins to overcome the effects of chemotherapeutic agents in clinical situations. A number of the proton-dependent multidrug efflux systems discussed in this review are clinically significant. For instance, azole resistance in clinical *Candida albicans* strains isolated from AIDS patients with oropharyngeal candidiasis is due to overexpression of the multidrug resistance *CaMDR1* gene (255). Resistance to fluoroquinolones in some clinical staphylococcal strains is partly due to overexpression of the multidrug resistance *norA* gene (126, 190, 324), and the staphylococcal *qac* genes confer resistance to a variety of antiseptic formulations. The *mexAB/oprM* efflux system appears to contribute significantly to the intrinsic drug resistance of *Pseudomonas aeruginosa*, a pathogen which is notoriously resistant to antimicrobial agents (222). Two well-known examples of clinically significant ATP-dependent multidrug efflux systems are human P-glycoprotein, which plays an important role in the development of resistance to chemotherapeutic agents used in the treatment of human cancers; and Pfmdr, a P-glycoprotein homolog which is amplified in chloroquine-resistant strains of *Plasmodium falciparum* (44, 71).

The emergence of strains of pathogens, such as *P. falciparum*, *Mycobacterium tuberculosis*, and *Staphylococcus aureus*, which are resistant to a wide range of chemotherapeutic agents, poses an increasingly significant hazard to human health because these strains are often recalcitrant to standard treatment regimens (48, 196). The evolution of such multiresistant strains has almost certainly been due to selective pressures imposed by antimicrobial chemotherapy. Pathogens have developed resistance by both undergoing chromosomal mutations and acquiring plasmid- and/or transposon-encoded resistance-conferring determinants (for a review, see reference 48). These drug-resistant pathogens utilize a range of mechanisms, including drug detoxification, target site alteration, bypass mechanisms, and single drug efflux (47, 116, 207).

The ability of pathogenic organisms to enlist either transport systems involved in the efflux of environmental toxins or other transport systems involved in unrelated metabolic operations as multidrug efflux systems capable of mediating resistance to a wide range of chemotherapeutic agents is a further disturbing development. This adaptability to multidrug resistance presents a challenge both to molecular biologists and to the pharmaceutical industry to understand the basis of multidrug efflux mechanisms and to design and develop new chemotherapeutic agents that are able to elude such systems.

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