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MINIREVIEW

Multidrug Efflux Pumps of Gram-Negative Bacteria

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INTRODUCTION

Gram-negative bacteria tend to be more resistant to lipophilic and amphiphilic inhibitors than gram-positive bacteria. Such inhibitors include dyes, detergents, free fatty acids, antibiotics, and other chemotherapeutic agents. This property is used in the selective enrichment of gram-negative bacteria, especially of members of the family *Enterobacteriaceae*, for example with MacConkey agar (containing crystal violet and bile salts), EMB agar (containing dyes), and deoxycholate agar (containing sodium deoxycholate). The fact that *Escherichia coli* K-12 can grow in the presence of 1% sodium dodecyl sulfate has been rediscovered many times. Many lipophilic antibiotics, such as penicillin G, erythromycin, fusidic acid, and rifamycin SV, are much less active against most gram-negative bacteria. In fact, a survey of recently reported antibiotics of natural origin showed that, among those compounds that showed activity against gram-positive bacteria, more than 90% lacked activity at a useful level against *E. coli* (51).

This intrinsic resistance of gram-negative bacteria has often been attributed entirely to the presence of the outer membrane barrier. This barrier does contribute to the resistance, as the narrow porin channels slow down the penetration of even small hydrophilic solutes, and the low fluidity of the lipopolysaccharide leaflet decreases the rate of transmembrane diffusion of lipophilic solutes (38, 40). However, the outer membrane barrier cannot be the whole explanation, even with species such as *Pseudomonas aeruginosa* which produces an outer membrane of exceptionally low permeability (1, 57). This is seen from the fact that equilibration across the outer membrane is achieved very rapidly, in part because the surface-to-volume ratio is very large in a small bacterial cell. Thus, the periplasmic concentrations of many antibiotics are expected to reach 50% of their external concentrations in 10 to 30 s in *P. aeruginosa* and in a much shorter time period in *E. coli* (34). Additional mechanisms are therefore needed to explain the level of intrinsic resistance. With the earlier β-lactam compounds, this second contributing factor is the hydrolysis by the periplasmic β-lactamases that are encoded by chromosomal genes in many gram-negative bacteria, and the levels of resistance can be explained quantitatively, in many cases, by the synergy between the outer membrane barrier and β-lactamase (37). However, with dyes, detergents, other classes of antibiotics, and even the β-lactams developed more recently that are not hydrolyzed easily by common β-lactamases, the second factor was completely unknown (34). Recent studies showed that multiple-drug efflux pumps, many with unusually broad specificities, play a major role in the intrinsic resistance of gram-negative bacteria.

Active efflux processes have been known to cause drug resistance in gram-negative bacteria, through the pioneering studies of S. Levy (19) on Tet-mediated tetracycline resistance. Each of these traditional efflux pumps excretes only one drug or one class of drugs (35). In contrast, a multidrug efflux pump can pump out a wide range of compounds, and it is often difficult to discern any common structural features among the substrates (20, 35).

EFFLUX ACROSS A SINGLE MEMBRANE LAYER

Classical efflux pumps such as Tet, as well as all gram-positive bacterial multidrug efflux pumps so far identified, such as QacA, NorA (Bmr), and Smr (see references 20 and 35), pump out solute molecules across a single cytoplasmic membrane layer (Fig. 1A and B). In gram-negative bacteria, pumps of this type will obviously move agents from the cytoplasm into the periplasmic space. This is an inefficient operation, because lipophilic inhibitor molecules are expected to diffuse back spontaneously into the cytoplasm through the lipid bilayer domains of the cytoplasmic membrane. The Tet pump has indeed been shown to pump out tetracycline into the periplasm of *E. coli* (50). It can create high levels of resistance only because it has such a high V_{max} that it can outcompete the spontaneous influx process.

Pumps of this type need to have a high throughput even in a gram-positive cell, as the spontaneous influx of the drug would also be rapid. It is possible that an extremely wide substrate specificity is not compatible with this essential requirement for a high turnover rate. This may be the reason why the substrate range of gram-positive bacterial multidrug efflux pumps appears to be somewhat limited. Thus, NorA (Bmr) pumps out basic dyes, puromycin, fluoroquinolones, and chloramphenicol, all of which are lipophilic cations except chloramphenicol. QacA and Smr both export quaternary ammonium compounds and basic dyes, again cationic lipophiles.

A few of the gram-negative multidrug efflux pumps appear to catalyze excretion across the cytoplasmic membrane (Fig. 1B). Examples are those of the Smr family, which are unusually small proteins that are predicted to span the membrane only four times. The *emrE* (*mvrC*) gene, whose disruption causes hypersusceptibility to tetraphenylphosphonium, methylviologen, and ethidium bromide, is a member of this family found in *E. coli* (see reference 20). The overproduction of EmrE protein makes *E. coli* slightly more resistant to tetracycline, erythromycin, and sulfadiazine (56).

EFFLUX ACROSS A DOUBLE-MEMBRANE SYSTEM

The majority of gram-negative bacterial multidrug efflux pumps are entirely different in their construction in that they traverse both the cytoplasmic (inner) and outer membranes by utilizing three protein components (Fig. 1D). These systems include the AcrAB system of *E. coli* and the MexAB-OprM system (earlier described as MexAB-OprK [42]) of *P. aeruginosa*. The complex of this type was first suggested by Wandersman and coworkers (52, 53) for the assemblies that secrete,

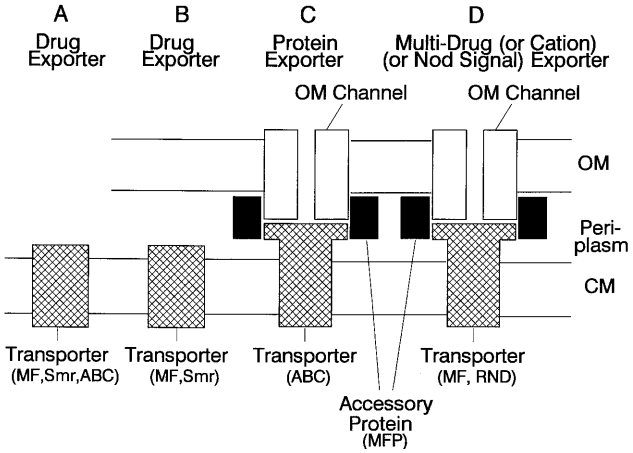


FIG. 1. Schematic representations of various types of exporters. (A) A single-component transporter commonly catalyzes the efflux of drugs across the cytoplasmic membrane (CM) in gram-positive bacteria. The pump may catalyze the efflux of drugs of one kind (as in Tet) or of diverse compounds (as in NorA [Bmr], QacA, and Smr). It may be energized by proton motive force (in MF- and Smr-type pumps) or by ATP hydrolysis (in ABC transporters). (B) A single-component transporter in gram-negative bacteria. Pumps of this type will excrete the drugs into the periplasm. They belong to either the MF (e.g., Tet protein) or Smr (e.g., MvrC) family. The latter are multidrug efflux pumps. (C) Protein exporters in gram-negative bacteria. These exporters consist of ABC transporters, outer membrane (OM) channels, and periplasmic linker proteins of a special class (MFP family) that connect these two classes of proteins. (D) Multidrug, multication, or nodulation signal efflux complexes of gram-negative bacteria. The construction of these complexes are similar to that of the protein exporters shown in panel C, except that the transporter belongs to either the MF or RND family and is energized by proton motive force. They pump out a wide range of drugs, several divalent cations (as in Cnr and Czc systems), or presumably nodulation signal lipooligosaccharides.

into the medium, hemolysin of *E. coli* (HlyB-HlyD-TolC), proteases B and C of *Erwinia chrysanthemum* (PrtD-PrtE-PrtF), and cyclolysin of *Bordetella pertussis* (CyaB-CyaD-CyaE) (Fig. 1C). In all these systems, the transporter protein located in the cytoplasmic membrane is thought to be brought into apposition with an outer membrane channel (such as TolC, PrtF, and CyaE) through the linker protein such as HlyD, PrtE, and CyaD, which were shown to make up the named MFP (membrane fusion protein) family (6). Members of this family have some homology with a paramyxovirus membrane fusion protein, a finding that reinforces the hypothesis that these proteins link or fuse the two membranes, the outer and inner membranes (Fig. 1C). Transporters in these systems (such as HlyB, PrtD, and CyaB) are all members of ABC (ATP-binding cassette) family, in contrast to the transporters of multidrug efflux systems, which are energized by proton motive force (see below).

Multidrug efflux pumps of the type found exclusively in gram-negative bacteria appear to share the same construction (Fig. 1D). First, the *mexA-mexB-oprM* genes, each coding for a component of this putative assembly, form an operon (42). Second, there is also biochemical evidence. Thus, the efflux of tetracycline from wild-type *E. coli*, presumably catalyzed by a pump of this type, AcrAB (and TolC) complex (26, 39), appears to bypass the outer membrane barrier (50). There is little accumulation of tetracycline in the periplasm of such cells, in contrast to the large accumulation seen in cells expressing the Tet protein. Furthermore, decreases in the rate of entry through the outer membrane, for example, by the mutational loss of porins, produce increases in tetracycline MIC. This is the result expected when the susceptibility is determined by the balance between the influx through the outer membrane barrier and efflux bypassing this barrier. In contrast, in cells expressing the Tet protein, decreases in outer membrane permeability had no effect on MIC, as the cytoplasmic concentration of tetracycline is determined solely by competition between efflux and influx across the cytoplasmic membrane.

The consideration described above suggests that the outer membrane barriers and the multicomponent efflux systems should act synergistically to lower the cytoplasmic and even the periplasmic (see below) concentrations of harmful agents. Organisms with a low-permeability outer membrane, such as *P. aeruginosa*, thus become intrinsically resistant to many antibiotics precisely because the direct efflux can easily outpace the slow influx of inhibitors across the outer membrane. This hypothesis is indeed borne out by the observation that *P. aeruginosa* mutants with deficient MexAB-OprM pumps are as susceptible as or even more susceptible than *E. coli* to a number of antibiotics (23). In contrast, although *E. coli* produces a MexAB homolog, AcrAB, the pump can produce intrinsic resistance only to large, lipophilic agents that have difficulty penetrating the efficient porin channels, such as erythromycin, fusidic acid, dyes, and detergents, and the organism remains susceptible to small antibiotics that are likely to diffuse rapidly through the channel, such as tetracycline, chloramphenicol, and fluoroquinolones. This efficient synergy also means that the multicomponent multidrug efflux systems can produce significant levels of resistance even when the V_{max}s of their transporters are very low, as long as the outer membrane functions as an effective barrier. Mathematical modeling suggests that a level of activity about 2 orders of magnitude lower than that of the Tet pump suffices to produce the same level of elevation of tetracycline MIC (50).

MULTICOMPONENT, MULTIDRUG EFFLUX SYSTEMS OF DIFFERENT CLASSES

Systems containing transporters of the resistance-nodulation-division (RND) family. The intrinsic resistance of *P. aeruginosa* to a wide variety of antibiotics cannot be explained solely by the low permeability of its outer membrane, as described above. Furthermore, the level of intrinsic resistance can be elevated to a much higher level. Thus, almost 80% of the carbenicillin-resistant clinical isolates from the British isles were of this type (54). It was difficult to explain this higher resistance phenotype, because there was no elevation of the periplasmic β-lactamase level and because they were more resistant not only to β-lactams but also to other agents, such as tetracycline, chloramphenicol, and fluoroquinolones.

The easiest explanation was the decreased permeability of the outer membrane. However, attempts to show this have not been successful. Decreased outer membrane permeability is expected to produce a slower accumulation of drug, which nevertheless will eventually reach the equilibrium concentration (Fig. 2A). Examination of drug accumulation in contrast revealed that the isolates with elevated resistance had lower steady-state accumulation levels of tetracycline, chloramphenicol, norfloxacin, and benzylpenicillin than the more-susceptible isolates and that the drug levels in the former strains increased upon the collapse of the proton gradient across the cytoplasmic membrane through the addition of an uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (21, 22). This was precisely the results expected for strains with increased levels of active drug efflux (Fig. 2B). Furthermore, an antibiotic-hypersusceptible mutant strain, whose phenotype had been believed to be due to a defective outer membrane, turned out to be deficient in active efflux of most drugs and to accumulate the drugs to the highest levels, which were approx-

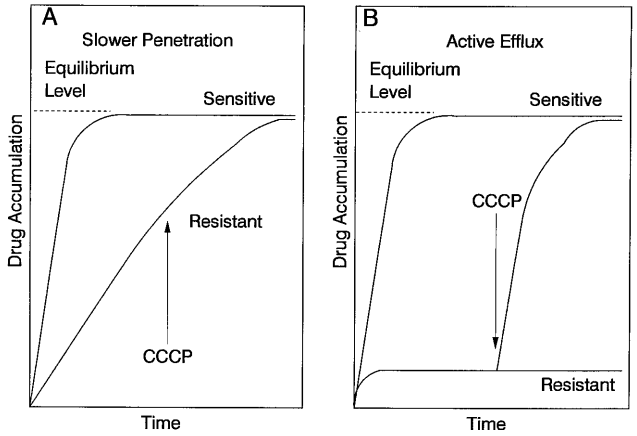


FIG. 2. Expected time course of accumulation of drugs in a pair of resistant and susceptible strains. (A) If the resistance were due entirely to the decreased outer membrane permeability, the accumulation would be slower in the resistant strain but would eventually reach the same steady state (which is in equilibrium with the external medium). (B) If the resistance were due entirely to the increased active efflux of the drug, the drug accumulation would reach a steady state at a much lower level in the resistant strain. Furthermore, deenergization of the pump by a proton conductor such as CCCP would rapidly increase the accumulation level to that of the susceptible strain.

imately equal to the levels expected for equilibration with external drug concentrations and which were not affected by the addition of CCCP (21, 22). These results suggested that various drugs are actively pumped out even by the wild-type strains of *P. aeruginosa*, an activity that occurs at an elevated level in carbenicillin-resistant isolates. It was further shown that the multidrug resistance phenotype, reminiscent of that of *nalB* mutants (43), could be produced by a single mutation of presumably regulatory nature (21).

At this point, independent work by K. Poole's group (42) showed the existence of an operon, *mexAB-oprM*, which coded for an MFP linker protein (MexA), a large transporter of about 112 kDa (MexB), and a putative outer membrane channel (OprM), and showed that disruption of these genes caused hypersusceptibility to several agents. This is the first multidrug efflux operon described containing genes for all three of the components expected for the system shown in Fig. 1D. The putative transporter, MexB, is predicted to be a proton antiporter containing 12 transmembrane α-helices, but unlike many other transporters of this class, it contains large periplasmic domains between helices 1 and 2 and helices 7 and 8. Such a sequence is characteristic of transporters already known or suspected to export heavy metal cations in *Alcaligenes* species (CzcA and CnrA) as well as nodulation signals in *Rhizobium* species (NolGHI), and these proteins were shown to compose a homologous family, the RND family (45). MexA is a member of the MFP family that usually occurs together with cytoplasmic exporter proteins and outer membrane channels, as described above. OprM (originally misidentified as OprK [42]) (12) shows homology with outer membrane channels of the export systems of a similar construction, such as NodT, PrtF, and CyaE, that are thought to function in the secretion of nodulation signals, alkaline proteases, and cyclolysin, respectively, in various gram-negative bacteria (42). The MexAB-OprM system appears to pump out a very wide range of compounds and to be overexpressed in the carbenicillin-resistant clinical isolates of *P. aeruginosa*. Thus, the multidrug-resistant phenotype of such isolates can now be explained as a consequence of the overexpression of this system, caused by *nalB* mutations (23).

P. aeruginosa produces additional homologs of MexAB-OprM. Thus, mutations in the regulatory gene *nfxB* (17) induce overexpression of three proteins, MexC, MexD, and OprJ, which are similar to MexA, MexB, and OprM both in size and in subcellular location (21, 30). MexCD-OprJ also appears to pump out a wide variety of antibiotics, but there is a significant difference in the specificities of the two systems (Table 1). Another potential homolog of MexAB-OprM is listed in Table 1 as MexEF-OprN. So far, only the outer membrane component of the system, OprN, which is similar to OprM and OprJ in size, has been identified (30). OprN is overproduced in *nfxC* mutants (10), which have the multidrug resistance phenotype. Interestingly they are the only mutants that appear to pump out carbapenems, such as imipenem (Table 1).

The *acr* mutation makes *E. coli* K-12 hypersusceptible to dyes, detergents, and lipophilic antibiotics. Until recently it has been thought to cause this phenotype by damaging the outer membrane barrier, yet no reproducible alteration has been found in the outer membrane of *acr* mutants (38, 51). In 1993,

TABLE 1. Multidrug efflux systems in gram-negative bacteria

Transporter	Linker	Outer membrane channel	Organism	Substrates[a]	Reference(s)
Smr-type					
EmrE			*E. coli*	Lipophilic cations (TPP, EB, methylviologen)	20
MF-type					
EmrB	EmrA	TolC	*E. coli*	CCCP, nalidixic acid, thiolactomycin	24
RND-type					
AcrB	AcrA	TolC	*E. coli*	TC, CP, FQ, β-lactams, Nov, EM, FuA, Rif, EB, AF, CV, SDS, DOC	25, 26
MexB	MexA	OprM	*P. aeruginosa*	TC, CP, FQ, β-lactams (except carbapenems), Nov, EM, FuA, Rif	21–23, 42
MexD	MexC	OprJ	*P. aeruginosa*	TC, CP, FQ, "fourth-generation" cephems[b] (but not conventional β-lactams or carbapenems)	29, 30
MexF	MexE	OprN	*P. aeruginosa*	CP, FQ, carbapenems (but not conventional β-lactams or fourth-generation cephems)	30
MtrD	MtrC	MtrE	*N. gonorrhoeae*	TC, CP, β-lactams, EM, FuA,Rif,EB,AF,CV,TX,DOC	14, 47

[a] Abbreviations: TPP, tetraphenylphosphonium; TC, tetracycline; CP, chloramphenicol; FQ, fluoroquinolones; Nov, novobiocin; EM, erythromycin; FuA, fusidic acid; Rif, rifampin; EB, ethidium bromide; AF, acriflavin; CV, crystal violet; SDS, sodium dodecyl sulfate; DOC, deoxycholate; TX, Tween X-100.

[b] Fourth-generation cephems are cephalosporins that contain aminothiazole-oxime at position 7 and a positively charged substituent at position 3 (see Fig. 2 of reference 29).

two laboratories independently cloned and sequenced the relevant gene and discovered that two genes, *acrA* and *acrB*, form an operon (26, 55). AcrA is a periplasmic protein that was classified later as a member of the MFP family, and AcrB is a 12-transmembrane α-helix transporter that belongs to the RND family. AcrAB are close homologs of MexAB of *P. aeruginosa*. The *acrAB* operon does not contain a gene coding for an outer membrane channel. However, the AcrAB system appears to catalyze direct efflux of tetracycline into the medium in the wild-type K-12 strain (50), which suggests that AcrAB is associated with an outer membrane channel. TolC appears to be the most likely candidate for this channel, as it is already known to serve as channels for more than one protein export system (7, 52), and as *tolC* mutants show a hypersusceptibility phenotype very similar to that of *acrA* or *acrB* mutants (31). In fact, the addition of the *tolC* mutation to the Δ*acrAB* mutation did not increase susceptibility further, suggesting that these genes function in the same pathway (9).

Ma et al. (26) showed that the dye acriflavine was maintained at a much lower steady-state concentration in the wild-type cells than in an *acrA* mutant, a finding that led to the conclusion that AcrAB is a multidrug efflux pump and solved the long-standing mystery of the hypersensitivity of *acr* mutants. Since then, however, we found that essentially the same observation was made 30 years ago by Nakamura (33); obviously at that time it was impossible to interpret the data correctly. Judged on the basis of the hypersusceptibility phenotype of *acrAB* mutants, AcrAB system pumps out an extraordinary wide variety of antibiotics, chemotherapeutic agents, detergents, and dyes (Table 1).

In the sequenced parts of the *E. coli* genome, there are two homologs of *acrAB*, *acrEF* (formerly *envCD*), and *yhiUV* (earlier called OrfAB), as well as an *acrB* homolog, *acrD*, which is not associated with any gene for an MFP (28). A mutant of the *envCD* locus was so named because of its defect in cell division, and this contributed to the name of the RND family. However, the newly constructed null mutations of *acrEF* show no change in the cell division process (28). Although *acrEF* does not appear to be expressed at significant levels in the wild-type K-12 strain, its overexpression can suppress the hypersusceptible phenotype of *acrAB* mutants (55), and thus AcrEF is also a multidrug efflux pump with a substrate range similar to that of AcrAB.

Homologs of *acrAB* and *mexAB-oprM* are apparently widespread among gram-negative bacteria and were found in *Neisseria gonorrhoeae* (MtrCDE [Table 1]) (14) as well as in the completely sequenced genome of *Haemophilus influenzae* (8). A gene coding for an outer membrane protein homologous to OprM has been identified in *Burkholderia cepacia* (3).

Systems containing transporters of the MF family. A gram-negative multidrug pump was described by Lomovskaya and Lewis in 1992 (24). These workers cloned an operon, *emrAB*, from the *E. coli* K-12 genome into a multicopy plasmid and found that the presence of plasmid produced a higher level of resistance to such uncouplers as CCCP, 2-chlorophenylhydrazine, and tetrachlorosalicylanilide, as well as to nalidixic acid and thiolactomycin (Table 1). The putative transporter gene of the operon is *emrB*, which codes for a protein with putative 12 transmembrane helices. However, EmrB lacks the large periplasmic domains found in RND transporters and in fact belongs to the major facilitator (MF) family that also uses proton motive force as energy. EmrA is a periplasmic protein of the MFP family.

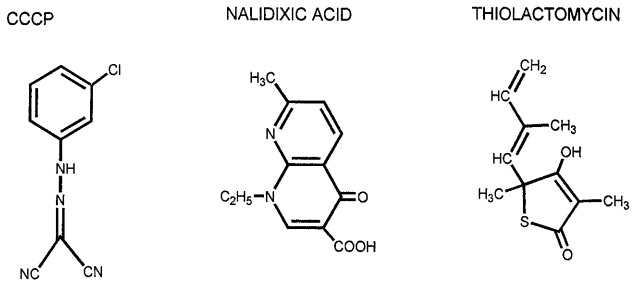


FIG. 3. Some representative substrates of the EmrAB efflux system of *E. coli*. All of these agents can also exist in charged (deprotonated) forms, but it is believed that under physiological conditions a substantial fraction of the population exists in uncharged forms shown.

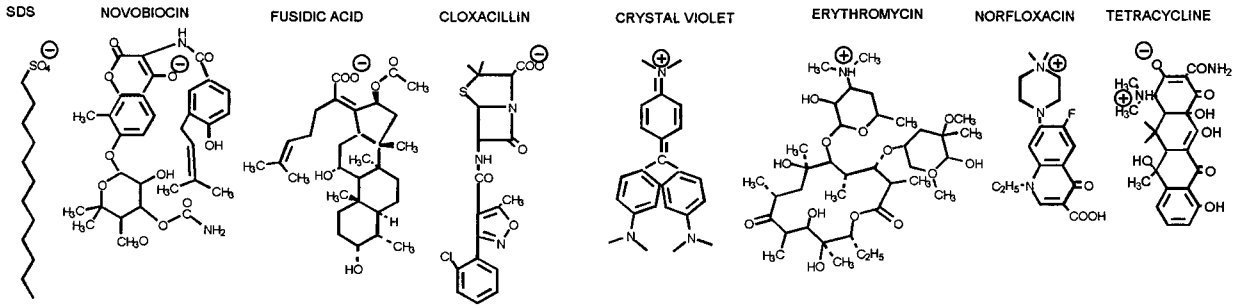
WIDE SPECIFICITY OF MULTICOMPONENT, MULTIDRUG EFFLUX SYSTEMS

Various toxic compounds are pumped out by multicomponent efflux machineries containing RND and MF transporters (Table 1). The range is often so wide that it is difficult to see the common structural features among the substrates. However, with some systems, working hypotheses can at least be proposed. For example, all of the known substrates of the EmrAB pump appear to be lipophilic compounds that exist largely in uncharged form at physiological pH values (Fig. 3).

The substrates of RND-based systems appear to have structures that cover a wider range (Table 1). With AcrAB and MexAB, many of the substrates carry net negative charge, but there are also compounds with positive charges, such as the cationic dyes and erythromycin. Perhaps the common characteristics among the substrates can be glimpsed by examining compounds that are not exported by these systems. Among antibiotics, there is no evidence for the efflux of aminoglycosides (21, 26). These are very hydrophilic compounds, whereas the substrates of AcrAB and MexAB seem to contain hydrophobic domains of significant sizes (Fig. 4).

Another surprising feature of these pumps is that they apparently catalyze the efflux of agents that are unlikely to cross the plasma membrane and to enter the cytoplasm. For β-lactam compounds containing additional charged groups, such as carbenicillin and ceftriaxone, we have experimentally shown that they do not traverse the cytoplasmic membrane within the time scale of our experiment (22). Yet the genetic inactivation of the MexAB-OprM pump makes *P. aeruginosa* hypersusceptible to these compounds, and overexpression of this pump often results in an impressive degree of resistance to them: for example, the MICs of carbenicillin are 0.25 μg/ml or less for *mexA* or *oprM* mutants, 32 μg/ml for the wild-type strain, and 1,024 μg/ml for an overexpression strain (23). This suggested strongly that the MexAB pump is capable of pumping out these compounds from the periplasm.

We have been able to gain some more insight into the nature of AcrAB substrates by comparison of β-lactam MICs for the wild type and an *acr* mutant (originally called SS-B [48]) of *Salmonella typhimurium*, a species that lacks *ampC*, the structural gene for the chromosomally coded β-lactamase (2). There was a good positive correlation between the hydrophobicity of the substituent at position 6 of penicillins (or position 7 for cephalosporins) and their ability to become substrates of this pump (36). These data seem to suggest that the only requirement for AcrAB and MexAB pumps is that the hydrophobic domain of the substrate molecule become inserted into the lipid bilayer of the membrane (Fig. 4); this idea will be expanded in the following section.



	SDS	NOVOBIOCIN	FUSIDIC ACID	CLOXACILLIN	CRYSTAL VIOLET	ERYTHROMYCIN	NORFLOXACIN	TETRACYCLINE
AcrAB	+	+	+	+	+	+	+	+
MexABOprM	?	+	+	+	?	+	+	+
MexCD-OprK	?	-	-	-	?	+	+	+

FIG. 4. Representative substrates of RND-containing multidrug pumps, AcrAB (TolC), MexAB-OprM, and MexCD-OprJ. The structures and charges of the substrates (top) and whether the substrates are used by the pumps (bottom) (+, used; ?, not known; −, not used) are shown. As seen, all these substrates are amphiphilic molecules that are expected to become inserted partially into the phospholipid bilayer, with head groups containing any charges (for AcrAB and MexAB-OprK) or at least one positive charge (for MexCD-OprJ). An exception to this rule is chloramphenicol, which is uncharged. However, this compound appears to be pumped out only inefficiently either by MexAB-OprM or MexCD-OprJ (23, 29). Whether a compound serves as a substrate was deduced by the examination of changes in MICs for overexpression mutants as well as in null mutants (23, 29, 36).

What is the difference between the MexCD-OprJ system and the MexAB-OprM system? The former does not pump out conventional cephalosporins but pumps out cephalosporins with quaternary-nitrogen-containing substituents at position 3 (29, 30). Possibly MexCD-OprJ requires that its substrate contains at least one positively charged group at its hydrophilic end, but there is no such requirement for MexAB-OprM. Indeed, MexCD-OprJ seems to excrete compounds with only one positive charges, such as erythromycin and rifampin (29), but not compounds with only a negative charge at its head group, such as novobiocin and conventional β-lactam compounds (29) (Fig. 4).

POSSIBLE MECHANISM AND ORIGIN OF RND-CONTAINING MULTIDRUG PUMPS

Two intriguing features of the RND-containing pumps are their ability to handle a very wide range of compounds and the efflux of agents that apparently cannot enter the cytoplasm. Mammalian multidrug efflux pumps (MDR) also excrete many compounds of diverse structure (13) and appear to capture their substrates while they are still associated with the bilayer of the plasma membrane, that is, before they traverse the membrane to enter the cytoplasm (18). MDRs thus show properties reminiscent of RND-containing systems, which could suggest possible mechanisms for the latter, although MDRs are coupled to ATP hydrolysis in contrast to the RND systems that are energized by proton motive force. Interestingly, the product of the mouse *mdr2* gene, a homolog of multidrug efflux protein gene *mdr1*, is a phospholipid flippase, which translocates phospholipid from the inner to the outer leaflet of the bilayer (44). The MDR2 protein must therefore capture phospholipid molecules from within a bilayer leaflet, as predicted earlier (16). It is possible then that a homolog of MDR2, MDR1, also captures its substrates from within a leaflet. If RND transporters function in a similar way, they would capture various amphiphilic, nonphospholipid molecules inserted into a leaflet of the cytoplasmic membrane. This hypothesis explains both the broad specificity and the efflux of membrane-impermeable compounds (Fig. 5). Furthermore, the observation that AcrAB pumps out only those β-lactams with lipophilic substituents, presumably capable of partial insertion into the bilayer, is consistent with this hypothesis. Other systems may bind substrates preferentially in the interior of the bilayer or in the inner leaflet, and this may explain their different substrate ranges. For example, the EmrAB system may prefer uncharged substrates buried in the interior of the bilayer.

Is the physiological function of multidrug efflux pumps to exclude harmful agents that exist in the environment or to pump out endogenous metabolites? The MexAB-OprM system was initially hypothesized as an export machinery for a siderophore, pyoverdine (42), but this is unlikely as its expres-

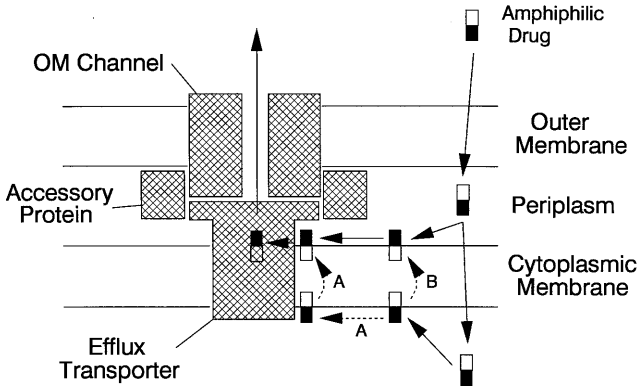


FIG. 5. A hypothetical mechanism for AcrAB, MexAB-OprM, and MexCD-OprJ pumps. It is proposed that the substrates are captured from within the bilayer. Available results suggest the existence of drug-capturing mechanism from the outer leaflet (see text). For drug molecules in the cytoplasm, two possible pathways of capture seem possible. In one pathway (A), the transporter captures the substrate from the inner leaflet and perhaps acts also as a flippase. In another pathway (B), the capture is limited to the substrates in the outer leaflet and only the molecules that have reached that location through spontaneous flipping across the bilayer will be excreted. No evidence is yet available to discriminate between these pathways (shown by broken arrows).

sion is not regulated by iron deficiency (41). However, a multicomponent export machinery containing an RND transporter, NolGHI, is thought to be involved in the export of nodulation signal lipooligosaccharides (46), which have an amphiphilic structure similar to the substrates of the AcrAB and MexAB systems shown in Fig. 4. It will be interesting to see whether the NolGHI system can also export other compounds.

ACCUMULATION OF FLUORESCENT PROBES

It has been known that deenergized cells of gram-negative bacteria are labeled much more efficiently by fluorescent probes such as *N*-phenylnaphthylamine (NPN) (5, 49). These effects were explained by assuming that the energization state of the cytoplasmic membrane somehow affects the permeability of the outer membrane (38), obviously a rather unlikely explanation. Now that we know that most lipophilic molecules are actively extruded out of these cells, it seems likely that these phenomena were simply due to the deenergization of the endogenous efflux pumps. Indeed, *acrAB* mutants of *E. coli* accumulate far higher levels of NPN than the parent strain (36).

REGULATION OF MULTIDRUG EFFLUX PUMPS

The wide specificities of multicomponent, multidrug efflux systems suggest that their expression may be carefully controlled, because their runaway overexpression might result in the efflux of essential metabolites. In terms of physiological regulation, the AcrAB system has been studied in the most detail (27). First, global regulatory systems such as *marRAB* and *soxRS* affect the antibiotic resistance of *E. coli*. Positive regulators MarA and SoxS are produced at a higher level in response to the presence of antibiotics and superoxide radicals, respectively, and these regulators are known to increase the efflux of several drugs (4, 11), mediated by AcrAB (39). In addition to the increased transcription of *acrAB* operon, MarA (and SoxS) down-regulates the synthesis of the major porin OmpF, through the increased production of an antisense RNA, *micF* (4). The decreased outer membrane permeability thus synergistically enhances the effect of an increased production of the efflux machinery. Second, other global stress conditions, such as the addition of 0.5 M NaCl or 4% ethanol, or entrance into the stationary phase, also increases the transcription of the *acrAB* operon. This effect is not mediated by *marRAB* or *soxRS*. Interestingly, under these conditions the transcription of the repressor gene, *acrR*, is also increased, a mechanism that may prevent the uncontrolled overexpression of the AcrAB pump (25).

Studying global regulatory systems produced another hint on a still unknown, possible multidrug efflux system. Thus, the overexpression of RobA regulator, another homolog of MarA and SoxS, was shown to make *E. coli* more resistant to kanamycin and phosphonomycin in addition to chloramphenicol and tetracycline (32). This is interesting because both kanamycin and phosphonomycin are very hydrophilic drugs that are not pumped out by any of the multidrug efflux systems so far studied. Furthermore, RobA overproduction (as well as that of SoxS) increased the resistance of *E. coli* to Ag^{+} and Hg^{2+}. These data are consistent with the presence of a multidrug, multication, efflux pump that excretes hydrophilic cations, although we cannot exclude the possibility that these solutes are pumped out by several different pumps.

Little is known about the physiological regulation of expression of multidrug pumps in other species. However, mutants overproducing these pumps are frequently isolated (see above). In *P. aeruginosa*, the mutation *nalB*, which causes the overexpression of the MexAB-OprM system, has recently been located in *mexR*, the local repressor gene for the system (41). *N. gonorrhoeae* mutants with increased expression of the MtrCDE multidrug efflux pump have short deletions in the promoter sequences of these genes (15).

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