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The Challenge of Efflux-Mediated Antibiotic Resistance in Gram-Negative Bacteria

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SUMMARY

The global emergence of multidrug-resistant Gram-negative bacteria is a growing threat to antibiotic therapy. The chromosomally encoded drug efflux mechanisms that are ubiquitous in these bacteria greatly contribute to antibiotic resistance and present a major challenge for antibiotic development. Multidrug pumps, particularly those represented by the clinically relevant AcrAB-TolC and Mex pumps of the resistance-nodulation-division (RND) superfamily, not only mediate intrinsic and acquired multidrug resistance (MDR) but also are involved in other functions, including the bacterial stress response and pathogenicity. Additionally, efflux pumps interact synergistically with other resistance mechanisms (e.g., with the outer membrane permeability barrier) to increase resistance levels. Since the discovery of RND pumps in the early 1990s, remarkable scientific and technological advances have allowed for an in-depth understanding of the structural and biochemical basis, substrate profiles, molecular regulation, and inhibi-

tion of MDR pumps. However, the development of clinically useful efflux pump inhibitors and/or new antibiotics that can bypass pump effects continues to be a challenge. Plasmid-borne efflux pump genes (including those for RND pumps) have increasingly been identified. This article highlights the recent progress obtained for organisms of clinical significance, together with methodological considerations for the characterization of MDR pumps.

INTRODUCTION

Antibiotic resistance has emerged as a major threat to public health in this century, as evident from global surveillance data (1). Indeed, with the ancient origin and widespread presence of diverse resistance genes (2, 3), the modern evolution of resistance has led to the global emergence and spread of a large number of resistant bacteria that possess sophisticated genotypes and phenotypes against antibiotics. This phenomenon is a consequence of the natural selection process in microorganisms and promotion by human activities over the past 70 years of the antibiotic era (4, 5). In 2013, the U.S. Centers for Disease Control and Prevention (6) listed current resistance threats, of which multidrug-resistant Gram-negative bacteria constitute a large proportion (e.g., *Enterobacteriaceae*, *Acinetobacter*, and *Pseudomonas*). Of the various molecular and biochemical mechanisms of resistance to antibiotics, active efflux of antibiotics in bacteria plays an important role in both intrinsic and acquired multidrug resistance (MDR) of clinical relevance. It also interplays with other resistance mechanisms, such as the membrane permeability barrier, enzymatic inactivation/modification of drugs, and/or antibiotic target changes/protection, in significantly increasing the levels and profiles of resistance.

Energy-dependent drug efflux was discovered in the 1970s, initially with P-glycoprotein in mammalian cells (7) and later with Tet proteins in *Escherichia coli* isolates resistant to the specific antibiotic class tetracyclines (8). The subsequent discovery in the early 1990s of MDR pumps in *E. coli* and *Pseudomonas aeruginosa*, represented by the resistance-nodulation-division (RND) superfamily exporters (9–13), has made an important contribution to our understanding of resistance mechanisms (14). Since then, with rapid technological advances in biochemistry and molecular biology, there have been ever-growing identification and characterization of MDR pumps in numerous bacterial species of public health concern (e.g., in the ESKAPE [*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* species] pathogens), which compellingly demonstrate their predominant role in clinical settings (15, 16). Meanwhile, efforts of scientists led to the understanding of not only the structural and functional basis of these drug transporters but also their regulation and inhibition. In this review, we aim to provide a comprehensive and up-to-date description of efflux-mediated antibiotic resistance in Gram-negative bacteria.

BIOCHEMISTRY AND GENETICS OF MULTIDRUG EFFLUX PUMPS

Classes of Efflux Pumps

Because there are so many different efflux transporters, the only feasible way for their classification is to use phylogenetic grouping, based on protein sequences. Such a classification for all transporter proteins has been established by Milton Saier's group (17–19) and is available in the Transporter Classification Database (http://www.tcdb.org/). Transporter genes in hundreds of sequenced bacterial genomes are classified in Ian Paulsen's database (20) for each of these genomes (http://www.membranetransport.org/). Among many families of transporters, several contain prominent members of efflux transporters: especially important in bacteria are the RND, MFS (major facilitator superfamily), MATE (multidrug and toxic compound extrusion), SMR (small multidrug resistance), and ABC (ATP-binding cassette) superfamilies or families. ABC transporters utilize ATP hydrolysis as the energy source, but all others are dependent on proton motive force and are thus secondary transporters or proton/drug antiporters.

The transporters also differ in their subcellular organization. The RND pumps, which are all exporters of drugs and toxic cations, are located in the inner membrane (IM) (cytoplasmic membrane) but must interact with the periplasmic adaptor protein (also called membrane fusion protein) and the outer membrane (OM) channel, thus producing a tripartite complex spanning the IM, the periplasm, and the OM (represented by *E. coli* AcrAB-TolC and *P. aeruginosa* MexAB-OprM) (see the multicomponent pump depicted in Fig. 1). Some members of the ABC superfamily (e.g., MacB), the MATE family (e.g., MdtK), and even the MFS (e.g., EmrB) (all from *E. coli*) also are organized in this manner. The tripartite transporters excrete drugs directly into the external medium so that the reentry of drugs requires the slow traversal of the OM, an effective permeability barrier (21, 22). For this reason, these pumps are far more efficient in creating detectable resistance to antibiotics (especially AcrB, a constitutive RND transporter of *E. coli* [9]) (see Gammaproteobacteria: *Enterobacteriaceae*, below). In contrast, the pumps that are not organized in this manner and exist as single-component or "singlet" pumps in the IM (Fig. 1), including the vast majority of MFS and SMR pumps, are less effective in producing a detectable decrease in susceptibility, because the drug molecules are excreted only into the periplasm and can spontaneously diffuse back into the cytosol, since most antibiotics are relatively lipophilic molecules that can cross the phospholipid bilayer region of the IM. However, RND pumps, which are thought to capture antibiotics mostly from the periplasm (23, 24), can collaborate with the singlet pumps and thus increase their efficacy (25, 26).

The most detailed information on the contribution of various pumps to drug susceptibility is available for *E. coli* K-12, and Table 1 lists data on known and predicted multidrug pumps identified in the Transporter Classification Database mentioned above. An obvious way to detect the contribution of individual pumps is to measure the MICs of drugs in defective mutants. This was done in 2001 by Sulavik and coworkers (27) and showed that the RND transporter AcrB (in cooperation with its periplasmic and OM partners AcrA and TolC) plays a truly predominant role in raising the MIC levels in a wild-type strain. This also creates a problem because deletion of other pumps rarely produces detectable changes in MICs in the presence of the active AcrB-AcrA-TolC system. A similar problem was reported in a study (28) examining the MIC values of nearly 4,000 deletion mutants of all nonessential *E. coli* genes (the "Keio collection" [29]). Thus, although that study showed that the functions of many metabolic genes have an unsuspected influence on drug sensitivity, in terms of transporter genes, it essentially identified the effect of only the *acrAB-tolC* complex and nothing else. One possible exception is the deletion

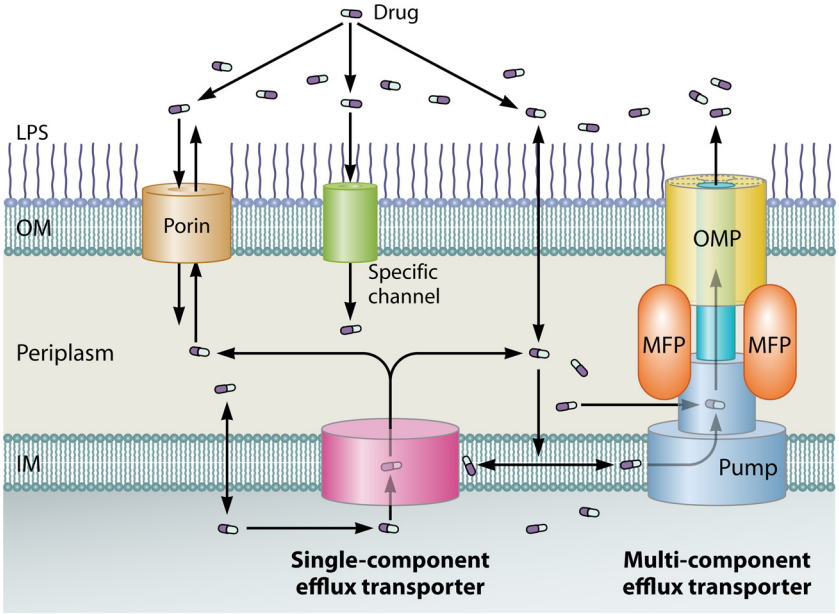

FIG 1 Location of drug efflux pumps and pathways of drug influx and efflux across the OM and IM in Gram-negative bacteria. The influx of drugs (shown as pills) through the OM occurs in one or more of the following three pathways: porin channels (e.g., OmpF of *E. coli* and OprF of *P. aeruginosa*), specific protein channels (e.g., CarO of *A. baumannii* and OprD of *P. aeruginosa* for carbapenems), and the LPS-containing asymmetric lipid bilayer region. After their entry into the periplasmic space, the drug molecules can further penetrate the IM via diffusion. However, these drugs can be extruded out of the cell by efflux transporters, which exist as either single-component pumps ("singlet"; e.g., Tet pumps) or multicomponent pumps (e.g., AcrAB-TolC and MexAB-OprM tripartite efflux systems that each typically contain a pump, an OM channel protein [OMP], and an accessory membrane fusion protein [MFP]). While the singlet pumps may take up the drug from the cytosol and the periplasm and function with porins or other types of protein channels to make the efflux process effective, the multicomponent exporters capture their substrates from the periplasm and the IM and directly pump them into the medium. The competition between the influx and efflux processes ultimately determines the steady state of drug molecules in bacterial cells. With the lipophilic drug molecules that cross the OM slowly or the hydrophilic drugs that penetrate the *A. baumannii*/*P. aeruginosa* low-permeability porins (i.e., "slow porins"), the efflux mechanism become very effective, thus being able to yield MDR. In contrast, with the less hydrophobic and smaller drug molecules that can rapidly penetrate, for example, *E. coli* porins, efflux is not effective to counteract drug influx, thus hardly decreasing the concentrations of the drug in the cell.

of the *ycdZ* gene, which produced hypersusceptibility to tetracycline and may code for an exporter. However, this conclusion is not supported by a study from the Carol Gross group, who quantitated the growth phenotype of the same set of mutants in the presence of sub-MICs of various drugs (30). This approach is more sensitive than the determination of MIC values and, indeed, as presented in Table 1, showed that the deletion of practically all known and suspected pumps produces hypersusceptibility to at least one agent tested. These results, however, must be interpreted with care, since this approach is very sensitive and could produce false-positive results in spite of efforts to avoid them (Table 1).

A completely different approach is the plasmid-based overexpression of putative efflux genes. This analysis by Nishino and Yamaguchi (31) indeed detected efflux activity in those genes whose activity was difficult to detect by deletion-based approaches. However, these data do not tell us whether the pumps are functioning in the wild-type or even mutant cells, although the level of expression of most pumps can be increased by regulatory signals (see Regulation of Multidrug Efflux Pumps, below).

Reviews describing various types of efflux pumps include those written by Poole (32, 33), Piddock (34–36), Paulsen et al. (37), Saier and others (38), Van Bambeke and others (39), and Higgins (40). A review by Alekshun and Levy (41) is useful, as it also emphasizes the contribution of nonefflux mechanisms of resistance.

RND Transporters

AcrB of *Escherichia coli*. The constitutively expressed pump AcrB of *Escherichia coli* plays a major role in raising the MICs of most antibiotics, due mostly to the fact that it exists as the AcrB-AcrA-TolC tripartite complex so that the exported drug molecules end up in the external medium, not in the periplasm, and thus cannot easily reenter the cells except by crossing the effective OM permeability barrier (24). Therefore, the effectiveness of RND pumps is intimately tied to the strength of the OM barrier; permeabilizing the OM destroys the effect of RND pump-mediated efflux almost as effectively as the inactivation of the pump itself (42, 43). Available reviews on RND pumps include those emphasizing the structure and mechanism (24, 44–49), computational approaches (50), roles in solvent tolerance (51), and functions other than drug resistance (34, 52–54).

AcrB has been studied most intensively as the prototype of RND pumps. It has an extremely wide specificity, including practically all types of antibacterial agents (except aminoglycosides), detergents, microbicides, dyes (Table 1), free fatty acids (55), and even simple solvents (56). In a reconstitution assay (57), AcrB was shown to also extrude modified phospholipids. A common property of these AcrB substrates is the presence of a hydrophobic domain (24, 58). Such a wide specificity appeared surprising at first. However, as pointed out by Neyfakh (59), this may be expected. Thus, when a typical soluble enzyme captures its hydro-

philic substrate from the aqueous medium, the process requires the removal of the substrate molecule, already stabilized by its numerous hydrogen-bonding interactions with the surrounding water. Stable binding in the binding site of the enzyme therefore must involve precise, strong interactions with the residues in the site, in order to overcome this energy barrier, and it requires that the site is small and carefully designed to bind stringently only one substrate species. However, with the multidrug pumps that capture drugs with sizeable hydrophobic domains, the drugs are not strongly stabilized in the aqueous environment, as their presence involves a large entropic cost accompanying the ordering of the surrounding water molecules. Hence, the drug binding to the transporter does not require the binding site to be small, tight, and stringent. It can be very large and can thus accommodate a large range of substrates with small decreases in the binding free energy.

For biochemical studies of any transporters, transport studies of membrane vesicles are usually the preferred approach. Nevertheless, with AcrB, this approach was not fruitful, presumably because most of the ligands transported are lipophilic and cannot be accumulated in the intravesicular space due to their readiness in crossing the phospholipid bilayer domain of the membrane. It is also possible that the absolute rate of transport does not need to be high because of the presence of the OM barrier, and this impedes the detection of transport in vesicles. Thus, the first major advance had to rely on the reconstitution of purified AcrB into proteoliposomes, accomplished by Zgurskaya and Nikaido in 1999 (57). In order to circumvent the problem of the spontaneous diffusion of most ligands across the lipid bilayer, this study used an innovative approach relying on the efflux of fluorescently labeled phospholipids into empty "acceptor" vesicles and detected the efflux of conventional ligands through competition with phospholipid efflux. In this way, drugs such as cloxacillin, erythromycin, novobiocin, and fusidic acid (but curiously not chloramphenicol) as well as various bile acids were shown to compete against phospholipid efflux. Furthermore, the half-maximal concentration for inhibition was lowest for bile acid taurocholate (~15 μM), suggesting that the properties of AcrB were optimized for the exclusion of bile salts, major toxic components in the mammalian intestine, the normal habitat for *E. coli*. Interestingly, the addition of AcrA to the aqueous phase (in the presence of Mg^{2+}) strongly stimulated phospholipid transport: because lipids had to be transported from one vesicle to another, we hypothesized that AcrA may act by bringing the two vesicles together. This approach also established that AcrB was a proton/drug antiporter, as the transmembrane pH gradient was dissipated accompanying the flux of ligands.

A similar reconstitution assay was successfully used for *E. coli* AcrD (23), an AcrB homolog that also works with AcrA and TolC as a tripartite transporter. This study is important, as AcrD transports aminoglycosides, which are very hydrophilic and not expected to diffuse spontaneously across the lipid bilayer. Thus, a conventional accumulation assay using radiolabeled aminoglycosides indeed proved their accumulation in proteoliposomes. When streptomycin was added as the substrate to either the more acidic, intravesicular space corresponding to the periplasm or the more alkaline external space corresponding to the cytosol, pumping activity (as detected by the flux of protons) was observed only in the former case, showing clearly that the pump captures its substrate only in the periplasm. Although other aminoglycosides appeared to stimulate the pump activity even when added to the external space, the activities were rather weak, and it seems likely that AcrD (and possibly also other RND pumps) at least prefers to capture its substrates from the periplasm. Interestingly, the addition of AcrA was necessary for the function of AcrD. Because the assay does not require the juxtaposition of vesicles, AcrA is likely to stimulate directly the function of AcrD (and AcrB) by simply binding to the transporter.

Since reconstitution assays are quite cumbersome, methods for quantitative, real-time determination of pumping activity in intact cells were needed. Fluorescent probes [e.g., *N*-phenyl-1-naphthylamine, ethidium bromide, and 2-(4-dimethylamino)styryl-*N*-ethylpyridinium iodide] were preloaded into bacterial cells deenergized by uncouplers, and efflux was monitored by fluorescence after reenergization by adding an energy source, such as glucose (60, 61). It is difficult to perform assays of this type in a reproducible manner because some uncouplers remain after reenergization. An optimized, semiquantitative method for *E. coli* using the fluorescent dye Nile red was reported in 2010 (62). A major step in the intact-cell assay of AcrB was the real-time assay of cephalosporin efflux in *E. coli* achieved by Nagano and Nikaido in 2009 (63). Those authors measured spectrophotometrically cephalosporin hydrolysis in intact cells by a periplasmic β-lactamase. By comparing the hydrolysis rate with the V_{max} and K_m of the enzyme, those authors calculated the periplasmic concentrations of the cephalosporins, overcoming the most serious problems in intact-cell assays of efflux. They then obtained the expected influx rate (V_{in}) of the drug across the OM from the permeability coefficient obtained from uncoupler-poisoned cells and from the difference in the external and periplasmic concentrations of the drug. The difference between V_{in} and the observed hydrolysis rate then corresponds to the rate of efflux. When the efflux rate of nitrocefin was plotted against the periplasmic concentrations, a Michaelis-Menten-type saturation curve was obtained, showing the V_{max} (0.024 nmol/mg/s) and K_m (5 μM) of the AcrB-catalyzed efflux process for the first time. Since the expression of AcrB was increased severalfold in the strain used, we estimate that the V_{max} is ~6 pmol/mg/s in wild-type *E. coli* K-12. This assay was used with conventional cephalosporins and penicillins (63–65); one surprising finding was that a sigmoidal kinetics was often observed in the plots of velocity versus periplasmic drug concentration, suggesting positive cooperativity. Recently, a sigmoidal kinetics was observed for the AcrB-catalyzed efflux of a compound of a very different nature, L-arginine-β-naphthylamide (A. Kinana, A. V. Vargiu, and H. Nikaido, unpublished data), indicating that this is a common feature of the AcrB-catalyzed transport process.

A major advance in the study of AcrB was made when Murakami et al. (66) solved the crystal structure of trimeric AcrB in 2002. This symmetric structure showed that each protomer of AcrB contained a large periplasmic domain, as predicted from the primary sequence. Furthermore, the periplasmic domain was seen to have a large cleft facing the surrounding periplasm. Although binding of ligands to the area close to the cleft was shown by cocrystallization (67–69), this observation did not immediately suggest the mechanisms of drug extrusion (see below). The next big advance in our understanding of AcrB structure and function came with crystallographic analysis of the asymmetric trimer structure, where each protomer takes a unique conformation slightly different from that of its neighbor, elucidated in three laboratories (Fig. 2) (70–72). The work by Murakami and co-

TABLE 1 Phenotypes of proven and putative efflux pump mutants of *E. coli*[a]

Family and efflux gene	Deletion phenotype[b] (agent[s], fold decrease in MIC)	Overexpression phenotype[c] (agent[s], fold increase in MIC)	Agent(s) that inhibited growth[d]	Other substrate(s) or information (reference)
RND				
acrB	ACR, 128; AMP, 4; BAC, 32; CHL, 8; CIP, 4; CLO, >2; DAU, >128; DEO, >2; EB, 256; ERY, 32; FUA, 128; MTX, >8; NAL, 2; NOV, 64; PUR, 64; R6G, 512; SDS, >128; TET, 8; TPP, 256	ACR, >16; BAC, 32; CHL, 8; CV, 8; DEO, >32; DOR, >64; ERY, 32; NAL, 4; NOR, 8; NOV, 64; R6G, 64; SDS, >8; TMP, >32;TPP, 16	ACR, AMP, AZM, AZT, BAC, BLE, BS, CER, CHIR, CHL, CHO, CIP, CLR, CPZ, DEO, DOX, EB, ERY, FUA, MEC, MIN, NAL, NIT, NOR, NOV, OXA, PUR, SDS, SPT, SPR, STG, TCH, TCS, TET, TMP, TRX, VER	
acrD	No change	DEO, >32; KAN, 2; NOV, 4; SDS, >8	PAR	
acrF	No change	ACR, 8; DEO, 4; DOX, 2; SDS, 4	PHL, VER	
yhiV (*mdtF*)	No change	CV, 2; DEO, 4; DOR, 8; EB, 4; ERY, 8; R6G, 16; SDS, 4; TPP, 4	FUA	
mdtB	No change	DEO, >32; FOF, 2; NAL, 2; NOR, 2; NOV, 16; SDS, 4	CIP, SDS	
mdtC	No change	DEO, >32; FOF, 2; NAL, 2; NOR, 2; NOV, 16; SDS, 4	BAT, CSD, NAL, PHL	
MFS				
bcr		ACR, 2; FOF, 4; KAN, 2; TET 4	CHO, ERY, SMZ	
cmr (*cmlA*, *mdfA*)	BC, 4; EB, 4	ACR, 8; CHL, 16; DOX, 4; EB, 4; NOR, 8; TET, 2; TMP, 4; TPP, 4	ACR, BAC, CHL, EB	Pentoses (1010)
emrB	No change	DEO, 32; PAR, 2; R6G, 2; SDS, 2	EB, SXT	CCCP, NAL, TSA (139); CER, TLM (15)
emrD	No change	BAC, 2; SDS, 2	AZM, GEN, NIT, OXA, SMT, SPR	Uncouplers (15); pentoses (1010)
emrY	No change		CEC, SMZ, TET	H_2O_2, MIT, NAL, UV irradiation (1011)
yajR			EB, NIT, OXA, SPR, TMP	
yceB			AMX, BAC, MEC, NAL, PAR	
yceE (*mdtG*)		FOF, 4	BAC, CAZ, MEC, MTX, PUR, SDS, TLM	
yceL (*mdtH*)			ATM, EB	
ydeB (*marC*)			ACT	
ydhC			FUA, SPT	Pentoses (1010)
yebQ			AMK, CHL, CHO, CSD, DEO, ERY, FOX, NOV, PER, PMB, TET, VER	
yegB (*mdtD*)			None with agents tested	Iron citrate (in *Salmonella*) (1012)
yidY (*mdtL*)			AMX	
yieO (*hsrA*)			ACR, BAC, CAR, CHIR, CSD, DOX, EB, FOX, GFF, MIT, SDS, STR	
yjiO (*mdtM*)			AZT, BLE, CAL, CHIR, CHL, CIP, CPZ, INH, OXA, SMZ, SPR, TET	Binds CHL (1013)
ynfM			AMP, DEO	Pentoses (1010)

MATE (MOP)				
mdtK (*ydhE*, *norM*)		BAC, 2; CHL, 2; DEO, 32; DOR, 8; EB, 2; FOF, 2; NOR, 8; PAR, 4; TMP, 4; TPP, 32	CPZ, FUA, NOR, PUR, STR	
yeeO			ACR, BAC, BIC, CAR, DOR, PUR, VAN, VER	
SMR (DMT)				
emrE	EB, 4; PAR, 8	ACR, 16; BAC, 2; EB, 8; PAR, 2	ACR, EB, FOF, PAR	EB (1014); PAR (1015); ERY (155); cationic osmoprotectants (159)
ydgE (*mdtI*)		DEO, 4; SDS, 2	ACR, BAC, CHL, DEO, EB, FUA, MTX, PMB, PUR, TRX	Spermidine (1016)
ydgF (*mdtJ*)		DEO, 4; SDS, 2	CHO, CSD, ERY, NAL, VER	Spermidine (1016)
ABC				
macB	No change	ERY, 8	BAT, BIC, BS, CEC, EB, FOX, NIT, SDS, TCH	
LysE				
argO			AMP, BS, GFF, INH, NAL, PHL, TMP	Arginine (1017)
Unknown				
ycdZ			NOR, SDS (not TET)	TET, 1.7- to 2.5-fold decrease in MIC compared to the wild type (28)

[a] Abbreviations: ACR, acriflavine; ACT, actinomycin D; AMK, amikacin; AMP, ampicillin; AMX, amoxicillin; ATM, aztreonam; AZM, azithromycin; AZT, azidothymidine; BAC, benzalkonium chloride; BAT, bacitracin; BIC, bicyclomycin; BLE, bleomycin; BS, bile salts; CAL, calcofluor; CAR, carbenicillin; CAZ, ceftazidime; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CEC, cefaclor; CER, cerulenin; CHIR, CHIR-900 (an LpxC inhibitor); CHL, chloramphenicol; CHO, cholate; CIP, ciprofloxacin; CLO, cloxacillin; CLR, clarithromycin; CPZ, chlorpromazine; CSD, cefsulodin; CV, crystal violet; DAU, daunomycin; DEO, deoxycholate; DOR, doxorubicin; DOX, doxycycline; EB, ethidium bromide; ERY, erythromycin; FOF, fosfomycin; FOX, cefoxitin; FUA, fusidic acid; GEN, gentamicin; GFF, glufosfomycin (fosfomycin plus glucose-6-P); INH, isoniazid; KAN, kanamycin; MEC, amdinocillin; MIN, minocycline; MIT, mitomycin; MTX, methotrexate; NAL, nalidixic acid; NIT, nitrofurantoin; NOR, norfloxacin; NOV, novobiocin; OXA, oxacillin; PAR, paraquat (methyl viologen); PER, peroxide; PHL, phleomycin; PMB, polymyxin B; PUR, puromycin; R6G, rhodamine 6G; SDS, sodium dodecyl sulfate; SMT, sulfamonomethoxine; SMZ, sulfamethizole; SPR, spiramycin; SPT, spectinomycin; STG, streptonigrin; STR, streptomycin; SXT, trimethoprim-sulfamethoxazole; TCH, taurocholate; TCS, triclosan; TDC, taurodeoxycholate; TET, tetracycline; TLM, thiolactomycin; TMP, trimethoprim; TPP, tetraphenyl phosphonium; TRX, Triton X-100; TSA, tetrachlorosalicylanilide; VAN, vancomycin; VER, verapamil.

[b] See reference 27. Numbers after the abbreviation for the agent name indicate the fold decrease in MIC in comparison with the wild-type strain.

[c] See reference 31. Numbers after the abbreviation for the agent name indicates the fold increase in MIC in comparison with the Δ*acrAB* parent strain.

[d] Only the antimicrobial agents, dyes, and detergents that significantly inhibited the growth of mutants more than that of the wild type, usually leading to growth scores of <-2 (30), are listed. Furthermore, we confirmed that the inhibition was reproducible by examining the data obtained with different concentrations of the same agent.

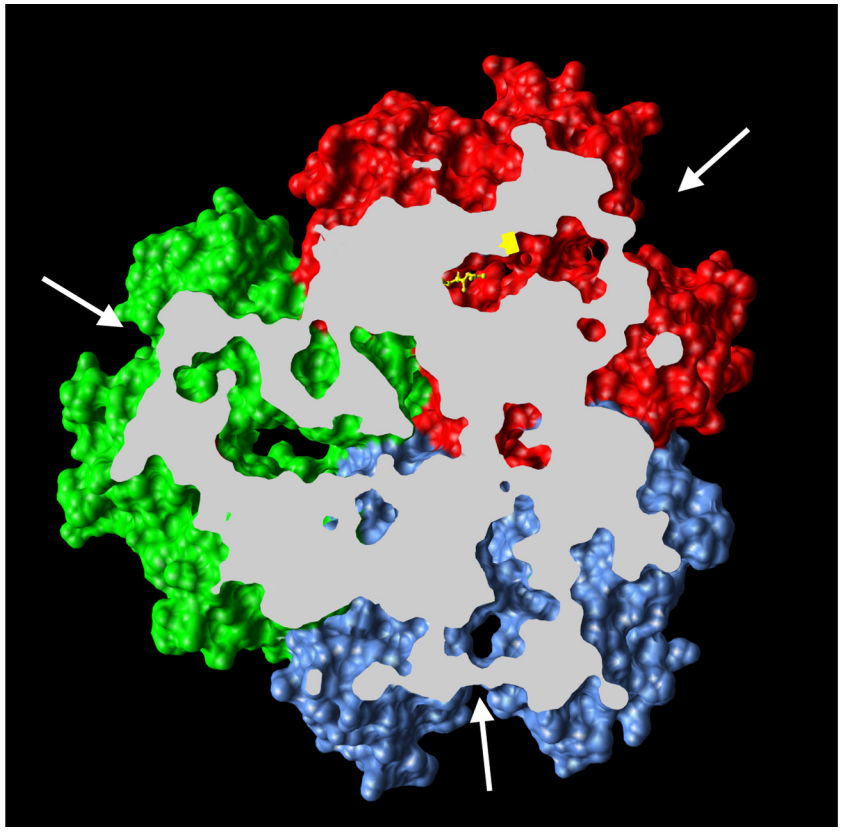

FIG 2 Drug transport mechanism of AcrB. Shown is the asymmetric crystal structure of AcrB (Protein Data Bank accession number 2DRD), viewed from outside the cell, with the top portion cut off for clarity. Conformational cycling of 3 AcrB protomers, in access (blue), binding (red), and extrusion (green), is seen by cocrystallization of AcrB with its substrate minocycline, shown in a yellow stick model.

workers (70) was especially important, because they succeeded in cocrystallizing AcrB with the substrate minocycline or doxorubicin. In both cases, the substrates were seen in a predominantly hydrophobic pocket within the periplasmic domain, now called the distal binding pocket, close to the center of the trimer and located in one particular protomer, called the binding protomer. The presence of three conformationally different protomers, the access, binding, and extrusion protomers (Fig. 2) (44), suggested a functionally rotating mechanism, in which each protomer goes through a succession of conformational alterations. The distal binding pocket becomes collapsed in the extrusion protomer, consistent with the movement of the drug to the exit gate close to the end of the TolC channel. This concept of conformational cycling or functional rotation was then substantiated by the finding that disulfide cross-linking of nearby residues, although apparently occurring in only one or two protomers, nearly completely inactivated the trimeric complex (73). (The AcrB homolog MexB of *P. aeruginosa* has been crystallized without [74] and with [75] an added inhibitor, a pyridopyrimidine derivative.) In a similar vein, when the AcrB trimer was produced as a covalently linked single protein, and only one protomeric unit was inactivated in the proton translocation pathway, the entire trimeric complex became inactive (76). Furthermore, when the Cys residues were introduced into only one of the protomeric units, their cross-linking immediately inactivated the function of AcrB trimers, showing that inactivation was not due to a failure of the trimeric assembly.

More recently, two laboratories (77, 78) showed that large substrates such as macrolides, rifampin, and a dimer of doxorubicin bind to a more proximal binding site in the access protomer, presumably before their eventual movement to the distal pocket concomitant with the conformational change of the protein into the binding protomer. Interestingly, this proximal binding site surrounded by residues Asp566, Phe664, Phe666, Glu673, Arg717, and Asn719 overlaps mostly the periplasmic binding site identified by Yu and collaborators several years earlier (67) in a symmetric AcrB crystal structure. Yu and associates further showed the importance of this binding site by site-directed mutagenesis, finding that a Phe666Ala mutation, for example, results in a drastic decrease of resistance to a wide range of substrates (67). The involvement of residues surrounding this site was also shown by the fact that their Cys-substituted mutants were strongly labeled by an AcrB substrate, boron-dipyrromethene (BODIPY)-maleimide (79). Another symmetrical crystal structure containing a substrate at this position has a deoxycholate molecule (80). The importance of this proximal binding pocket was further emphasized by recent studies (81, 82). The preference of AcrD for β-lactams containing multiple anionic groups, such as carbenicillin, sulbenicillin, and aztreonam, is essentially due to the residues within the proximal pocket (81), while a region in the MexY aminoglycoside pump that corresponds to a proximal binding pocket of AcrB plays a role in aminoglycoside recognition and export (82).

Yet another binding site for drugs was identified within the central cavity of the trimer by cocrystallization (67, 83). Possibly, the initial binding here is followed by the eventual translocation of

the substrates to the distal binding pocket, perhaps through the "vestibules" between the protomers (84). Although the functional significance of this binding site could not be ascertained by site-directed mutagenesis, symmetric cocrystals of AcrB with drugs in the central cavity have been reported for ampicillin (68) and linezolid (85).

Dastidar et al. carried out the first effort to use substrate competition for covalent labeling of selected residues for the elucidation of the path of the drug molecules within the large periplasmic domain of AcrB (86). Some residues of *Haemophilus influenzae* AcrB were converted to Cys and were labeled with fluorescein maleimide. The labeling of Ala288Cys (corresponding to Gly290 of *E. coli* AcrB and close to the distal binding site) was decreased by the presence of all substrates tested, except ethidium. We followed up on this work by selecting 48 residues that lie on the presumed path(s) of the drugs, converting each residue to Cys, and labeling the Cys residue in intact cells with a hydrophobic, covalent-labeling probe, BODIPY-maleimide (79). Residues outside the predicted path were not labeled at all, even when they were located in the middle of hydrophobic patches. In contrast, most of the tested residues in the distal binding pocket were strongly labeled, as were the residues lining the proximal pocket as well as the entrance and the bottom of the large external cleft between two subdomains (PC1 and PC2 [66]). Finally, by using bulky covalent-labeling reagents, with some residues, we have been able to "clog" the substrate path so that the efflux of a substrate, Nile red, could be blocked.

This study reinforced the importance of the drug binding to the distal binding pocket as a major step in efflux. Site-directed mutagenesis of Phe residues in this pocket (87) indicated that these residues are important for efflux, with the Phe610Ala mutation showing the most widespread effect on many substrates. A molecular dynamics (MD) simulation study of this mutant protein (88) revealed that a substrate, doxorubicin, still bound to the pocket with a strong affinity; the interpretation of these results is described below. Site-directed mutagenesis based on the sequence difference between AcrB and MexB, which show different proficiencies in macrolide efflux, led to the discovery of the importance of Gly616 in AcrB for this function (89); interestingly, this residue is a part of the Gly-rich loop (also called the switch loop), which separates the distal pocket from the proximal pocket (77) and is thought to be critical for translocation of the substrates, especially large molecules such as macrolides. More recently, Eicher et al. showed the coupling of remote alternating-access transport mechanisms for protons and AcrB substrates through a mechanism involving two remote alternating-access conformational cycles within each promoter (90).

Although these mutagenesis studies are valuable, they do not tell us how various substrates bind to the AcrB transporter. As stated above, only a few crystal structures of drug-AcrB complexes are currently available. Thus, computational analysis of drug-AcrB interactions was first initiated with the docking software Autodock Vina (91). Various known substrates of AcrB, including minocycline, docked to the upper part (closer to the exit gate) of the distal binding site, which contains a characteristic crevice (Fig. 3). This is where minocycline and doxorubicin bound in the crystal structures (70). Cefazolin, a nonsubstrate (63), gratifyingly did not bind to the binding pocket. However, other substrates failed to bind to this upper portion of the pocket; as an example, chloramphenicol and solvents such as cyclohexane were predicted to "bind" with a significantly lower binding energy to the lower part of the pocket, which we called a "cave" (Fig. 3) (91).

To get further insights into the binding-and-efflux process, we examined potential competition between substrates. It had been nearly impossible to show competition among substrates of AcrB by using an MIC assay (92). Still, with a real-time efflux assay with the dye Nile red, we showed that doxorubicin, minocycline, and other tetracyclines as well as tetraphenylphosphonium, but not chloramphenicol, macrolides, deoxycholate, nafcillin, or novobiocin, inhibited dye efflux (62). Also, when a real-time efflux assay of nitrocefin (63) was used, we showed strong inhibition by minocycline, predicted to bind to the upper part of the pocket, like nitrocefin (91). In contrast, a substrate that is not predicted to bind to this part of the pocket, i.e., chloramphenicol, did not inhibit nitrocefin efflux, and actually, there was some hint of stimulation instead (91). The latter phenomenon is discussed in more detail below.

Docking programs, however, have been optimized by using the binding of small, hydrophilic substrates mostly to the binding sites within enzymes. Binding of hydrophobic or amphiphilic ligands to the large binding pockets of transporters is predicted to occur in significantly different ways (59). Thus, we examined in detail the binding of 9 substrates, 2 inhibitors, and 2 nonsubstrates to the distal binding pocket of AcrB by extensive MD simulations (93). This introduced two major improvements over the docking approach. First, water molecules now became a part of the system so that the interaction of amphiphilic and more hydrophilic ligands could be predicted in a much more realistic manner. Second, the distal binding pocket is composed of residues that are on several loop segments in a relatively loosely constructed area of the protein, so movement and rotations of the chains were expected. Indeed, with many ligands, there was an extensive alteration in the shape of the binding site to better accommodate diverse substrates. Interestingly, some of those "cave binders" in the docking approach left the lower area and were found to favor the upper area of the pocket, although the binding appeared to be weak.

Chloramphenicol was found to slightly accelerate the efflux of nitrocefin (91). This was confirmed by a subsequent careful study, and it was found that solvents such as benzene or cyclohexane produced much more pronounced stimulation of nitrocefin efflux (94). MD simulations suggested that benzene interacts primarily with the Phe-rich hydrophobic domain that comprises the lower portion of the binding pocket and not with the upper subpocket that binds minocycline or nitrocefin (94). Interestingly, the lower portion of the binding pocket is where the hydrophobic part of the inhibitor D13-9001 binds tightly and was named a "hydrophobic trap" by Nakashima et al. (75) (see Efflux Pump Inhibitors, below). Furthermore, recent MD simulations showed that other efflux pump inhibitors (EPIs), such as phenylalanine-arginine-β-naphthylamide (PAβN), 1-(1-naphthylmethyl)-piperazine (NMP), and the new, potent inhibitor MBX2319 (95), all bind tightly to the hydrophobic trap and thereby distort the shape of the rest of the pocket, closing the crevice where minocycline or nitrocefin becomes bound (96). These observations suggest the following. (i) The distal binding pocket is very large, and different ligands prefer different areas of the pocket for binding. Thus, a "typical" substrate, like minocycline or nitrocefin, which has a number of hydrophilic groups, tends to bind to the upper "crevice" area, which is rich in hydrophilic and charged residues and was indeed shown to be involved in substrate binding (93). In

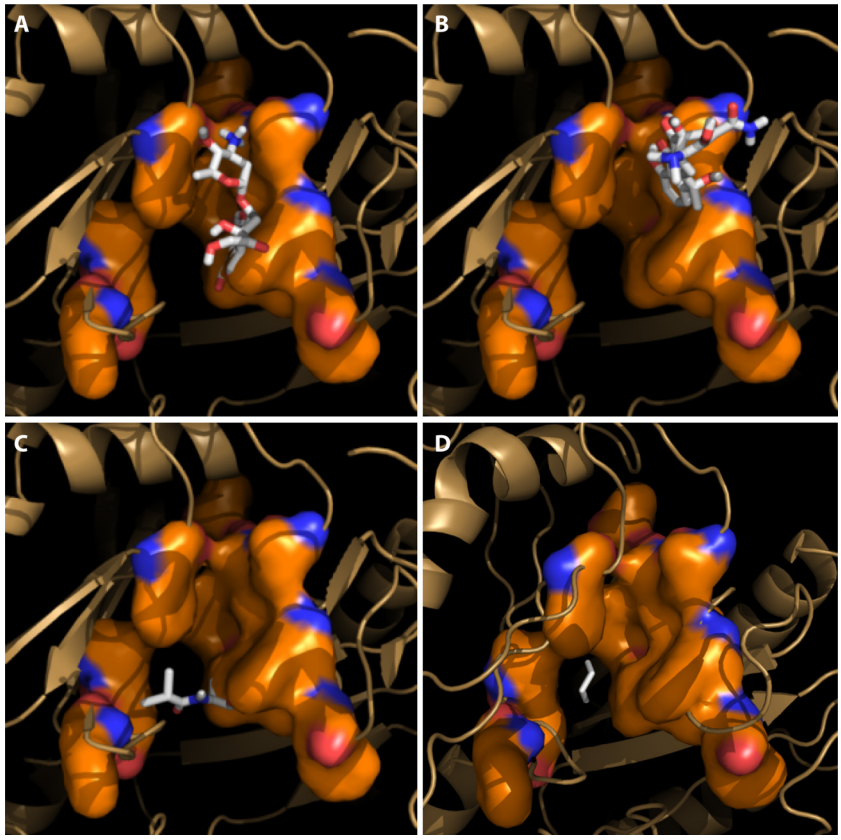

FIG 3 Interaction of drug substrates and the AcrB-binding protomer analyzed with Autodock Vina docking software. Substrates are shown to bind to either the upper part (groove binder) (doxorubicin [A] and tetracycline [B]) or the lower part (cave binder) (chloramphenicol [C] and cyclohexane [D]) of the distal binding site. (Modified from reference 91.)

contrast, for hydrophobic ligands such as cyclohexane or chloramphenicol, binding to this area is difficult. (ii) A tight interaction with the hydrophobic trap distorts the structure of the crevice, inhibiting the efflux of typical substrates. (iii) A loose interaction with the hydrophobic trap, on the other hand, may enhance the efflux of typical substrates, by either facilitating the interaction of such substrates with the pocket, speeding up the sequence of conformational changes needed for the export of substrates, or both. In any case, the interaction between the AcrB transporter and its substrates/inhibitors/enhancers appears quite complex. In assessing the binding of drugs to the pocket of transporters such as AcrB, we now realize that binding follows the same principles elucidated by the pioneering early crystallographic studies by Brennan and coworkers (for example, see references 97 and 98), carried out by using soluble regulators of MDR pumps, such as QacR, rather than the pumps themselves.

Computer simulation has now become an important approach for studying the mechanism of AcrB function. The movement of a substrate, doxorubicin, from the distal binding pocket to a position close to the exit gate, accompanying the closure of the pocket, was shown by a targeted MD simulation (99). Large substrates, found in the proximal binding pocket in AcrB cocrystals (77), moved substantially in the direction of the distal binding pocket during MD simulation (100). Movement of water molecules was analyzed by simulation (101, 102), and coarse-grained models were used to analyze the conformational transitions (103) as well as the drug pathways (104) within AcrB.

After the binding of the substrate to the distal binding pocket in the binding protomer, the proton(s) must come in from the periplasm to bind to the Asp residue(s) in the transmembrane domain, causing the conformation of the protein to change into the extrusion protomer, thereby squeezing out the substrate to the exit gate by the collapse of the pocket. Asp407 and Asp408 appear to be essential for the energy transduction of AcrB, together with Lys940 and Arg971 (105) as well as Thr978 (106). In the binding protomer, both Asp407 and Asp408 appear to be deprotonated, as the presumably protonated Lys940 side chain is situated between the two Asp side chains. In the extrusion protomer, Lys940 is moved away from the Asp residues and now faces the Thr978 side chain (107). The carboxyl group of Asp408 was indeed shown to have an unusually high pK_a of 7.4, which would help in the facile binding and release of the proton under physiological conditions (107). A recent MD simulation study (108) suggests that in the extrusion protomer, Asp408 becomes protonated, but Asp407 remains deprotonated. In this scheme, the translocation of one proton across the IM would be sufficient to cause the conformational changes in AcrB, resulting in the extrusion of the drug molecule(s). The folding and assembly of the AcrB trimer were studied mainly by Wei and associates, who showed that the folding of the monomeric unit precedes trimerization (109) and analyzed the function of a protruding loop that inserts deeply into the neighboring subunit (110).

In 2007, when AcrB was crystallized without amplification from *E. coli*, the symmetric crystals were found to contain a small

(110-residue) protein, YajC, associated with the transmembrane domain (68). The *yajC* gene occurs in the *secDF* operon (111), but the significance of this association is unclear. Deletion of *yajC* appeared to make *E. coli* marginally more susceptible to penicillins (68). Five years later, Hobbs et al. (112) found that a 49-residue protein, renamed AcrZ, associates with the transmembrane domain of AcrB. Deletion of *acrZ* renders *E. coli* moderately more susceptible to chloramphenicol and tetracycline. Importantly, AcrZ expression is regulated in the same manner as that of AcrB, by the global regulators MarA, SoxS, and Rob (see below).

AcrB functions as a member of the tripartite machinery including the periplasmic adaptor protein AcrA and the OM channel TolC. The structure of TolC, solved by the Koronakis group (113), shows a trimer containing an OM-spanning β-barrel and a contiguous, long, 12-stranded α-barrel. Among the adaptor proteins, the MexA structure was elucidated first (114). The AcrA structure is similar (115), but only three domains (the long α-hairpin, a lipoyl domain, and a short β-barrel) were elucidated; the membrane-proximal domain had to wait for work by Symmons and others in 2009 (116). Those authors showed by chemical cross-linking that the three domains other than the long α-hairpin interact closely with the periplasmic domain of AcrB. This suggested that the remaining α-hairpin domain was connected to the lower part of the α-barrel of TolC, completing the tripartite assembly. The top of the periplasmic domain of AcrB was cross-linked to the tip of the TolC α-barrel (117), and a model of the tripartite complex was proposed (116). This complex was shown to be stable enough to withstand cell disruption (118). The interaction of AcrA and other adaptor proteins with TolC has been studied by surface plasmon resonance (119), and a similar approach with immobilized AcrB showed that AcrB interacted with TolC, in the absence of AcrA, with a relatively high affinity (K_D [equilibrium dissociation constant] of 90 nM) (120).

The number of AcrA molecules per assembly was uncertain in early models. With MacA, another adaptor protein that functions with the ABC transporter MacB (see below), a hexameric crystal in which MacA forms a closed barrel was found (121). Because the diameter of the end of this MacA barrel (composed of α-hairpins) was similar to the diameter of the α-barrel of TolC, and because the tip of the hairpin had amino acids that are conserved among various adaptor proteins, it was proposed that MacB does not directly touch the end of TolC and that the MacA tunnel acts as a bridge between these two proteins (121). A cocrystal of the Cu efflux RND pump CusA with its cognate adaptor protein CusB (122) also shows that the top of the transporter trimer interacts with the lower end of the CusB hexamer to form a channel that is likely to allow the partial insertion of the α-barrel domain of the OM channel CusC. In this model, again, the end of the α-barrel of the OM channel is not in contact with the top of CusA. Finally, in the recently reported electron micrographic structure of the AcrB-AcrA-TolC complex elucidated by the use of an AcrB-AcrA fusion protein, again, the top of AcrB is not in contact with the end of the TolC channel (123). However, there is evidence that these two domains are likely to come into contact in intact cells, as mentioned above, and it seems possible that the tripartite assembly is a dynamic one that could become shorter during the efflux cycle, as was suggested by Su and coworkers (122).

A rather close homolog of AcrB appears to exist in all members of the *Enterobacteriaceae* and also in many other species (e.g., MexB in *P. aeruginosa*). The *acrB* gene forms an operon with the *acrA* gene coding for the adaptor protein, and a similar arrangement is common with other RND pumps and in other species, especially in *Enterobacteriaceae*, where the OM component TolC, also serving other transporters, is encoded elsewhere. In contrast, in *P. aeruginosa* and *A. baumannii*, where each RND pump tends to operate with its specific adaptor and OM channel, a three-gene operon coding for all three components is more common (see sections on *Pseudomonas*, *Acinetobacter*, and *Stenotrophomonas*, below).

Other RND transporters in *E. coli*. AcrF appears to have a wide substrate specificity, similarly to AcrB (124, 125). AcrD is an aminoglycoside efflux pump that works with AcrA and TolC (23). MdtF (YhiV) is likely involved in the extrusion of toxic metabolites during nitrosative stress, such as the nitrosyl derivative of indole, produced during anaerobic growth of *E. coli* (126). MdtBC is unusual because it contains two different transporter proteins, MdtB and MdtC, and appears to function only as a B_2C heterotrimer (127). When overexpressed, it pumps out norfloxacin, novobiocin, cloxacillin, and deoxycholate (15, 31, 128). Site-directed fluorescein maleimide modification studies suggest that MdtC binds the substrate, but MdtB probably functions in other ways, such as initiating the conformational alteration for drug efflux (129).

MFS Transporters

MFS transporters can be classified into at least 74 families on the basis of sequence homology (130). *E. coli* K-12 contains 70 MFS transporters, 15 of which may be considered drug exporters, as they belong to families 2 and 3 (http://www.membranetransport.org/), which are composed of 12-TMS (transmembrane segment) and 14-TMS members, respectively, of drug/H^+ antiporters (37, 130). Most of them, however, are free-standing transporters located in the IM and transport drugs from the cytosol to only the periplasm. Because most antimicrobial agents reach the cytosol usually by diffusion across the membrane bilayer, the pumped-out drug molecules have a good chance of reentering the cytosol through this free-diffusion process, and the transporters of this class are not expected to create high-level resistance. However, constitutive RND pumps, such as AcrAB-TolC and MexAB-OprM, may capture such pumped-out drug molecules in the periplasm and thus synergistically enhance the activity of singlet pumps in producing resistance (Fig. 1). This was first shown in *P. aeruginosa* (25) and was rediscovered in *E. coli* nearly a decade later (26). The latter study (26) also made an important point that the contribution of some singlet pumps may have escaped detection because of overlapping specificities; thus, the double deletion of an MFS pump, MdfA, and an SMR pump, EmrE, made *E. coli* as susceptible as or even more susceptible than the AcrB deletion mutant to cationic agents with intracellular targets, like acriflavine or ethidium. This finding suggests that RND pumps are usually rather inefficient in capturing drugs from the cytosol (although some contrary views have been presented [131]) and that the singlet pumps often play an important role in resistance to agents with intracellular targets. The fact that the plasmid-encoded TetA pump creates significant tetracycline resistance (8) suggests that this synergistic mechanism can sometimes be quite effective.

Although the MFS-type MDR pumps usually do not play a predominant role in resistance, as described below, the singlet drug pumps of the Tet group, usually plasmid encoded, are clinically important in creating tetracycline-specific resistance in many bac-

terial species. In the current nomenclature, TetA refers to the MFS exporter, and the phylogenetic group to which it belongs is specified by the group within parentheses, as in TetA(B). Currently, the 12-TMS TetA pumps, present in Gram-negative bacteria, contain 13 phylogenetic groups, whereas the 14-TMS Tet pumps, present in Gram-positive bacteria, contain at least 3 groups (132). The plasmid-encoded TetA pumps were the first bacterial drug efflux pumps identified (8, 133). Biochemical studies by the Yamaguchi group showed that their substrate was the magnesium salt of tetracycline (134), and cysteine-scanning mutagenesis followed by labeling studies identified the residues important for substrate binding and proton translocation (135). Interestingly, the Gram-positive pumps Tet(K) and Tet(L) were found to transport monovalent cations, such as Na^+, and cation transport was hypothesized to be the original function of such pumps (136). Finally, glycylcyclines such as tigecycline were developed by selecting for derivatives that withstand the presence of Tet pumps and are indeed poor substrates for these tetracycline-specific transporters (137). However, tigecycline is a substrate for the RND pumps of many species, including *E. coli*, such as AcrAB or AcrEF (138).

A few MFS pumps, however, occur with their own periplasmic adaptor proteins and with OM channels, such as TolC, and presumably produce an efficient tripartite efflux system. In *E. coli*, these pumps include EmrB (occurring with the cognate adaptor EmrA) and EmrY (with EmrK), which indeed appear to be involved in the efflux of uncouplers and other substrates (Table 1) (15, 31, 139, 140). Importantly, the crystallographic structure of EmrD was determined (141). It is similar in general to those of the other MFS transporters but has a larger central cavity surrounded by hydrophobic and aromatic side chains. It was also noted that the loops connecting H4 and H5, and H10 and H11, protrude into the cytoplasm much more than in the inward transporters of the MFS, such as LacY and GlpT, and that these loops may play a role in substrate recognition and capture (141). A pH-dependent conformational change was also established for EmrD (142). EmrB that occurs with the periplasmic adaptor EmrA appears to assemble *in vitro* into a dimer of EmrAB dimers (143). If EmrA forms an intermediary channel similar to the AcrA and CusB channels (see above), perhaps the discrepancy between the trimeric TolC and dimeric EmrB may not matter. Alternatively, the dimeric arrangement could be an artifact of the *in vitro* assembly of the proteins. The EmrA protein was shown to form dimers and trimers *in vitro*, and interestingly, it bound an efflux substrate, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (a proton uncoupler), with a reasonable affinity (K_D of ~1 μM) (144).

Among the singlet MFS transporters, MdfA, which confers MDR when overproduced (145), has been studied extensively in terms of biochemistry (146). However, its clinical relevance remains unknown, although it plays a major role with the SMR-type transporter EmrE in the efflux of cationic dyes (and presumably other cationic agents, such as quaternary ammonium compounds) (26).

ABC Transporters

In fungi and animal cells, most of the transporters involved in drug efflux belong to the ABC family (7, 147). In Gram-negative bacteria, there are only few examples of ABC family drug efflux pumps, although MsbA, the exporter of biosynthetic intermediates of lipopolysaccharide (LPS), was shown to pump out drugs, including erythromycin, when overexpressed in *Lactococcus lactis* (148).

The best-studied bacterial ABC drug exporter is MacB of *E. coli*, which functions together with the periplasmic adaptor MacA and the OM channel TolC (149). MacAB-TolC raises macrolide MIC values when overproduced (149). The isolated MacB shows only trace ATPase activity, which is not stimulated by substrates. However, ATP hydrolysis is very strongly stimulated by the simultaneous presence of MacA (150); these results were confirmed by another laboratory (151), which showed that MacB is a dimer, as expected for an ABC transporter. Finally, an analysis using surface plasmon resonance led to the conclusion that MacA binds to MacB with a nanomolar affinity, and the complex remains stable during the ATP hydrolysis cycle (152); the authors of this study assume that the MacA channel connects a TolC trimer and a MacB dimer, with no direct connection between the latter two. The expression of the MacAB system is stimulated by the heat shock sigma factor σ^{32} (153). Its physiological function might be related to the export of LPS or its biosynthetic intermediate (154).

SMR Transporters

The proton-motive force-driven SMR transporters belong to the drug/metabolite transporter (DMT) superfamily and are very small, each containing only four TMSs. Thus, unlike MFS transporters, which presumably function as monomers, SMR transporters, which typically exchange incoming H^+ with the pumping out of either monocationic (ethidium and tetraphenylphosphonium, etc.) or dicationic (e.g., paraquat) compounds (155), must function as a dimer. They also appear to decrease susceptibility to aminoglycosides when the proteins are overproduced from plasmids (156). However, there was controversy on the issue of whether this was a parallel dimer in which each component monomer was embedded in the same direction within the bilayer or an antiparallel dimer. A crystallographic study clearly shows the antiparallel arrangement within an EmrE dimer (157), but chemical cross-linking favors a parallel arrangement, and it appears that the direction of insertion of the monomeric unit really does not matter for the efflux function (158).

EmrE is one of just a few transporters that produce a drug-hypersusceptible phenotype when the gene is deleted in wild-type *E. coli* still containing AcrAB (Table 1). One of the characteristic substrates of EmrE is a quaternary ammonium compound, including the endogenous osmoprotectant of *E. coli*, betaine (159), and thus, EmrE overproduction makes cells more susceptible to hyperosmolarity conditions as well as alkaline-pH media. Using these phenotypes, one study found that OmpW, an OM protein, apparently helps in the removal of such compounds pumped into the periplasm by EmrE (160). This is rather unexpected, as OmpW forms an 8-stranded β-barrel, which usually contains a channel too narrow for solute diffusion. Indeed, its structure shows that its central channel is truncated, although it may open up sideways into the interior of the OM (161). If (and how) quaternary ammonium compounds could diffuse through OmpW is thus an open question; however, we note that AcrAB-TolC is not needed for full resistance to paraquat (26).

There are a few reports suggesting the possible involvement of SMR transporters in resistance in clinical isolates of organisms other than *E. coli*. The deletion of *abeS* resulted in significant decreases in MICs of chloramphenicol, ciprofloxacin, and erythromycin in *A. baumannii* (162), and the deletion of a pair of genes,

kpnEF, in *K. pneumoniae* makes cells hypersusceptible to a wide range of antimicrobials (163). An EmrE homolog contributes to MDR in *P. aeruginosa* (164). The contribution of SMR transporters to resistance seems to be an important future area of study for clinical microbiologists.

MATE Transporters

The MATE transporters have now become a part of a new superfamily, the multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) flippase superfamily (19), because of their connection to transporters like the LPS flippase RfbX. These transporters are widespread in bacteria and are also found in higher animals and plants.

The first of these transporters identified was the Na^+/cationic agent antiporter NorM from *Vibrio parahaemolyticus* (165), with 12 TMSs. Its homologs pump out cationic dyes, fluoroquinolones, and aminoglycosides into the periplasmic space. Many of these transporters use a gradient of Na^+, H^+, or both as the energy source. The crystal structure of NorM from *Vibrio cholerae* indicates an outward-facing conformation with two portals open to the outer leaflet of the IM (166). The structures of NorM from *Neisseria gonorrhoeae* (167) and its distant, proton-driven *Pyrococcus furiosus* homolog (168) bound to several substrates show a wide, central substrate-binding cavity. With the latter, protonation of Asp41 bends TMS1 so that the part of the cavity located in the N-terminal half of the protein becomes collapsed, suggesting that this will produce the extrusion of substrates. Furthermore, cyclic peptides representing prototype inhibitors were also cocrystallized, and the best inhibitor appears to bind tightly to the cavity, preventing the bending of TMS1 (168). Finally, the mechanism of the NorM pump was examined with MD simulation (169).

Although genes for the MATE pumps have been cloned from many pathogens, their contribution to resistance in the organisms of origin has not been studied in most cases. One exception is a transporter from *Enterobacter cloacae*, EmmdR, and its gene disruption contributed to susceptibility to fluoroquinolones and cationic dyes (170).

SYNERGY WITH THE OUTER MEMBRANE BARRIER

Pathways of Drug Influx across the OM

In Gram-negative bacteria, antimicrobial agents must first traverse the OM barrier in order to exert their action (Fig. 1). The OM usually functions as a very effective permeability barrier, because the porin channels are narrow, being only 7 by 11 Å in the *E. coli* OmpF porin at its constriction point (171), and the bilayer domain of the OM is asymmetric, with its outer leaflet composed only of LPS (172), producing an unusually impermeable bilayer. Furthermore, because several basic amino acid residues are on one side and acidic amino acids are on the opposite side at the constriction zone of the porin channel, the water molecules inside are thought to be strongly oriented in one direction, and this presumably hinders the diffusion of lipophilic drugs, as they must disorganize this assembly of water molecules for penetration (173). Indeed, measurement of influx rates of cephalosporins showed that lipophilicity strongly hinders diffusion through porin channels (174). These considerations suggest that only those drugs that are relatively small, and preferably not too lipophilic, pass through the porin channels relatively rapidly. This group includes β-lactams, fluoroquinolones, tetracycline, chloramphenicol, cycloserine, and aminoglycosides. Aminoglycosides can be fairly large, but as polycations they are likely to become "sucked into" the periplasm by the presence of the interior-negative Donnan potential (175). Aminoglycosides and β-lactams are also essentially prohibited from diffusion through the bilayer region because of the presence of multiple cationic groups and a strongly acidic group, respectively. β-Lactams allow us to measure their OM permeation process precisely by coupling their influx with their subsequent hydrolysis by the periplasmic β-lactamase (174), and the importance of the porin pathway can be ascertained by using porin-deficient mutants in this assay. For other classes of drugs, quantitative assays of the OM penetration process are difficult. However, one can artificially increase the permeability of the OM bilayer region, either by using an agent that disorganizes the LPS leaflet (polymyxin B nonapeptide) (176) or with mutants with partial defects in LPS synthesis (177). The MIC values of the agents mentioned above showed little change under these conditions, suggesting that they predominantly permeate through the nonbilayer pathway, i.e., through the porin channels, at least in *E. coli*.

The preference for the OM permeation pathway, however, is not absolute. We note in particular that among β-lactams, those that are more hydrophobic (e.g., oxacillin and cloxacillin) or larger (such as some third- or fourth-generation cephalosporins) tend to be hindered in their penetration through porin channels, and for them, permeation through the bilayer region, although slow, may become significant (176, 177).

It should be mentioned here that the situation is very different for organisms such as *P. aeruginosa* or *Acinetobacter* species, which do not produce classical *E. coli*-type trimeric porins that provide a fast influx of small drugs. The major nonspecific porin in these organisms is a homolog of *E. coli* OmpA, and its major function is structural, that is, to connect the OM to the underlying peptidoglycan (178). The porin function is produced by the alternative folding of only a small fraction of the protein (perhaps ~2% of the population) to produce ~16 transmembrane β-strands, and we proposed to call this class of porins "slow porins," in order to distinguish them from the classical trimeric porins, in which every molecule produces an open channel (179, 180). Because the number of open channels is small, OM permeability is very low, and β-lactams cross the OM of *P. aeruginosa* at a rate ~100 times lower than that for the *E. coli* OM (181, 182). Because of this slow permeation through slow porins, the endogenous RND system MexAB-OprM can compete well with the influx of hydrophilic β-lactams as well as other antibiotics (Fig. 1). Thus, the deletion of a component of this pump complex decreases the MICs of many antibiotics drastically (Table 2). The situation is similar for *A. baumannii* (183). Hence, with these organisms, even efflux at moderate rates is expected to produce significant increases in β-lactam MICs, and indeed, the genetic deletion of major efflux pumps decreases β-lactam MICs substantially (Table 2) (13, 184). If we increase the OM permeability of *P. aeruginosa* by adding polymyxin B nonapeptide, we see impressive decreases in antibiotic MICs, comparable to those obtained by the genetic deletion of MexAB-OprM (42).

Because LPS contains about six, usually all saturated, fatty acid chains in a single molecule, it is expected to produce a strong permeability barrier when organized into an LPS-only leaflet, as in the OM (22). Indeed, when the permeability of the OM bilayer to steroid probes (which are too large and too hydrophobic for pas-

TABLE 2 Effect of efflux pump gene inactivation on antibiotic MICs[a]

Agent	MIC (μg/ml)			
	P. aeruginosa PAO1		*E. coli* K-12	
	Wild type	*mexA*::*tet*	Wild type	Δ*acrAB*
Norfloxacin	>8	1	0.004	0.004
Ciprofloxacin	2	0.1	0.01	0.0025
Levofloxacin	0.25[b]	0.015[b]	0.063[c]	0.016[c]
Besifloxacin	1[d]	0.06[d]	0.25[d]	0.015[d]
Moxifloxacin	0.8[e]	0.05[e]		
Tetracycline	8–16	0.5[f]	1.25	0.156
Tigecycline	8[g]	0.25[g]	0.5[h]	0.125[h]
Chloramphenicol	16	4	6.25	0.78
Novobiocin	128	16	100	1.56
Erythromycin	512[i]	64[i]	50	1.56
Azithromycin	100	6.25	8[j]	0.5[j]
Benzylpenicillin	>1,024	512	16[k]	8[k]
Cloxacillin	5,120[l]	2.56[l]	256[k]	2[k]
Ampicillin			12.5	3.12
Carbenicillin	32	0.25	4[k]	1[k]
Azlocillin	4	0.5	16[k]	4[k]
Piperacillin	3[m]	0.4[m]	4[c]	0.25[c]
Cefoperazone	4	0.5	0.03[k]	0.015[k]
Ceftriaxone	64	8	0.0015[k]	0.0015[k]
Cefepime	2	1	0.0075[k]	0.0075[k]
Cefpirome	4	2	0.015[k]	0.015[k]
Imipenem	2	1–2	0.12[k]	0.12[k]

[a] The MIC data for *P. aeruginosa* are from reference 13, and those for *E. coli* are from reference 27, unless otherwise indicated.
[b] Data from reference 411, where a *mexAB-oprM* mutant was used.
[c] Data from reference 95.
[d] Data from reference 1009, where an *oprM* mutant was used for *P. aeruginosa*.
[e] Data from reference 392.
[f] Because the mutant strain contains the *tet* marker, the value for an *oprM* mutant is used (13).
[g] Data from reference 393.
[h] Data from reference 138.
[i] Data from reference 187.
[j] X.-Z. Li, unpublished data.
[k] Data from reference 188.
[l] Data from reference 390, where a *mexAB-oprM* mutant was used.
[m] Data from reference 391, where an *oprM* mutant was used.

TABLE 3 Molecular sizes and lipophilicities of antimicrobial agents and their relation with the effect of *acrAB* deletion on MICs in *E. coli* K-12[a]

Agent	Molecular weight	Lipophilicity (XlogP3)[c]	MIC (μg/ml) for strain	
			acrAB$^+$	Δ*acrAB*
Lipophilic and/or large agents				
Clotrimazole	345	5.0	>32	16
Cloxacillin[b]	436	2.4	256	2
Erythromycin	734	2.7	50	1.56
Fusidic acid	517	5.5	400	3.125
Methotrexate	454	−1.8	>640	80
Novobiocin	613	3.3	100	1.56
Puromycin	472	0.0	100	1.56
Rifampin	823	4.0	5	2.5
SDS	288	4.7	>12,800	100
Small and hydrophilic agents				
Ampicillin	349	−1.1	12.5	3.12
Cephalothin[b]	396	−0.4	4	4
Cefoxitin[b]	427	0.0	4	1
Ceftazidime[b]	547	0.4	0.12	0.12
Imipenem[b]	317	−0.7	0.12	0.12
Nalidixic acid	232	1.4	3.13	1.56
Norfloxacin	319	−1.0	0.004	0.004
Ciprofloxacin	331	−1.1	0.01	0.0025
Chloramphenicol	323	1.1	6.25	0.78
Tetracycline	444	−2.0	1.25	0.156

[a] The MIC data are mostly from reference 27.
[b] MIC data are from reference 188.
[c] The values of log*P* (the logarithm of the partition coefficient between *n*-octanol and water) are from PubChem (http://pubchem.ncbi.nlm.nih.gov/) based on the calculation by the XlogP3 method.

sage through porin channels) was examined, it was found to be ~2 orders of magnitude lower than that of the conventional phospholipid bilayer membranes (185). At the time when this study was carried out, the existence of multidrug efflux transporters was not known. More recently, however, such derivatives of steroid hormones were shown to be the substrates of AcrB (186). Thus, the permeability difference between the OM bilayer and the phospholipid bilayer might not quite reach 100-fold, yet it seems clear that the OM bilayer is an unusually impermeant barrier. Nevertheless, for large, lipophilic agents, the bilayer is the only possible pathway for OM permeation. These agents include macrolides, rifamycins, novobiocin, and fusidic acid (Table 3). Glycopeptides such as vancomycin and teicoplanin are large but not lipophilic. Nevertheless, because of their size, their only possible path to cross the OM is through the bilayer. Since these agents have so much difficulty in crossing the OM, they are usually considered agents effective against only Gram-positive bacteria. These agents can be active against Gram-negative bacteria if the LPS leaflet is breached (176, 177) or if the RND pump is inactivated (13, 187).

Drugs Traversing the OM Mainly through Porin Channels

The RND transporters, which play a predominant role in raising the MIC values of most antibiotics, pump out drugs mostly from the periplasmic space, as mentioned above (see Biochemistry and Genetics of Multidrug Efflux Pumps). They cannot create resistance if the drugs flow into the periplasm across the OM rapidly enough to counteract the rate of efflux. This is especially so because the RND pumps appear to have a relatively low velocity, for example, with AcrB having a velocity of ~0.3 nmol/s/mg cells (dry weight) for penicillins (65) (or turnover rates of the order of 100/s [see reference 63]). (In *E. coli*, the chromosomally encoded class C β-lactamase is expressed at a low, constitutive level, and thus, β-lactam MIC values are essentially determined by the balance between influx and active efflux.) On the other hand, ampicillin was measured to cross the OM with a permeability coefficient (*P*) of 2.8×10^{-4} cm/s (64) or at a rate of $P \times A \times \Delta c$, where *A* is the area of cell surface (~128 cm^2/mg) and Δc is the concentration difference of the drug across the OM. If the external concentration of ampicillin is 10 μg/ml (3×10^{-5} M), the influx rate is expected to be ~10 nmol/s/mg, which is much higher than the V_{max} of efflux. Thus, efflux has only a barely visible effect on the MIC of this drug in *E. coli*, as has been ascertained by the use of Δ*acrAB* mutants (Table 2) (188). Similarly, for relatively small antibiotics such as fluoroquinolones and tetracycline, which are expected to diffuse through the trimeric porin channels rapidly, AcrAB inactivation decreases their MIC values only minimally (Table 2).

Even among the β-lactams, however, more hydrophobic compounds, such as oxacillin or cloxacillin, diffuse through the *E. coli*

porin channels presumably rather slowly. Consequently, active efflux by AcrB strongly affects the MIC values, and the cloxacillin MIC decreases 128-fold, from 256 to 2 μg/ml, upon the deletion of *acrB* (Tables 2 and 3) (188). Because the *acrB* deletion hardly affects the ampicillin MIC, we thought previously that cloxacillin must be an exceptionally good substrate of AcrB and that ampicillin was a very poor substrate. Nonetheless, quantitative determination of efflux kinetic parameters (65) showed that these two drugs have similar affinities for AcrB and that the V_{max} is higher for ampicillin by only ~2-fold.

In contrast, in *P. aeruginosa*, where even small antibiotics must diffuse across the OM slowly via its slow porin, active efflux becomes very effective in increasing MICs, as seen from the fact that MICs of practically any antibiotic are drastically decreased upon the deletion of its major RND pump MexAB-OprM (Table 2). Here the situation with β-lactams becomes somewhat more complex, because with early compounds, hydrolysis by the powerful, inducible chromosomal β-lactamase plays a significant role. The relative lack of an effect of pump deletion on the fourth-generation cephems cefepime and cefpirome (Table 2) may suggest that they are poor substrates of the pump; it may also reflect the extreme stability of these compounds with the chromosomal class C enzyme (189). The imipenem MIC is hardly affected by the pump deletion, but this is because imipenem permeates across the OM much more rapidly than other compounds, by utilizing a specific channel, OprD (190). Thus, the efflux pump, even if it were capable of pumping out imipenem, would be overmatched by the rapid influx of the substrate (Fig. 1).

Drugs Traversing the OM through the Lipid Bilayer Region

Large molecules that cannot diffuse through the porin channels must penetrate the OM by slowly diffusing through the asymmetric bilayer domains, which have similarly low permeability in *E. coli* and *P. aeruginosa* (185, 191). Because of their slow influx, active efflux can become extremely effective, particularly when these molecules are preferred substrates for the efflux pumps, as can be seen in the huge decreases of MICs upon genetic inactivation of the main RND pumps (novobiocin and erythromycin) (Tables 2 and 3).

To recapitulate, the multidrug pumps work in synergy with the OM barrier. The pumps can make Gram-negative bacteria resistant only when the influx of the drug across the OM is relatively slow, and thus, efflux should always be considered in relation to the OM penetration process (Fig. 1). This also underscores the problems presented by organisms that produce slow porins, such as *P. aeruginosa* and *Acinetobacter*, because the efflux processes there become extremely efficient in increasing the resistance level.

GAMMAPROTEOBACTERIA: *ENTEROBACTERIACEAE*

Drug efflux pumps are widely distributed in bacterial species. The contribution of representative pumps to resistance and their synergistic interplay with other resistance mechanisms in clinical settings are further described below. The members of the *Enterobacteriaceae* family discussed in this section all produce high-permeability trimeric porins in their OM, although there are likely differences in the sizes of the channels.

E. coli

E. coli is a commensal resident of human and animal intestinal tracts but also includes various intestinal pathogenic types (enterotoxigenic, enterohemorrhagic, enteroinvasive, enteropathogenic, enteroaggregative, and diffusely adherent *E. coli*) as well as extraintestinal pathogenic *E. coli* (192). The major, constitutively expressed RND-type multidrug transporter is AcrB, although *E. coli* possesses a number of drug pumps of various families (Table 1) (15, 16). AcrB is described above as the prototype example for the structural and biochemical elucidation of RND pump transport mechanisms. The effect of *acrAB* genetic deletion on antimicrobial susceptibility is shown in Tables 1 to 3. Lipophilic (or large) compounds (e.g., erythromycin, novobiocin, fusidic acid, and cloxacillin) cannot diffuse easily through porins, and consequently, AcrB-catalyzed efflux becomes very effective in raising their MIC values to a range outside clinical utility (Table 3). Thus, efflux is responsible, in synergy with the OM barrier, for making *E. coli* intrinsically resistant to such compounds. In contrast, compounds that are smaller and usually hydrophilic (with the exception of nalidixic acid and chloramphenicol) (such as ampicillin, cephalothin, imipenem, and fluoroquinolones) can penetrate the OM barrier rapidly through porin channels. Therefore, in wild-type cells, AcrB cannot raise MIC values to a significant extent, although many of these drugs are likely to be good substrates of AcrB (for the case of ampicillin, see reference 65).

These small agents are of course useful in the treatment of *E. coli* infections. Regarding treatment options, a major advance in the 1960s was the introduction of semisynthetic penicillins and cephalosporins active against Gram-negative bacteria, such as ampicillin and amoxicillin, and the first-generation cephalosporins (e.g., cephalothin). Their efficacy decreased drastically with the spread of plasmids coding for class A β-lactamases (usually of the TEM or SHV type), which could hydrolyze these drugs rapidly. To counter this problem, in the 1980s, extended-spectrum cephems (third-generation cephalosporins) were introduced. They could withstand the assault of class A β-lactamases but were hydrolyzed at sufficient rates by overproduced, chromosomal, class C AmpC β-lactamases in *Enterobacter* and *Proteus*, etc. (but not in *E. coli*). In *E. coli*, these extended-spectrum cephems eventually became less useful because of the spread of plasmids producing TEM or SHV derivatives as extended-spectrum β-lactamases (ESBLs) that acquired a broadened substrate specificity. More recently, however, plasmids encoding class A CTX-M-type enzymes, which apparently originated from a chromosomal gene in an obscure genus called *Kluyvera*, have become so prevalent as to replace the older ESBLs (193). One common type, CTX-M-15, hydrolyzes a third-generation agent, cefotaxime, much faster than it does a first-generation compound, cephalothin. Other agents also entered the market around the time of the introduction of the extended-spectrum cephems or somewhat later. These agents include fluoroquinolones, semisynthetic aminoglycosides, and carbapenems.

It should be noted that there has been a steady increase in the resistance of *E. coli* isolates to the agents mentioned above. In a survey covering 30 years of isolates in Sweden (194), the prevalence of isolates showing "non-wild-type" MICs of ciprofloxacin increased from 0% to 40% in 2009. Drugs that have become essentially useless in recent years include ampicillin (70% showing non-wild-type MIC values), tetracyclines, and trimethoprim (up to 60%). It is even more alarming that these statistics are from Sweden, a country with one of the lowest frequencies of drug-resistant bacteria. The prevalence of *E. coli* isolates resistant to extended-spectrum cephalosporins was 4.4% in Sweden in 2012 but was much higher in some other European countries, e.g., 31%

in Slovenia (195). Efflux mechanisms likely contribute to such a rapid emergence of resistance in the presence of antimicrobial selection pressure, as discussed below.

Fluoroquinolones. Because fluoroquinolones are not hydrolyzed in bacteria, resistance to these agents involves the OM permeation barrier, active efflux, and mutational alterations to their targets, DNA topoisomerases. The target mutations are well known and are indeed present in practically all resistant strains of clinical origin. However, the first two factors also make frequent contributions, especially in strains exhibiting very high MIC values. Already in 1996, a pioneering study by Everett et al. (196) showed that among 36 *E. coli* strains with ciprofloxacin MICs of >2 μg/ml, 22 accumulated smaller amounts of ciprofloxacin than the wild-type strains in an energy-dependent manner, suggesting active efflux. That study also found alterations in porin expression, although their identities were not conclusively established. The involvement of efflux was also determined by the finding that fluoroquinolone-resistant isolates tended to be resistant to solvents (197). Since both fluoroquinolones and solvents are substrates of AcrB, this suggests strongly the involvement of this pump, an idea that was later confirmed by the discovery that these isolates had mutations in the *marR* repressor gene, which resulted in the overproduction of the MarA activator of *acrAB* transcription (198). The strong overexpression of MarA was also found in most levofloxacin-resistant clinical strains from Japan (199), and the overexpression of AcrA/AcrB was correlated with high levels of norfloxacin resistance among clinical isolates from the United States (200). Since MarA represses the expression of the larger channel porin OmpF (201), the downregulation of OmpF in some strains might have been caused in this manner. Among isolates resistant to amoxicillin, co-trimoxazole, and quaternary ammonium disinfectants, TolC overproduction was common, and this appeared to be caused by MarA or SoxS overproduction (202). High-level fluoroquinolone-resistant strains from China contained mutations in the *acrR* gene (203). In any case, numerous studies have now confirmed the important role of AcrAB overproduction in high-level fluoroquinolone resistance (188, 200, 204). Although the levels of porins were not examined frequently, a study showed that 10 out of 11 highly fluoroquinolone-resistant isolates had a strongly decreased expression level of OmpF (205). Additionally, a plasmid-encoded fluoroquinolone-specific MFS pump, QepA, was reported in 2007 (206).

β-Lactams. Most cephalosporins and penicillins are substrates of AcrB. With sophisticated methodologies using intact cells, the kinetic constants for efflux have been determined for about a dozen β-lactams (63–65). Older compounds (e.g., ampicillin, cephalothin, and cephaloridine) were effective against *E. coli* partly because they were relatively small and hydrophilic and thus penetrated rapidly through the OmpF and OmpC porins (174, 207, 208). As described above (see Synergy with the Outer Membrane Barrier), active efflux cannot increase the MICs of these rapidly penetrating compounds, and indeed, the deletion of *acrAB* has no or very minor effects on their MICs (188). However, if porin permeability is decreased, efflux would produce a more visible effect. In a study reported in 1981, selection with carbenicillin easily enriched for mutants lacking the wider-channel OmpF and producing only the more restrictive OmpC porin (209). Since carbenicillin is not easily hydrolyzed by the chromosomal AmpC β-lactamase (210), from the vantage point of 2014, we can conclude that the mutant lacking OmpF became resistant to carbenicillin because the balance between influx and active efflux was perturbed. (The reader should be reminded here that even a very slowly penetrating drug would reach a half-equilibrium concentration in the periplasm within 1 min [21]. Thus, counteracting mechanisms such as efflux or enzymatic hydrolysis are absolutely necessary to raise the β-lactam MIC beyond the range needed for inhibiting their targets.) Indeed, one study (209) showed that the mutant became much more resistant to good substrates of AcrB, such as benzylpenicillin (65) or cephaloram, but not to poor substrates of AcrB, such as cefazolin and cephaloridine (63). A similar *in vitro* selection of porin-deficient strains was achieved by using cefoxitin (211), which is also only slowly hydrolyzed by the chromosomal β-lactamase. Laboratory selection using ceftazidime or ceftibuten, starting from an *E. coli* strain containing a TEM-1-producing plasmid, also resulted in the loss of OmpF or OmpF and OmpC (212). A clinical example involving the loss of a porin during therapy with a first-generation cephalosporin is described below in the section on *Salmonella* spp.

The loss or downregulation of porins thus seems an important mechanism for increased resistance to β-lactams in *E. coli* (as with *Enterobacter* and *Klebsiella*, described below), but unfortunately, this has not been examined in most studies of clinical isolates of this species. One would predict (see also above) that such a mechanism may be even more important with the extended-spectrum cephems because they tend to be larger and tend to penetrate more slowly through wild-type porins (208). In rare studies where the porin pattern was examined, its alteration was found. In 1991, seven out of the eight amoxicillin-clavulanate-resistant strains examined were found to produce reduced levels of or no OmpF protein (213). In the era dominated by extended-spectrum cephems, a study of clonally related cefoxitin- and ceftazidime-resistant strains from a Spanish hospital in 2000 showed that all strains were deficient in OmpF and produced AmpC β-lactamase at a high level (214). In a 2003 study of *E. coli* strains resistant to extended-spectrum cephems, most strains not producing ESBLs had altered patterns usually involving the apparent loss of OmpF (215). The absence of OmpF was also seen in a ceftazidime-resistant strain (216). In these porin-deficient strains, efflux is likely to contribute to the increased MIC values, although an even stronger contribution would probably come from enzymatic hydrolysis.

More recently, the widespread use of carbapenems appears to have selected porin-deficient mutants in *E. coli* (217, 218), as is also seen in other species of *Enterobacteriaceae* (see below). This could be because the widely disseminated enzymes cannot yet hydrolyze carbapenems very efficiently. The prevalence of porin-deficient strains is also consistent with the fact that imipenem, a frequently used agent, is most probably a poor substrate of AcrB; thus, porin loss, but not pump overproduction, raises the MIC values. However, other carbapenems, containing larger and often hydrophobic side chains, behave as the substrates of RND pumps (see reference 219, for example).

A priori, a strong overproduction of AcrAB-TolC should be able to increase the MIC values of β-lactams that are the substrates of this pump. However, AcrB is already constitutively expressed, and mutations in the global regulatory systems Mar, Sox, and Rob (see Regulation of Multidrug Efflux Pumps, below) can increase its expression only severalfold and may not produce a strong effect on the MIC, especially when enzymatic hydrolysis also plays a role in resistance. Perhaps this might be why pump overproducers were often not noted among clinical resistant isolates.

Other drugs. Given its extremely wide specificity, the AcrAB-TolC system obviously makes a major contribution to the increased resistance to other drugs (Tables 1 to 3). Even for rifamycins, which are large lipophilic molecules and expected to cross the OM via its lipid bilayer, the *acrAB* status has shown a modest impact on rifampin susceptibility. A relatively low level (20 μg/ml) of PAβN, an EPI of the AcrB pump, also decreased the MIC (>128-fold) of rifaximin, a rifamycin derivative, for resistant strains with no target mutations (21, 220), suggesting that efflux can contribute to rifamycin resistance under certain conditions (e.g., strain or species specific) (see also the section on *Neisseria* for rifampin as an MtrCDE pump substrate, below). However, as expected, the susceptibility of *E. coli* to rifampin has also been known to be impacted by the OM status (177).

Salmonella spp.

A clinical *Salmonella enterica* serovar Typhimurium isolate that lost OmpC and became resistant to the earlier-generation cephems was reported in 1987 (221). A patient who received a kidney transplant developed septicemia involving this organism, which developed cephalexin resistance only 3 days after this drug was added to the regimen. The pretherapy strain produced OmpF and OmpC in the laboratory, but the posttherapy isolate produced only OmpF. Presumably, the synthesis of the OmpF porin was strongly repressed by the relatively high salt content within the human body and was thus irrelevant during therapy, and hence, the posttherapy strain allowed only a minimal influx of drugs, which made it more resistant to cephalexin, which was hydrolyzed readily by the plasmid-encoded TEM β-lactamase in both isolates.

In *Salmonella*, OmpF and OmpC are the major porins, and AcrB is also the major, constitutive multidrug pump, although there are a total of 5 chromosomally encoded RND systems identified (i.e., AcrAB-TolC, AcrA-AcrD-TolC, AcrEF-TolC, MdtABC-TolC, and MsdABC or MsdAB-TolC) (58, 222–224). Importantly, RamA, a positive regulator of the *acrAB* transcription that does not exist in *E. coli*, seems to play a major role in AcrAB overproduction (see Regulation of Multidrug Efflux Pumps, below). Thus, for fluoroquinolones, we would expect situations similar to those that have been found for *E. coli*. In 1993, Piddock and associates found that posttherapy, ciprofloxacin-resistant isolates accumulated much less drug inside the cells (225), a phenomenon that is now interpreted to be a result of AcrB-driven efflux. Although mutations in target DNA topoisomerases are important, very high-level resistance to fluoroquinolones seems to require the additional contribution of increased efflux (188, 226), and this was the case in the DT204 clone (227). Among nontyphoid *Salmonella* isolates from Spain, ~40% were nalidixic acid resistant, although their ciprofloxacin MICs were below the clinical resistance breakpoint; in all of these strains, MICs of nalidixic acid (and ciprofloxacin) decreased strongly in the presence of the AcrB inhibitor PAβN at 20 μg/ml (228) (see Efflux Pump Inhibitors, below). In laboratory-selected ciprofloxacin-resistant *S. enterica* serovar Enteritidis mutants, AcrB overproduction was present, and in one mutant, a deficiency of the OmpF porin was also present (229). About 5% of *S. enterica* serovar Typhi strains isolated in South Africa were nalidixic acid resistant, and their nalidixic acid and ciprofloxacin MICs decreased strongly in the presence of PAβN, although this inhibitor was used at a high concentration of 40 μg/ml (230). With β-lactams, one might also expect a situation similar to that found in *E. coli*, i.e., decreased porin expression and efflux contributing to resistance together with hydrolysis by β-lactamases encoded by R plasmids. In fact, in a plasmid-free strain, the contribution of efflux is more pronounced because of the total absence of enzymatic hydrolysis, caused by the absence of the chromosomal *ampC* gene in this species (231). Thus, in *S. enterica* serovar Typhimurium, inactivation of *acrAB* decreases MIC values of penicillins and cephalosporins quite strongly (58).

Efflux is a significant factor for most β-lactams, with the exception of cefazolin, which is too hydrophilic to be a substrate for AcrB (58, 63). These data agree with the actual measurements of AcrB-catalyzed efflux of β-lactams (63–65). However, changes in porins and efflux have been rarely reported for β-lactam-resistant *Salmonella* isolates. These results may be related to the ecology of these organisms. *Salmonella*, as a pathogen of farm animals, tends to exist in a strongly clonal manner (232). Therefore, once a clone acquires a resistance plasmid, it will remain and can become the major mechanism of resistance. During the recent period in which extended-spectrum cephems were among the most important agents for therapy, strains containing plasmid-encoded AmpC β-lactamases (e.g., CMY enzymes) and ESBL β-lactamase (e.g., CTX-M) were prevalent among resistant strains (233). They have an advantage over porin-depressed or efflux-enhanced strains, which may have to pay heavily for these unfavorable metabolic changes. In addition, with *E. coli* (bloodstream) infections, resistant mutants can be selected during the course of therapy, thus favoring porin or efflux mutants. Infections with nontyphoid *Salmonella* may be caused frequently by strains already containing R plasmids (232, 234). In this regard, there are increasing numbers of reports of multidrug-resistant or extensively drug-resistant isolates of *Salmonella* that carry the plasmid-encoded OqxAB RND pump with other resistance determinants such as *aac(6′)-Ib-cr* and/or CTX-M genes (228, 235, 236).

Regarding porin mutants, there are a few reports implicating porin downregulation in β-lactam resistance. Armand-Lefèvre et al. (237) showed that OmpF was completely deficient in an imipenem-resistant, CMY-4 β-lactamase-producing isolate of *S. enterica* serovar Wien and that the resistance can be reproduced in an OmpF-deleted *E. coli* strain. Su et al. (234) reported that treatment of a patient infected by *S. enterica* serovar Typhimurium with ertapenem led to the development of carbapenem resistance concomitant with the mutational inactivation of OmpC, which was the only strongly expressed porin in the parent strain; this confirms the above-mentioned prediction that porin loss would be one mechanism of resistance development during the course of therapy. In a report with results more difficult to interpret, the downregulation of OmpD was correlated with an increased MIC of ceftriaxone (238). Unfortunately, nothing is known about the permeability of the channels of OmpD, although its *E. coli* homolog, NmpC, showed less single-channel conductance in NaCl than with OmpF and OmpC (239).

Citrobacter spp.

The genus *Citrobacter* is closely related to *Salmonella*, and one would expect rather similar situations. Thus, some fluoroquinolone-resistant isolates of *Citrobacter freundii* were found to accumulate less drug (240). *In vitro* selection of fluoroquinolone-resistant mutants showed the importance of increased efflux in practically all cases (241). Intrinsic resistance to linezolid is mediated by AcrB in this species as well as others (242). A *C. freundii*

isolate containing the plasmid-encoded RND pump was reported recently (243) (see Drug Efflux Genes on Plasmids, below).

Enterobacter aerogenes and *Enterobacter cloacae*

In contrast to *E. coli*, the chromosomally encoded *ampC* β-lactamase in *Enterobacter aerogenes* and *Enterobacter cloacae* is strongly inducible, and thus, penicillins and the first-generation cephalosporins were ineffective for them. The third-generation cephalosporins were initially thought to be resistant to AmpC-catalyzed hydrolysis. However, strongly overproduced AmpC turned out to have a low V_{max} but a very high affinity for these drugs (244) and to be capable of producing significant resistance. The fourth-generation cephems and carbapenems are quite effective against nonplasmid-containing strains. *Enterobacter* spp. produce the constitutive AcrB pump as in *E. coli*. (Another RND pump gene, *eefB*, was cloned from *E. aerogenes*, but its inactivation had no effect on the MICs of antibiotics [245].) They also possess the *E. coli*-like porins OmpF and OmpC, and hence, one would expect situations in these organisms similar to those seen in *E. coli* regarding their mechanisms of resistance to β-lactams and fluoroquinolones.

When a large number of β-lactam-resistant strains of *Enterobacteriaceae* from French hospitals was examined for the production of porins by Pagès and associates in 1996 (246), 6% of resistant strains of *E. cloacae* and 44% of resistant strains of *E. aerogenes* lacked detectable porins. The very high fraction of porin deficiency in *E. aerogenes* is striking. As these bacteria are commensal organisms, they presumably had less chance to acquire R plasmids than did obligatory pathogens like *Salmonella*, at least up to 1996 (although the plasmids producing ESBLs have become increasingly common in recent years). Furthermore, the resistant strains were probably selected mostly during therapy, favoring mutational mechanisms. Still, the difference in the frequency of porin-deficient strains between *E. aerogenes* (20 out of 44 resistant strains) and *E. cloacae* (1 out of 17 resistant isolates) begs for an explanation. The porins of at least one strain of *E. cloacae* had a five-times-lower permeability than did those of *E. coli* (244), so perhaps, in this organism, the influx of drugs across the OM is already limited, even without any mutation. Cephaloridine permeation of *E. cloacae* was found to be 25-fold lower than that of *E. coli* by another laboratory (247). (The OM permeability of *E. cloacae* to β-lactams, measured by a novel method [248], was reported to be 20 to 1,000 times lower than that of *E. coli*. However, the *E. coli* values were not obtained in the same way, and it is uncertain if a quantitative comparison is warranted.) In contrast, *E. aerogenes* was found to have a permeability very similar to that of *E. coli* (249), and thus, the creation of significant levels of resistance may need the downregulation of porins in addition to drug hydrolysis and/or efflux. (OmpF and OmpC from *E. aerogenes* were studied in detail, and the repression of OmpF biosynthesis by high osmotic pressure resulted in MIC increases of 8- to 16-fold [250] for slowly penetrating compounds such as cefotaxime, ceftazidime, and ceftriaxone [208].) An imipenem-resistant isolate of *E. cloacae* was deficient in both OmpF and OmpC (247). A carbapenem-resistant mutant of *E. cloacae*, selected during imipenem therapy, lacked both OmpF and OmpD porins (251); furthermore, ertapenem and meropenem MIC values were decreased strongly in the presence of 40 μg/ml PAβN, although overexpression of AcrB was not evident. (Imipenem does not appear to be a substrate of the *P. aeruginosa* MexAB-OprM system [13], but meropenem, a derivative with a lipophilic side chain, is a substrate [252]. Ertapenem, another carbapenem with a lipophilic side chain, is also expected to be a substrate for MexAB-OprM and homologous AcrAB.) *In vitro* selection for *E. cloacae* mutants with increased MICs of ceftazidime, cefepime, or cefpirome often resulted in strains defective in OmpF (253, 254). A study of ertapenem-resistant isolates from Taiwan (255) found that 43% were altered in the expression of porins (and 96% expressed a multidrug pump). In another study of carbapenem-resistant isolates (256), both OmpF and OmpC porins were totally lacking in two *E. aerogenes* isolates, whereas variable decreases in porin levels were found in *E. cloacae* isolates. Similarly, an imipenem-resistant *E. aerogenes* isolate lacked both OmpF and OmpC (257). A more recent study demonstrated that porin expression often becomes altered when *E. aerogenes* infection is treated with imipenem (258).

The Pagès group continued their work on *E. aerogenes* clinical isolates, which resulted in several studies. In one cephalosporin-resistant isolate, there was a missense mutation leading to an altered porin with decreased permeability (259). They also identified an increased efflux of fluoroquinolones and chloramphenicol in some multidrug-resistant strains (260), which is likely to be due to AcrB (261). Finally, frequencies of chloramphenicol-resistant isolates susceptible to the AcrB inhibitor PAβN appeared to have increased between 1995 and 2003, suggesting a larger role for the broad-specificity efflux mechanism in recent years (262).

As would be expected from results with *E. coli* and *Salmonella*, a large fraction of fluoroquinolone-resistant isolates of *E. cloacae* appeared to overproduce a multidrug pump, presumably AcrB (263). Deletion of *acrA* predictably decreased the MICs of oxacillin, erythromycin, clindamycin, linezolid, ciprofloxacin, chloramphenicol, tetracycline, and tigecycline (264). All multidrug-resistant *E. aerogenes* strains tested (also resistant to β-lactams) were found to overproduce AcrAB (265), but the effect of an EPI on β-lactam MICs was not examined. Among the large, lipophilic agents that have little activity against these species, macrolides are AcrAB substrates, but a ketolide, telithromycin, appeared to also be pumped out by an additional pump that is susceptible to PAβN inhibition (266). *Enterobacter* spp. develop resistance to tigecycline through AcrAB overproduction (during ciprofloxacin treatment in one study [267]), and this was caused by the overproduction of the RamA regulator (268), although one study concluded that the overexpression of RamA, an not necessarily that of AcrAB, correlated with the resistance, suggesting the involvement of other efflux pumps (269).

Klebsiella pneumoniae

Klebsiella pneumoniae produces the *E. coli*-like trimeric porins OmpK36 (an OmpC homolog) (270) and OmpK35 (an OmpF homolog) (271). Their permeability has not been measured in a way that could be compared with that of *E. coli* porins. However, our recent measurement of ampicillin and benzylpenicillin permeation in strain ATCC 11296 showed that the OM of this species is vastly more permeable to benzylpenicillin than *E. coli* OmpF, yet the permeability to ampicillin is actually similar (S. Kojima, E. Sugawara, and H. Nikaido, unpublished data), an observation that explains why porin deficiency is needed for resistance in *K. pneumoniae*. It also produces the ubiquitous AcrAB-TolC pump, whose deletion predictably produces hypersusceptibility to erythromycin, chloramphenicol, nalidixic acid, fluoroquinolones, cefoxitin, and cefotaxime and surprisingly produces hypersuscepti-

bility to gentamicin (272). Thus, some aspects of the resistance mechanisms are expected to be similar to what was found for the species discussed above. Indeed, the loss of porins was reported frequently to be the contributing mechanism of resistance, beginning with a 1985 report by Gutmann and associates (273) that one-step selection with nalidixic acid, trimethoprim, or chloramphenicol yielded mutants showing cross-resistance to all three agents and with detectable changes in OM protein patterns. Later, the selection of a porin-deficient mutant in a patient treated with cefoxitin was reported by the same group (274). During the 1990s, *in vitro* selection of porin mutants by cefoperazone-sulbactam (275) or cefoxitin (276) was confirmed, and clinical porin-deficient isolates resistant to cefoxitin and extended-spectrum cephems (277, 278) were reported. Porin loss is often caused by the insertion of an insertion sequence (IS) element (279). In the present century, reports on porin loss are so numerous that it is impossible to give an exhaustive list of the literature; we give only a few representative examples (280, 281). We note, however, that clinical isolates resistant to carbapenems often lack porins (257, 282, 283). The deletion of OmpC alone has little effect on antibiotic MICs, but the loss of OmpF has a large effect, as predicted (284). When OmpC or OmpF was expressed from plasmids in a strain expressing an ESBL but lacking both porins, MIC values for most agents decreased significantly, but the extent of the decrease was greater with the expression of OmpF (OmpK35) (271). The difference was larger with more bulky agents: for cefpirome (515 Da), the MIC of 512 μg/ml in the porin-deficient strain was reduced to 4 μg/ml by the expression of OmpC, whereas OmpF expression reduced it to 0.5 μg/ml. The MIC of a large agent, ceftazidime (547 Da), which was >512 μg/ml in the porin-deficient parent strain, was reduced only to 256 μg/ml by the expression of OmpC, whereas OmpF expression decreased it to 2 μg/ml. On the other hand, for a smaller agent, meropenem (383 Da), the expression of either porin decreased the MIC of 4 μg/ml in the parent strain to the same value, 0.03 μg/ml. These results suggest that OmpF produces a more permissive, possibly larger, diffusion channel than does OmpC. (This concept may also explain why there have been some confusing data on the role of OmpF versus OmpC for carbapenem susceptibility [see reference 285].) A similar explanation was invoked to explain the higher level of resistance to a more bulky ceftazidime than a smaller cefotaxime in a strain lacking only OmpF (286); this seems to be a valid hypothesis since the strain produced an ESBL enzyme that hydrolyzed cefotaxime much better, on the basis of V_{max}/K_m values.

In recent years, *K. pneumoniae* has become a major nosocomial pathogen causing outbreaks of multidrug-resistant clones. Such clones typically express ESBLs (more recently of the CTX-M type) and/or AmpC-type enzymes. These enzymes obviously make predominant contributions to general β-lactam resistance, thus making carbapenems some of the few remaining effective drugs. Recently, however, porin deficiency combined with enzymes with marginal carbapenemase activity has attracted attention. One of the often encountered types combines a frameshift null mutation of OmpF with a mutational change in OmpC, which inserts a few residues into loop 3 that create the narrow constriction of the channel (for example, see references 283, 285, 287, and 288). The role of porin deficiency is often underestimated. For example, Zhang et al. (285) conclude that porin alterations play only a minor role because the removal of the KPC-2 plasmid reduces the carbapenem MICs drastically. Still, unlike the presence of β-lactamase, the porin deficiency by itself cannot raise the carbapenem MICs substantially. (The proper experiment should have been the addition of the same plasmid to the wild-type and porin-deficient strains.) Considering that active efflux contributes to resistance to agents other than imipenem in organisms with a low-permeability OM, such as *P. aeruginosa* (252), efflux is likely to also make a contribution in porin-deficient *K. pneumoniae*, but this remains a topic for future study. Finally, LamB appeared to allow some permeation of carbapenems in a strain deficient in both OmpF and OmpC (289).

The genome of *K. pneumoniae* shows the presence of a large number of drug efflux pumps similar to those observed in *E. coli* (290). The overexpression of efflux pumps, most probably that of AcrAB, is important in resistance to certain agents, such as chloramphenicol and tetracycline but especially fluoroquinolones (see references 291 and 292). In an isolate lacking porins and showing high-level fluoroquinolone resistance, an efflux pump was apparently expressed strongly, as judged from the low level of norfloxacin accumulation in the absence of energy poison (293). In an important study of ESBL-producing strains from outbreaks of nosocomial infections (294), ciprofloxacin resistance (caused in part by AcrB overproduction) was strongly correlated with cefoxitin resistance, suggesting that for β-lactam resistance, not only the β-lactamases and porin depletion but also efflux may make a significant contribution. Similarly, ESBL-producing strains often lack porins and show a stronger efflux of fluoroquinolones (295). The significance of efflux in β-lactam resistance was pursued later by the Pagès group (296), who examined cefoxitin-resistant but ceftazidime-susceptible clinical isolates. Although β-lactam resistance is most frequently caused by the plasmid-encoded ESBLs, the ceftazidime susceptibility of these strains did not fit with this idea. Indeed, all the strains examined were devoid of plasmid-encoded β-lactamases and produced only the chromosomal class A enzyme. However, in the presence of PAβN (50 μg/ml [unfortunately an excessive concentration]), the MICs of cefoxitin (and cloxacillin), erythromycin, chloramphenicol, nalidixic acid, and ofloxacin decreased strongly, implicating active efflux as a major factor in resistance. There was little decrease in the ceftazidime MIC; as we mention above, ceftazidime is too hydrophilic to be a good substrate of AcrB. Similarly, studies by Källman et al. (297, 298) are important because they show that AcrAB-mediated efflux contributes to cefuroxime resistance in both *K. pneumoniae* and *E. coli*, together with the porin deficiency and β-lactamase-catalyzed hydrolysis. Additionally, *in vitro* selection with cefoxitin and fluoroquinolones, starting from the drug-susceptible revertants of the clinical strains described above, resulted in AcrB overproduction caused mainly by mutations in *ramR* and in one case in *soxR* (299). A recent study linked *ramR* mutation-driven overexpression of AcrAB to tigecycline resistance in KPC-producing strains (300), although one study suggested that RamA-AcrB overexpression occurred only in about one-half of the tigecycline-resistant isolates (301).

Finally, efflux pumps other than AcrB also contribute to resistance. In addition to MDR mediated by the chromosomally encoded OqxAB pump (302–304), overexpression of the newly identified RND-type KpgABC pump was involved in tigecycline nonsusceptibility due to an IS element insertion in the promoter region of *kpgABC* (305). The KexD RND pump expressed by cloning provides MDR with the requirement of AcrA accessory and KocC OM proteins (306). KpnEF, an SMR pump, contributes to

MDR and may be important in the infection process, because its expression is regulated by the Cpx regulatory system, which also affects capsule production (163). Inactivation of the MFS-type KpnGH pump results in 4- to 10-fold MIC reductions of the third- and fourth-generation cephalosporins, spectinomycin, streptomycin, and tetracycline (307).

Proteus, *Providencia*, and *Morganella* spp.

In an extensive study in 1987 (308), it was shown that two major OM proteins (presumably porins) are produced in *Proteus mirabilis*, *Proteus vulgaris*, *Morganella morganii*, *Providencia rettgeri*, and *Providencia alcalifaciens* and that the major porin in every case had channel properties similar to those of *E. coli* OmpF. Furthermore, *in vitro* selection with cefoxitin led to the isolation of mutants deficient in the major porin in each species. Since the chromosomal AmpC β-lactamases of these species can hydrolyze cefoxitin only extremely slowly and cefoxitin passes through the porin channel only slowly, this selection is similar to what has been achieved with carbenicillin and *E. coli* K-12, resulting in the loss of the porin with a larger channel. Interestingly, in 4 out of 5 strains, the MIC of tetracycline also increased upon selection with cefoxitin, suggesting that strains derepressed for a multidrug pump, presumably AcrB, were also selected. In a fluoroquinolone-resistant *M. morganii* isolate selected in the laboratory, there was a downregulation of what appeared to be OmpF and increased norfloxacin uptake in the presence of CCCP, indicating active efflux (309).

The substrate range of AcrB from *P. mirabilis* includes, as expected, a number of antibiotics, dyes, and detergents (310, 311). A similar range was found in a tigecycline-resistant clinical isolate of *M. morganii* overexpressing AcrAB, whose inactivation has led to hypersusceptibility to multiple agents, including a 130-fold reduction of the tigecycline MIC value (312). In a 1992 study (313), a cefoxitin-resistant laboratory isolate of *P. vulgaris* not only lacked OmpF but also accumulated smaller amounts of most fluoroquinolones; although multidrug pumps were not known at that time, it seems clear that resistance was the result of synergy between the decreased OM permeability and AcrB-catalyzed efflux. This study found that porin loss did not diminish the intracellular accumulation of two agents, sparfloxacin and tosufloxacin. Since it is difficult to imagine that these compounds cross the OM by a unique, unconventional route, this result, if confirmed, may indicate that the major pump discriminates between these substrates. Isolates of *P. rettgeri* from larvae of the oil fly have shown a correlation between natural resistance to a variety of antimicrobials and organic solvent tolerance, a phenomenon indicating strong efflux involvement (314).

Serratia marcescens

Porins of *Serratia marcescens* are similar to OmpF/OmpC of *E. coli* in terms of cephalosporin permeation (315). *S. marcescens* isolates selected in increasingly higher concentrations of cephalosporins, often those compounds that are not easily hydrolyzed by the endogenous β-lactamase, were found to lack one or more porins (316, 317). Similar porin-deficient *S. marcescens* isolates were also selected by using chloramphenicol, nalidixic acid, or trimethoprim and showed cross-resistance to β-lactams (273); from the vantage point of today, it seems quite likely that at least some mutants were overexpressing efflux pumps. Selection with moxalactam yielded a mutant strain lacking a "42-kDa" porin, or OmpF, and its OM showed a permeability to cephaloridine >100-fold lower than that of the parent strain, in spite of the presence of the "40-kDa" or OmpC porin (318). In contrast, OmpC deletion had little influence on antibiotic susceptibility. Salicylate induction of fluoroquinolone resistance was ascribed to the decreased expression level of OmpF (319); however, increased production of AcrB is likely to have played a more important role.

As expected, fluoroquinolone-resistant strains produced higher levels of an RND pump in *Serratia* (15). Cosmid cloning of the genes responsible for this phenotype identified *sdeA* and *sdeB*, which confer resistance to fluoroquinolones, chloramphenicol, sodium dodecyl sulfate (SDS), and ethidium (320). SdeAB's function is dependent on the TolC-like OM protein HasF, and *sdeB* inactivation increases the susceptibility to fluoroquinolones and other drugs listed above (321). Exposure to a biocide (cetylpyridinium chloride) led to a mutational upregulation of SdeAB and thus antibiotic resistance (322). SdeAB expression is controlled by a BadM-type repressor, SdeS (323), and a putative MarA-like regulator, SdeR (321). Another RND system (SdeCDE) requires two paired pump genes, *sdeDE*, but their deletion did not seem to affect the MICs of common antibiotics (320).The RND pump SdeXY produces resistance to fluoroquinolones as well as many substrates of AcrB (16). Its overexpression (with HasF participation) is responsible for tigecycline and fluoroquinolone resistance (324). SdeY, however, has a sequence 84% identical to that of *E. coli* AcrB and should more properly be called AcrB. Thus, at present, the relative importance of AcrB and SdeB in fluoroquinolone efflux remains unclear, and the expression levels of *sdeB* in clinical isolates had no clear correlation with their fluoroquinolone resistance levels (325). An MFS pump (SmfY), an SMR pump (SsmE), and an ABC pump (SmdAB) increased the MICs of several drugs when expressed from a plasmid in *E. coli* (16).

Shigella flexneri

For *Shigella flexneri*, the involvement of efflux, including AcrAB overproduction, leading to high-level resistance to fluoroquinolones, was reported (326, 327). Inactivation of the MFS pump MdfA-encoding gene led to an 8-fold decease in the norfloxacin MIC (328).

Yersinia enterocolitica and *Yersinia pestis*

The genus *Yersinia*, although relatively distantly related to *E. coli*, produces the classical trimeric porins OmpF and OmpC (329). It also contains genes for RND pumps. In one study, all 41 nalidixic acid-resistant isolates of *Y. enterocolitica* showed significant decreases in nalidixic acid MICs in the presence of 20 μg/ml PAβN, suggesting a strong contribution of efflux (330). A Mar homolog was identified in *Y. pestis*, and its overexpression increased, albeit to a small degree, the MICs of several antibiotics, including tetracycline and rifampin (331), presumably by the increased expression of the AcrB-type pump. Moreover, a homolog of a well-known activator of *acrAB* transcription, Rob, increased ofloxacin MICs >10-fold (332). Indeed, the *acrAB* deletion resulted in large decreases in MICs of many antimicrobials, including aminoglycosides (333). Lastly, an MFS pump, together with a KefC-like exporter, pumped out cationic antimicrobial peptides in *Y. enterocolitica*, and their deletion made cells more susceptible to novobiocin and tetracycline (334).

OTHER GAMMAPROTEOBACTERIA: *VIBRIO*, *AEROMONAS*, *LEGIONELLA*, AND *PASTEURELLACEAE*

This section examines genera and species that are known or suspected to produce high-permeability trimeric porins and thus are similar to *Enterobacteriaceae* in this respect.

Vibrio spp.

Vibrio spp. are outside the *Enterobacteriaceae* but not very far in terms of phylogeny. *V. cholerae* has attracted attention as the causative agent of cholera. Its OM appears to be significantly different from that of *Enterobacteriaceae*. First, its outer leaflet appears to contain phospholipids in addition to LPS (335), which should allow an unusually rapid permeation of large, lipophilic antibiotics. Indeed, *V. cholerae* is far more susceptible to such agents, e.g., erythromycin, novobiocin, and rifampin, than are members of the *Enterobacteriaceae* (336). Second, its major porins, OmpU and OmpV, albeit belonging to the OmpF/OmpC family, are quite different from their enterobacterial representatives in sequences (22) and in permeability. An early study on OmpU (337) found that its single-channel conductivity was ~3 times higher than that of *E. coli* OmpF, consistent with the results of a liposome swelling assay revealing a much larger channel than that of OmpF (338). Finally, some of the bile salts apparently bind to the channel interior and block permeation (339), suggesting that these large, planar, lipophilic molecules diffuse through the channel, at least up to the constriction zone. These high-permeability characteristics of the *V. cholerae* OM are surprising for a gastrointestinal pathogen but may be related to the ecology of this organism as a water dweller.

Because of their genetic relatedness to *E. coli*, cloning of *Vibrio* genes in *E. coli* was not difficult, and a number of efflux systems have been identified in this manner. The *V. cholerae* genome contains 6 RND transporters (VexAB, VexCD/BreAB, VexEF, VexGH, VexIJK, and VexLM), and most of them require TolC for drug efflux (340–345). VexAB seems to play a predominant role in the efflux of antibiotics (benzylpenicillin, erythromycin, and polymyxin B) and detergents (cholate, SDS, and Triton X-100) (341), although among the 6 RND systems cloned from a non-O1 strain, VexEF conferred the strongest resistance to an *E. coli* host (343). VexCD (confusingly "renamed" BreAB) is involved in bile efflux (340) and is induced by bile (342). A drug-susceptible phenotype could also be created by adding EPI PAβN or NMP to the parent strain (346). VexGH shows an overlapping substrate specificity and also contributes to the production of cholera toxin (344). In an interesting study (345), it was shown that not only was the expression of VexAB and VexGH enhanced by the CpxAR system, but the expression of this system also was stimulated by the deletion of efflux pumps, presumably due to the accumulation of intracellular metabolites. In *V. parahaemolyticus*, 12 RND pumps were identified, and when expressed in hypersusceptible *E. coli*, two-thirds of them produced an MDR phenotype (347–349), although the deletion of one of them, VmeAB, produced little increase in drug susceptibility (347). The isolation of laboratory mutants resistant to deoxycholate showed that VmeTUV is important for the efflux of bile salts (349).

Among the MFS transporters, VceB was the earliest discovered drug transporter in *V. cholerae* (350); it occurs together with a periplasmic accessory protein, VceA, and a TolC-like OM protein, VceC (16), and contributes to the intrinsic levels of resistance to deoxycholate, CCCP, pentachlorophenol, and nalidixic acid (350). Another MFS transporter, called EmrD-3, was found by cloning in a drug-hypersusceptible *E. coli* host and produced resistance to various lipophilic agents, such as linezolid (351). Five MFS transporters (each under the control of a Lys-type MfsR regulator) whose deletion causes tetracycline and bile salt hypersusceptibility are known (352). *V. parahaemolyticus* was the organism in which the first MATE family transporter, NorM, was discovered by cloning in *E. coli* (353), and in *V. cholerae*, 5 members of this family were described (16), including the NorM homolog VcmA (15), which increases MICs of fluoroquinolones, ethidium bromide, acriflavine, and doxorubicin when overproduced in *E. coli*. Two MATE transporters from a multidrug-resistant *V. fluvialis* isolate provided 2-fold increases in MIC values of ciprofloxacin and norfloxacin when expressed in *E. coli* (354).

Although many potential efflux transporters are known, there is not much knowledge on their role in clinically relevant situations, possibly because for *V. cholerae*, antimicrobial chemotherapy plays only a subsidiary role to fluid repletion therapy. The incidence of drug-resistant *V. cholerae* appears to be increasing, but the mechanisms presumably involve plasmids and integrons in many cases. In a study of fluoroquinolone-resistant *V. cholerae* clinical isolates, decreased accumulation of norfloxacin was found together with target gene mutations, suggesting the involvement of efflux (15).

Aeromonas spp.

Aeromonas belongs to another order, *Aeromonadales*. Although the pore-forming activity of an OmpA-like, monomeric protein has been reported (355), this protein presumably contributes only a minor activity to OM permeability, because OmpF/OmpC-like trimeric porins appear to exist in the genome sequences of *Aeromonas salmonicida* and *Aeromonas hydrophila*. The genome of *A. hydrophila* contains 10 RND pump genes as well as genes for MFS, MATE, SMR, and ABC efflux transporters (16). Apparently, an RND system, AheABC, plays a major role in the maintenance of the basal level of intrinsic resistance to most antimicrobials, which is rather similar to that of *E. coli*, except that *A. hydrophila* is more susceptible to macrolides like erythromycin and pristinamycin (16). An SMR pump, SugE, produces resistance to tributyltin, a compound that was used in the past as a biocide to prevent biofouling of ships; when introduced into *E. coli*, this gene increased resistance to chloramphenicol, tetracycline, as well as ethidium bromide (356). Fluoroquinolone-resistant isolates were often seen to have enhanced efflux activity (16). In contrast, another study found little evidence of the contribution of efflux to fluoroquinolone resistance (357), perhaps not surprisingly, because most strains had relatively low (<0.4 μg/ml) MICs of ciprofloxacin.

Legionella spp.

Legionella pneumophila contains the major oligomeric OM protein of 28 kDa (358). Although its single-channel conductance was low (0.1 nS) compared with that of *E. coli* OmpF (0.7 nS), this may have been caused by the low salt concentration (0.1 M rather than 1 M), and the channel size is difficult to ascertain. *L. pneumophila* strains show high levels of *in vitro* susceptibility to a variety of antibiotics, including macrolides, rifamycins, fluoroquinolones, aminoglycosides, and β-lactams (359), probably suggesting a limited role of either OM permeability or an efflux mechanism in

intrinsic resistance. However, the fact that *in vitro* exposure of *Legionella* to erythromycin or ciprofloxacin selects mostly low-level resistance may be an indication of efflux participation (360), while target mutation-derived moxifloxacin resistance causes high-level MIC increases (8- to 512-fold) (361). The genomes of *L. pneumophila* strains (3.4 to 3.5 Mb) are smaller than that of *E. coli* K-12 (4.64 Mb) but still possess a large number of genes encoding efflux pumps, membrane fusion proteins, and OM channel proteins (362, 363). Inactivation of the TolC efflux channel (36% identity to *E. coli* TolC) increases the susceptibility to erythromycin (16-fold reduction in the MIC) and to benzalkonium chloride, deoxycholate, ethidium bromide, norfloxacin, novobiocin, and rhodamine 6G (2- to 8-fold decreases in MIC values) (362, 363). Moreover, TolC is also involved in the secretion of a lipid-containing surfactant that promotes *Legionella* motility and displays activity against *Legionella* (363). Given that intracellular killing of *L. pneumophila* by antibiotics is required for legionellosis therapy, drug efflux could be an important factor affecting the efficacy of *in vivo* antibiotic regimens. Particularly, the multiplication of *L. pneumophila* within macrophages has already limited the choice of antibiotics to those that can penetrate phagocytic cells, such as macrolides, rifamycins, and fluoroquinolones, which are generally good substrates of typical drug pumps.

Pasteurellaceae

Pasteurella multocida. *Pasteurella multocida* produces a trimeric porin, OmpH, that is remotely related to the enterobacterial porins in sequence (364). OmpH is similar to *E. coil* porins in terms of permeability on the basis of the reported single-channel conductivity (364, 365). The genome of this organism contains one AcrB-like transporter, and the inactivation of *tolC* makes the organism susceptible to a wide range of agents (366). An Msr(E) ABC transporter, together with the phosphotransferase Mph(E), mediates clinically relevant resistance to macrolides, including new veterinary ones, gamithromycin and tildipirosin (367). Efflux-based tetracycline resistance genes from *Pasteurella* are also usually found on plasmids (16).

Haemophilus influenzae. Because of its clinical significance and its natural ability to be transformed by naked DNA, many early studies were carried out by using *Haemophilus influenzae*. *H. influenzae* produces one trimeric porin, OmpP2, which allows the passage of larger oligosaccharides (up to 1,400 Da) than the *E. coli* porins (typically excluding solutes of >600 Da) (368). The large size of the porin channel is probably responsible for making this organism susceptible to large, hydrophobic agents such as macrolides. Deficiency in this porin accounts for, at least in large part, resistance to chloramphenicol (369) and some other agents. *H. influenzae* contains a homolog of *E. coli acrB*, and its disruption made the organism more susceptible to erythromycin, rifampin, novobiocin, and other toxic agents (370). A later study suggested that AcrAB acted together with TolC and showed that the system also pumps out additional compounds such as ampicillin, fusidic acid, linezolid, puromycin, trimethoprim, cholate, Triton X-100, rhodamine 6G, and hexadecyltrimethylammonium bromide (371). A MATE family pump was cloned and produced resistance when expressed in an efflux-deficient *E. coli* host (16); however, its inactivation in *H. influenzae* produced little effect on antimicrobial susceptibility (371).

When a number of clinical isolates were studied for the accumulation of azithromycin with and without the proton conductor CCCP, azithromycin-resistant strains tended to show a higher level of accumulation with CCCP, suggesting a large contribution of efflux (372). Similar results were also obtained for the efflux of telithromycin (16). There has been a rapidly increasing prevalence of ampicillin-resistant clinical isolates that do not produce β-lactamase. Although most of them have mutations in the target penicillin-binding proteins, in a good portion of strains, frameshift mutations in the negative regulator AcrR were discovered, thus suggesting the increased production of AcrAB (16, 373). Isolates showing reduced susceptibility to the formylase inhibitor LBM415 often had mutations in *acrR*, and such mutations were selected by this agent *in vitro* (374).

GAMMAPROTEOBACTERIA: *PSEUDOMONAS*, *ACINETOBACTER*, AND *STENOTROPHOMONAS*

The gammaproteobacteria *Pseudomonas*, *Acinetobacter*, and *Stenotrophomonas* are very different from those discussed above, as they do not produce the high-permeability trimeric porins, and the effective OM permeability barrier makes their RND efflux systems highly efficient.

Pseudomonas aeruginosa

In its natural habitat as well as in the hospital, the notorious opportunistic pathogen *Pseudomonas aeruginosa* is often exposed to fluctuating and hostile external conditions. To maintain cell homeostasis and thrive in challenging environments, it has evolved a strong and selective OM permeability barrier whose effectiveness is reinforced by broad-spectrum exporters. No fewer than 12 different RND-type drug efflux systems have been recognized and characterized in strain PAO1. Although most of these multispecific pumps accommodate antibiotic molecules and confer some degree of resistance when overexpressed from recombinant plasmids, only a minority of them appear to be therapeutically relevant. Indeed, significant constitutive or drug-induced expression levels are required for efflux pumps to contribute to intrinsic and/or acquired resistance to antimicrobial agents. Involvement of other families of transporters in resistance to antibiotics appears to be restricted to a few examples (MATE-type transporter PmpM and SMR-type transporter EmrE) (16, 164).

OM permeability. *P. aeruginosa* is characterized by its very low OM permeability due to the presence of the slow porin OprF instead of the classical OmpF/OmpC trimeric porins, as mentioned above (179, 181, 182) and as discussed elsewhere (22). However, this low-OM-permeability property alone does not sufficiently explain the intrinsic MDR of this organism (11, 12, 375) and may also explain (together with the envelope-stabilizing function of OprF) the lack of or rare detection of OprF deficiency in clinical isolates (376–378). On the other hand, *P. aeruginosa* possesses specific channels, such as OprB, specific for glucose uptake, and OprD, specific for the diffusion of basic amino acids and peptides (22). The latter channel is the primary channel for the entry of carbapenems across the OM, and the reduced expression or loss of OprD has been frequently observed in carbapenem-resistant clinical isolates (379–384), which may also display upregulated drug efflux systems, such as MexEF-OprN, due to the shared regulation of the expression of OprD and MexEF-OprN (carbapenem susceptibility is unlikely affected by MexEF-OprN) (see Regulation of Multidrug Efflux Pumps, below) (385–387).

RND efflux pumps. (i) MexAB-OprM. Constitutively ex-

pressed, the MexAB-OprM pump was the first RND system characterized in *P. aeruginosa* (10, 13, 388), although a link between the development of MDR and increased amounts of an OM protein, OprM, had previously been established in *in vitro*-selected mutants (389) and was also analyzed together with other overproduced 50-kDa OM proteins (11). Reminiscent of *E. coli* AcrAB-TolC, MexAB-OprM displays an incredibly wide substrate specificity that encompasses structurally very different antibiotics (e.g., β-lactams, including β-lactamase inhibitors and certain carbapenems [except imipenem], aminoglycosides [with low-ionic-strength medium], [fluoro]quinolones, tetracyclines, tigecycline, macrolides, amphenicols, novobiocin, sulfonamides, trimethoprim, cerulenin, and thiolactomycin) (10–13, 16, 164, 389–394) as well as a series of amphiphilic molecules, disinfectants, dyes, solvents, detergents, and C_8 to C_{14} 3-oxo-acyl-homoserine lactones involved in quorum sensing (395–400). (A recent study also showed the quorum sensing inhibitors of nonnative *N*-acylated L-homoserine lactones and derivatives as the substrates of MexAB-OprM [401].) This system thus provides *P. aeruginosa* with protection against multiple inhibitors that can be encountered in different environments.

MexAB-OprM-overproducing mutants can be readily generated by *in vitro* selection in the presence of an antibiotic(s) (11, 389, 402), and *in vitro* studies on reference strains have shown that any mutational event inactivating the *mexR* (also called *nalB*), *nalC*, or *nalD* gene or impairing the activity of their respective products (MexR, ArmR, and NalD [see Regulation of Multidrug Efflux Pumps, below]) results in the overexpression (≥3-fold) of *mexAB-oprM* with a concomitant increase in resistance (2- to 16-fold MIC increases) to all the pump substrates compared to baseline levels, with *nalC* mutants being in general 2-fold more susceptible than the *nalB* and *nalD* mutants (393, 403–409). Similar findings were reported for phenotypically but non-genetically characterized multidrug-resistant mutants selected *in vitro* with various antibiotics (11, 12, 392, 402, 410, 411).

Because clinical strains often accumulate multiple resistance mechanisms, MexAB-OprM overproducers may have quite atypical drug susceptibility profiles and therefore may be underrecognized by medical microbiologists. Methods aimed at measuring the intracellular accumulation of pump substrates are not amenable to routine laboratory practice and not specific because of the large overlaps between RND pump substrate specificities. In several studies, systematic quantification of *mexA* and/or *mexB* transcripts by reverse transcription-quantitative PCR (RT-qPCR) demonstrated that these mutants are very prevalent among multiresistant non-cystic fibrosis strains (16, 412–419), including those producing reduced susceptibility to carbapenems (380, 381, 383, 420, 421) and those producing ESBLs or metallo-β-lactamases (385, 422). Rates of MexAB-OprM overproducers of near 50% were recorded even in subpopulations of isolates exhibiting a reduced susceptibility to ticarcillin (≥32 μg/ml) (423). These results agree with those of other experimental approaches based on the use of EPIs (e.g., PAβN and MC-04,124) (60, 424). Interestingly, a detailed analysis of 12 multidrug-resistant MexAB-OprM-overproducing strains revealed an equivalent distribution of *nalB*, *nalC*, and *nalD* mutants among them, highlighting the fact that all these mutant types may emerge in patients (413). Similar data were reported by different investigators (380, 425).

In the absence of other known resistance mechanisms (e.g., enzymatic drug inactivation and drug target alterations), MexAB-OprM dysregulation decreases the susceptibility of clinical isolates to substrate antibiotics from 2- to 8-fold compared to baseline levels (394, 415, 426). According to the susceptibility breakpoints from the Clinical and Laboratory Standards Institute (CLSI) (1029), changes in strain categorization resulting from a maximal effect of the efflux mechanism (8-fold) are limited to a small number of antipseudomonal agents, such as ticarcillin (from S [drug susceptible] to I [intermediate] or R [resistant]), aztreonam (from S to I or R), meropenem (from S to I), ciprofloxacin (from S to I), and levofloxacin (from S to I). Whether this modest impact of MexAB-OprM on drug MICs is therapeutically relevant still awaits to be clarified by clinical investigations (427). Nevertheless, it can reasonably be assumed that even low-level-resistant mutants will survive chemotherapy better than wild-type bacteria if inappropriate agents are used to treat the infection or if only suboptimal drug concentrations reach the infection site because of limited diffusion *in vivo* or an insufficient antibiotic dosage. As demonstrated in an animal model of infective endocarditis, management of difficult-to-treat infections requires higher doses of a β-lactam if MexAB-OprM-upregulated mutants develop (428).

Another important therapeutic issue relates to whether overexpressed MexAB-OprM can potentiate other resistance mechanisms and thus can enable *P. aeruginosa* to become recalcitrant to more antibiotic treatments. This does not seem to be the case, as interplays between the pump (active drug efflux) and the β-lactamase AmpC (drug inactivation) or mutations in type II topoisomerases (reduced drug target affinity) result mostly in cooperative rather than synergistic effects on drug resistance levels (383, 412, 415, 417, 429). As shown in *in vitro* mutants, the overproduction of MexAB-OprM causes only a slight increase in the MIC of carbenicillin when the enzyme AmpC is derepressed (430) (however, since either MexAB-OprM or derepressed AmpC production alone has resulted in high-level carbenicillin resistance, it is apparently difficult for these combined mechanisms to further increase the carbenicillin resistance level). Similarly, the coexpression of two RND pumps simultaneously (e.g., MexAB-OprM plus MexXY or MexEF-OprN) at the most tends to produce additive effects on the MICs of shared substrates, as exemplified with fluoroquinolones (25, 377, 413, 431). Synergistic interactions are expected to occur when multicomponent efflux pumps (e.g., RND systems) and singlet pumps (e.g., TetA/C) operate coordinately to extrude substrates from both the cytoplasm and the periplasmic space up to the external medium (25). Since MexAB-OprM is able to accommodate some carbapenems such as meropenem (252, 402, 430, 432), its contribution to the acquired resistance of clinical strains to these agents was investigated and found to be modest with respect to other mechanisms such as the loss of OprD and carbapenemase production (380, 381, 383, 384). Thus, the role of the pump appears to be obscured by more efficient drug-specific resistance mechanisms in terms of the resistance phenotype of the mutants. However, their role with respect to resistance emergence should not be underestimated, for example, in the development of resistance to pump substrate antimicrobials such as fluoroquinolones (433).

(ii) MexXY-OprM(OprA). The MexXY proteins, which are encoded by a two-gene locus, need to interact with an OM component in order to form a functional tripartite pump (434–436). Despite the fact that MexXY may accommodate various OM channels (e.g., OpmB) to actively export substrates, OprM appears to be the primary partner in most strains (434, 435, 437). In

the phylogenetically distinct clade PA7, the *mexXY* operon contains a third gene encoding an OprM-like protein, named OprA, which somewhat surprisingly shows a higher level of sequence similarity with OM channels of *Burkholderia* efflux pumps than with those of *P. aeruginosa* (438). In the PA7-related strains, the MexXY proteins seem to cooperate with both OprM and OprA, as each of the latter can compensate for the genetically engineered suppression of the other. While the MexXY-OprM/OprA system is able to transport aminoglycosides, fluoroquinolones, macrolides, tetracyclines, tigecycline, and zwitterionic cephalosporins (cefepime and ceftobiprole) (393, 434–436, 439), its contribution to natural resistance is restricted to those agents able to induce *mexXY*(*oprA*) expression (219, 434). This occurs when bacteria are exposed to subinhibitory concentrations of ribosome-targeting antibiotics, including chloramphenicol, a poor substrate, if at all, of the pump (440, 441).

Stable overexpression of the MexXY proteins provides *P. aeruginosa* a 2- to 16-fold-higher level of resistance to all the pump substrates, with OprM being present in amounts apparently sufficient to stoichiometrically cooperate with both the MexXY and MexAB proteins in non-PA7-related strains. The weak promoter identified ahead of *oprM* within the *mexAB-oprM* operon (442) could possibly account for an excess of OprM molecules relative to MexAB, thus allowing the interaction of OprM with other extrusion systems without impacting the activity of MexAB-OprM by titration. MexXY(OprA)-overproducing mutants can be easily selected *in vitro* and *in vivo* by substrate antibiotics (393, 439, 443, 444) or protein synthesis inhibitors (445), which is consistent with the increased prevalence, sometimes >80%, of such mutants in cystic fibrosis (436, 446–451) and non-cystic fibrosis (384, 385, 414, 416, 417, 420–423, 429, 431, 452) patients worldwide. The abundance of reactive oxygen species in the cystic fibrosis lung environment might explain the high rates of resistant mutants with this pathology (453). Supporting this notion, it was observed that prolonged exposure of *P. aeruginosa* to H_2O_2 promoted the emergence of MexXY overproducers *in vitro* (454). Differential resistance (MIC ratio, ≥4) to cefepime and ceftazidime, which are good and poor substrates of MexXY/OprM(OprA), respectively, has been attributed to pump derepression in some non-cystic fibrosis isolates (425, 455, 456).

Three types of MexXY-overproducing mutants have been characterized so far. In so-called *agrZ* mutants, the *mexZ* gene and its product are compromised by a number of nonspecific genetic events (e.g., indels and point mutations) (425, 439, 443, 446–448, 450, 457). In addition to mutations causing *mexZ* disruption, some generate single amino acid substitutions in the DNA-binding domain, the dimerization domain, or the structure of MexZ, abrogating its repressor activity (416, 452, 458, 459). A second group of mutants, dubbed *agrW1*, was defined in line with various defects in ribosomal proteins such as L1 (436), L25 (460), L21 and L27 (461), or components of the Fmt bypass (methionyl-$tRNA^{fMet}$ formyltransferase FolD) (445) that ultimately affect protein synthesis. Reminiscent of the effects of ribosome-targeting inhibitors, any mutation impairing the translation process seems to be able to induce PA5471 (ArmZ) expression and subsequently the *mexXY*(*oprA*) operon via the MexZ-ArmZ interaction. Finally, in the third group of mutants, named *agrW2*, mutational activation of the sensor ParS or the response regulator ParR of the two-component system ParRS leads to constitutive expression of the efflux operon (384, 444). A detailed analysis of a collection of non-cystic fibrosis isolates showing moderate, nonenzymatic resistance to aminoglycosides demonstrated the occurrence of the three types of mutants (*agrZ*, *agrW1*, and *agrW2*) among clinical strains (452). In cystic fibrosis patients, the *agrZ* type seems to predominate over the other two (446–448).

With providing a 2- to 16-fold increase in resistance, the MexXY-OprM(OprA) pump is not expected to change the classification of most clinical strains from S to I or R regarding the pump substrates, unless additional mechanisms are expressed. Therefore, the clinical impact of this low to moderate level of resistance remains uncertain, and as for MexAB-OprM, it is likely to depend upon individual patient conditions and treatment options. Very few studies have examined the potential role of MexXY/OprM(OprA) in clinical outcomes. In a rabbit experimental model of pneumonia treated with intravenous administration of tobramycin, the pump was considered to have a modest influence on animal survival and posttreatment bacterial loads (462). However, tobramycin itself appeared to have poor bacteriological efficacy in this model, contrasting with the quite high survival rates. The increased prevalence of MexXY(OprA) overproducers in the clinical setting, as reported above, can be interpreted as resulting from a positive, adverse effect of the efflux system on either the resilience of *P. aeruginosa* to chemotherapy (i.e., drug resistance) or its adaptation to the host (e.g., resistance to the immune system or improved fitness). Reinforcement of efflux activity due to *mexXY*(*oprA*) derepression may be just one of the multiple means by which the pathogen is able to combine to gradually increase its resistance to potent antimicrobials (460). For instance, the simultaneous overexpression of multiple efflux pumps (e.g., MexAB-OprM, MexXY, and MexEF-OprN) in conjunction with other resistance mechanisms is common in hospital strains (11, 413, 431, 464).

(iii) MexCD-OprJ. The MexCD-OprJ system is generally not expressed in wild-type strains (465) but is inducible by membrane-damaging agents (see Regulation of Multidrug Efflux Pumps and "Bacterial Stress Responses," below) (466, 467). Overproduction of the MexCD-OprJ pump in *nfxB* mutants causes increased resistance to fluoroquinolones, zwitterionic cephalosporins (cefepime and cefpirome), macrolides, chloramphenicol, and tetracyclines concomitant with hypersusceptibility to aminoglycosides and other β-lactams (15, 440, 465, 468). This overexpression results in an apparently deficient production of MexAB-OprM and/or impaired drug inducibility of the intrinsic β-lactamase AmpC, the effects of which were each proposed to account for the higher level of susceptibility of the *nfxB* mutants to β-lactams (469, 470), although this issue seems to be controversial (471). In dense bacterial communities such as those occurring in cystic fibrosis patients, the *nfxB* mutations could be less detrimental to *P. aeruginosa* than in planktonic cells. Some data indeed suggest that in *nfxB* mutants, the enzyme AmpC leaks out of the cells and concentrates in the surrounding milieu rather than in the periplasm, thus generating whole protection for cells of the biofilm (472). This may be relevant *in vivo*, as important extracellular AmpC activities have been measured in the sputa of cystic fibrosis patients (473). Whereas the *mexXY* genes (and their products) are expressed at similar levels in *nfxB* mutants and wild-type strains, MexCD-OprJ appears to compromise the MexXY/OprM(OprA) drug transport activity and associated resistance to aminoglycosides by downmodulating the production of the protein OprM

(472). Analysis of fluoroquinolone-resistant clinical strains identified only a minority of *nfxB* mutants by comparison with mutants harboring quinolone target alterations (GyrA/B and ParC/E) or overexpressing other efflux pumps [e.g., MexAB-OprM, MexXY/OprM(OprA), and MexEF-OprN] (15, 431, 474). Likewise, MexCD-OprJ overproduction was infrequent in β-lactam-resistant strains (380, 421, 423), including those exhibiting increased MICs of the pump substrate cefepime relative to those of ceftazidime (425, 455). Microbiological follow-up of one mechanically ventilated patient treated with two substrates of MexCD-OprJ, namely, ciprofloxacin (14 days) and cefepime (19 days), revealed the emergence of *nfxB* mutants over the time, which accounted for a change of bacteria from S to I or R with regard to their susceptibility to fluoroquinolones (CLSI breakpoints) (475). Interestingly, the overexpression of MexCD-OprJ, as with that of MexAB-OprM or MexXY, occurred in 60% of carbapenem-resistant clinical isolates (476). Moreover, although MexCD-OprJ overproduction can be part of the complex resistance mechanisms (464, 476, 477), the genotypic alterations may not correlate with the phenotype (478), likely attributed at least partly to global changes in the physiology and metabolism caused by *nfxB* mutations (479).

(iv) MexEF-OprN. *In vitro* selection of *P. aeruginosa* mutants cross-resistant to (fluoro)quinolones, chloramphenicol, trimethoprim, and carbapenems (imipenem) while being hypersusceptible to other β-lactams and to aminoglycosides was reported in the early 1990s (480, 481). This type of mutant, named *nfxC* mutants, is readily selected by fluoroquinolones and chloramphenicol but not by carbapenems (433, 481, 482). The observed resistance phenotype partly relies upon the overproduction of MexEF-OprN, which exports only (fluoro)quinolones, chloramphenicol, trimethoprim, and tetracycline (483, 484). *nfxC* mutants have rarely been reported in clinical settings (421, 431, 485) and even failed to be detected in several studies systematically investigating clinical resistance mechanisms (385, 414). Such a low prevalence can be attributed to β-lactam hypersusceptibility and/or the impaired virulence of these bacteria, although additional resistance mechanisms may mask the loss of β-lactam resistance in some strains (431). However, these mutants have been found in cystic fibrosis and other patients (478, 486).

(v) Other RND pumps. There are also additional RND systems in *P. aeruginosa*, and they are known to often require OprM for efflux activity (16). However, their clinical relevance remains essentially unknown in spite of their involvement in resistance or virulence. MexJK functions with OprM or another OM protein, OpmH, for pumping out erythromycin and triclosan, respectively (16). Several cloned RND pumps were able to confer resistance to a *P. aeruginosa* or *E. coli* host deficient in major RND pumps: MexMN-OprM for resistance to macrolides and fluoroquinolones (487); MexPQ-OpmE for resistance to amphenicols (487); MexVW-OprM for resistance to macrolides, chloramphenicol, fluoroquinolones, and tetracycline (488); and MuxABC-OpmB (with two RND components, MuxBC) for resistance to aztreonam, macrolides, novobiocin, and tetracyclines (489). The inactivation of MuxABC-OpmB increases resistance to carbenicillin (490). TriABC-OpmH, with an unusual property of requiring two periplasmic adaptor proteins, TriA and TriB, pumps out triclosan (491), while the CzcCBA (CzrCBA) system is involved in resistance to cadmium, cobalt, and zinc salts (492, 493).

Acinetobacter spp.

Acinetobacter spp. and particularly those belonging to the *A. baumannii-A. calcoaceticus* complex have emerged globally as common nosocomial and community pathogens with high levels of MDR or pandrug resistance (494, 495). The modest genome size of *A. baumannii* of ca. 4 Mb has shown the acquired genetic diversity (including at least two dozen genomic resistance islands [*A. baumannii* resistance islands {AbaR}] of 22 to 121 kb) that provides the molecular basis of almost all types of resistance mechanisms and renders the organism significantly resistant to a large number of antibiotics, biocides, and heavy metals (496–502). Multidrug-resistant isolates or their epidemic clones are frequently isolated from patients after treatment with ciprofloxacin, co-trimoxazole, colistin, imipenem, and/or tigecycline (503–506), highlighting the rapid *in vivo* evolution of MDR in *Acinetobacter*.

OM permeability. *Acinetobacter* has an OM of exceptionally low permeability that is similar to that of *P. aeruginosa* and ~100-fold lower than that of *E. coli* (to cephalosporins) (183, 507). This is attributed to the lack of a high-permeability trimeric porin found in *Enterobacteriaceae* (183, 507). Being a close homolog of *E. coli* OmpA and *P. aeruginosa* OprF, the major OM protein of *A. baumannii* (508), monomeric OmpA, was experimentally shown to be the principal nonspecific slow porin (183). However, as with other slow porins such as OprF (180), the majority conformer of OmpA folds as a two-domain protein that is needed for the stabilization of the cell envelope. Thus, the porin function of OmpA is difficult to decipher by using OmpA deletion mutants, which paradoxically show reduced MICs of most antibiotics (183, 509) due to the destabilization of the envelope. It required the precise determination of the OM permeation rates of zwitterionic cephaloridine, which is unlikely to permeate across the bilayer domain of the OM, to show that *ompA* deletion results in a decrease of OM permeability (183). Plant extracts from *Holarrhena antidysenterica* permeabilize the OM of extensively drug-resistant isolates and thus restore certain activities of antibiotics (510), similar to those observed with other plant extracts, including coriander oil, geraniol, and ginger compounds (511, 512).

A. baumannii also possesses channel proteins specific to some substrates. CarO (for carbapenem resistance-associated OM protein) functions as an influx channel for carbapenems with a demonstrated imipenem binding site (513–516), similar to *P. aeruginosa* OprD, which is specific for the uptake of basic amino acids and carbapenems, although OprD and CarO share no recognizable homology (190, 514). CarO is also involved in the influx of L-ornithine and basic amino acids (517). Indeed, the absence of CarO expression, due to gene disruption by an IS*Aba10* or IS*Aba825* insertion, correlates well with carbapenem resistance in clinical isolates (502, 514, 518). The observation of extensive genetic diversity of *carO* within clinical populations suggests horizontal gene transfer as well as assortative gene recombination, likely providing a strategy for *A. baumannii* survival under different environmental conditions (519). The synergistic interplay between the loss of CarO and pump overproduction contributes to MDR phenotypes (502, 506). Moreover, the loss of additional OM proteins of 31 to 36 kDa is also reported to be associated with carbapenem resistance (520–522). In response to a physiological level of 200 mM NaCl, there is an upregulation of 14 distinct transporter genes, while the expression of *carO* and 31- to 36-kDa OM protein genes is downregulated. NaCl can induce significant

tolerance to aminoglycosides, carbapenems, quinolones, and colistin (523).

RND efflux pumps. There are a large number of studies that have investigated the role of *Acinetobacter* efflux pumps in resistance to clinically relevant antibiotics and also to biocides, dyes, and detergents (16, 184). Three RND systems, AdeABC, AdeFGH, and AdeIJK, have been well characterized. AdeABC is apparently not well expressed in wild-type strains (524) but contributes significantly to acquired MDR (including biocide resistance) in clinical isolates obtained worldwide (506, 525–534). These contributions include resistance to tigecycline, a major alternative drug for treating *Acinetobacter* infections, in isolates covering epidemic clones (502, 526, 531, 533, 535–538). *In vitro* exposure of susceptible isolates to tigecycline resulted in a >10-fold tigecycline MIC increase that was accompanied by AdeABC hyperproduction (536). There was a difference of tigecycline MICs of 16-fold between isogenic parental and AdeABC-hyperproducing strains (184). A nearly 30-fold overexpression of *adeB* was observed for tigecycline-nonsusceptible isolates (533). Thus, although tigecycline displays activity against isolates possessing tetracycline-specific resistance mechanisms (ribosomal protection and efflux) (539), broad-substrate-specificity RND pumps play a key role in the emergence of tigecycline resistance in *Acinetobacter* spp., similar to observations for many other Gram-negative bacteria (16, 138, 268, 310, 312, 324, 393, 540). (Additionally, non-pump-mediated tigecycline insusceptibility occurred due to a deletion mutation in the *trm* gene that encodes *S*-adenosyl-L-methionine-dependent methyltransferase [541], showing the complexity of tigecycline resistance mechanisms.) AdeABC overproduction is also seen in carbapenem-resistant *A. baumannii* isolates (504, 538).

AdeIJK contributes to both intrinsic and acquired MDR (502, 528, 530, 533, 542, 543). Its inactivation in a wild-type strain caused mostly 4- to 16-fold reductions of MICs of β-lactams (aztreonam, ceftazidime, cefepime, and ticarcillin), chloramphenicol, clindamycin, erythromycin, fluoroquinolones (norfloxacin and ciprofloxacin), and tetracyclines (tetracycline, minocycline, and tigecycline) (184, 542). AdeIJK also mediates resistance to biocides, including the frequently used biocides chlorhexidine and triclosan and other disinfectants in hospitals (530, 544). *In vitro* selection of AdeIJK-overproducing mutants by triclosan was recently demonstrated (544). A synergistic interplay between AdeIJK and AdeABC was observed with resistance to chloramphenicol, fluoroquinolones, and tetracyclines, including tigecycline (184). Recently, the efflux properties of the AdeABC and AdeIJK systems were compared with those of the *E. coli* AcrAB-TolC pump, and important activity and substrate differences were identified (545). Expressed in a heterologous *E. coli* host, the AdeABC and AdeIJK pumps were both able to pump out β-lactams, and this activity is masked in *Acinetobacter* due to endogenous β-lactamases. AdeABC is more effective than AcrAB-TolC in the extrusion of tetracycline but weaker in the efflux of lipophilic β-lactams, novobiocin, and ethidium bromide. AdeIJK is remarkably more active in pumping out multiple agents (except erythromycin) (545).

AdeFGH was initially identified through a microarray assessment of *in vitro*-selected multidrug-resistant mutants deficient in AdeABC and AdeIJK (546). This pump mediates acquired MDR and shows a broad substrate profile, including fluoroquinolones and tigecycline (184, 547). Although its clinical significance remains largely unknown, AdeFGH was the most overexpressed RND pump in isolates from Canadian hospitals (532).

AdeA-AdeA2-AdeB (containing a pair of AdeAs) is a newly identified AdeAB homolog involved in tigecycline resistance (548). Another putative RND pump, AdeT, possibly involved in aminoglycoside resistance (16), was further confirmed to be present in the genomes of two multidrug-resistant isolates (500). Many other non-*A. baumannii* species of *Acinetobacter* also often possess homologs of RND pumps (16, 528, 543, 549).

The critical role of RND pumps in MDR in *Acinetobacter* emphasizes the need to identify drug candidates that bypass or inhibit efflux mechanisms. Several studies have investigated the potentiation of anti-*Acinetobacter* activity by EPIs, which often included PAβN and NMP, which themselves have MIC values of ≥400 and 200 to ≥400 μg/ml, respectively (527, 550, 551). At a lower concentration of 25 μg/ml, their effect on reversing resistance is quite limited, while at a higher concentration of 100 μg/ml, NMP has a stronger effect on restoring drug susceptibility than that of PAβN (550); this difference is likely to be due to the poor permeation across the OM by NMP, in contrast to PAβN, which permeates this membrane by perturbing its structure (see Efflux Pump Inhibitors, below). In other studies, PAβN at 10 μg/ml decreased the MIC values of chloramphenicol, clindamycin, and trimethoprim against clinical isolates regardless of the *adeFGH* expression status mostly 2- to 4-fold (552), while PAβN at 20 μg/ml reduced the nalidixic acid MIC up to 16-fold but displayed little effect on ciprofloxacin susceptibility (553). PAβN and NMP, each at 100 μg/ml, restored susceptibility to fluoroquinolone (2- to 16-fold reduction of MICs) and tigecycline (mostly by a 2-fold MIC decrease) (554). Apparently, these EPIs have a stronger effect on resistance reversal with agents that have relatively high MIC values, such as chloramphenicol, clarithromycin, clindamycin, linezolid, rifampin, and trimethoprim (552, 553, 555), agents whose OM penetration is likely to be slow. Intriguingly, one study revealed that NMP at 64 μg/ml increased the tigecycline MIC 2-fold, although no effect on susceptibility to doxycycline, minocycline, and tetracycline was observed (551). While PAβN at 25 μg/ml decreased rifampin MIC values against rifampin-resistant isolates containing no mutations in the *rpoB* gene by 16- to 32-fold, NMP at 100 μg/ml showed little effect on rifampin susceptibility (555).

By using a multidrug-resistant isolate, one study tested the effects of a large number of EPIs (at 0.5× MIC), including not only CCCP (not an EPI *per se*), PAβN, and NMP but also omeprazole, phenothiazines (chlorpromazine, prochlorperazine, and promazine), reserpine, and verapamil (556), on antibiotic susceptibility. Although all agents tested showed decreased ethidium bromide accumulation in intact cells, PAβN, NMP, and phenothiazines were the only agents that restored susceptibility to certain antibiotics by an ≥8-fold decrease in the MIC values. Omeprazole and verapamil showed either no effect on antibiotic susceptibility or an antagonistic impact on tigecycline susceptibility (i.e., an increase of up to 128-fold MIC) (556). Similarly, three mammalian proton pump inhibitors, omeprazole, lansoprazole, and pantoprazole, increased tigecycline MIC values (up to >128-fold) for all six ESKAPE pathogens, including *A. baumannii*, in a concentration-dependent manner (557). Although the mechanism of this increase in tigecycline MICs is not clear, it should be noted that some compounds can actually enhance the efflux of other drugs through AcrB (94).

Non-RND efflux pumps. An MFS pump, AmvA, shows a broad

substrate specificity, including both antibiotics and disinfectants, and its expression is detectable in clinical isolates (558). AbeM and AbeS pumps that belong to the MATE- and SMR-type exporters, respectively, also accommodate a number of drug substrates (16, 162). AbeM hyperexpression (with moderately increased expression levels of AdeABC and AdeIJK) was observed in imipenem-resistant isolates (559). Three additional AbeM homologs, AbeM2, -3, and -4, were also identified, with no demonstrated role of AbeM4 in MDR (560). Although the clinical importance of AbeM pumps remains unknown, the *abeM* gene was used with other resistance genes (*adeB*, *adeR*, *ampC*, and *ompA*) to assess the genetic linkage of multidrug-resistant endemic isolates (529).

Besides these MDR pumps, the MFS pump CraA specifically provides resistance to chloramphenicol (>128-fold MIC increase) (561). Its elevated expression occurs in response to NaCl induction (523). Tetracycline-specific pumps such as Tet(A), Tet(B), and Tet39 were also found (16) and were frequently encoded both in chromosomal genomic resistance islands and by plasmids (496, 500, 562). Plasmid-borne *tet*(B)-*tetR* genes were associated with the IS*CR2* mobile element in multidrug-resistant isolates (562), suggesting possible rapid horizontal resistance spread. The copresence of the Tet(A) pump and Tet(M) for ribosomal protection was also noted (16). Lastly, a new type of efflux pump, AceI, was found to mediate resistance to chlorhexidine (563). Chlorhexidine itself also induces the expression of AceI and AdeABC. AceI belongs to the bacterial transmembrane pair family and is grouped as a prototype member of the proteobacterial chlorhexidine efflux family (563). Similarly, the exposure of *Acinetobacter baylyi* to chlorhexidine also induced resistance to chlorhexidine and oxidants (564), which could potentially be attributed to efflux pumps.

Stenotrophomonas maltophilia

Stenotrophomonas maltophilia, found in various environments, including hospital patients and animal sources, is a key emerging opportunistic pathogen in humans that is highly versatile and adaptable. Its genome contains genes encoding numerous major mechanisms of resistance, including drug efflux pumps (565, 566). The MDR phenotypes are attributed to the interplay between low OM permeability (567) and efflux mechanisms (15). The reduction of LPS synthesis is associated with modestly increased susceptibility to several antibiotics (568). The efflux mechanism was initially suggested by the selection of multidrug-resistant isolates by any of several structurally unrelated agents (565, 569). Eight RND-type Sme systems and several other types of drug exporters have been identified (570).

The first RND pump, SmeABC, identified in this species via the construction of a cosmid-based genomic library (571), is attributable to acquired MDR (572, 573). The subsequently characterized SmeDEF pump plays a major role in both intrinsic and acquired MDR in clinical isolates from various sources (392, 572, 574–576), and its overexpression can be readily selected *in vitro* by conventional antibiotics and also by biocides such as triclosan (565, 577, 578). Inactivation of SmeDEF usually leads to a 2- to 8-fold reduction of the MIC values of fluoroquinolones (including ciprofloxacin, clinafloxacin, and moxifloxacin), tetracyclines (including minocycline and tigecycline), macrolides (erythromycin and azithromycin), chloramphenicol, novobiocin, dyes, and SDS against wild-type and multidrug-resistant isolates (574). In mutants carrying a genetic inactivation of the class B L1 metallo-β-lactamases and class A L2 β-lactamases, the role of RND pumps in resistance to aztreonam, piperacillin, cefepime, and cefpirome (4-fold MIC increases) was demonstrated (565, 571, 574). SmeC overexpression was also associated with L2 β-lactamase production (571). Intriguingly, SmeDEF disruption has little or only a minimal impact on susceptibility to penicillins, cephalosporins, carbapenems, and monobactams (i.e., no or merely a 2-fold MIC decrease in mutants that are also deficient in L1 and L2 β-lactamases) and does not alter rifampin and trimethoprim susceptibility (574).

There are 4 additional RND Sme pumps that all lack a genetically linked OM component (i.e., SmeGH, SmeIJK [paired SmeJK pump], SmeMN, and SmeYZ) and 2 RND pumps containing an OM protein (SmeOP-TolC and SmeVWX) (570, 579). Hypersusceptibility data with the selective inactivation of the SmeC or SmeF OM protein suggest that these OM proteins may function in multiple drug exporters (571, 574), similar to the situations observed for OprM of *P. aeruginosa* or TolC of *E. coli*. Although a TolC homolog was also identified in *S. maltophilia* (580), it is phylogenetically distinct from the SmeC, SmeF, and SmeX OM channels, and hence, its role in any Sme system (except SmeOP [579]) remains unknown. The *tolC* gene and an upstream *pcm* gene (encoding protein-L-isoaspartate *O*-methyltransferase) likely form the *pcm-tolC* operon, but only TolC inactivation renders the organism susceptible to aminoglycosides and macrolides (580). In fact, the Smlt3926 gene, encoding a TetR repressor, SmeRo, of SmeOP is located immediately upstream of the *pcm-tolC* operon, and thus, there is a 5-gene cluster comprised of *tolC-pcm-smeRo-smeO-smeP*. SmeOP-TolC was recently demonstrated to provide resistance to several antibiotics, CCCP, dyes, and detergents (579). The simultaneous hyperexpression of SmeJK (forming one exporter) and SmeZ pumps increases the substrate profiles of a clinical isolate (581). Inactivation of SmeJK significantly increases the susceptibility to aminoglycosides (amikacin, gentamicin, kanamycin, and tobramycin) and the macrolide leucomycin (8- to 16-fold reduction in MIC values), yet the disruption of either SmeJ or SmeK produces merely a 2-fold decrease in the MICs of the tested aminoglycoside agents (582), likely suggesting that SmeJ or SmeK alone may still be functional.

S. maltophilia also possesses ABC and MFS transporters (570). The ABC-type tripartite FuaABC system mediates fusaric acid-inducible resistance to fusaric acid, and this resistance is dependent on the FhuR regulator, which functions as a repressor in the absence of fusaric acid but as an activator in its presence (583). ABC-type MacABC causes intrinsic resistance to aminoglycosides, macrolides, and polymyxins, as its inactivation resulted in 2- to 8-fold reduction of the MIC values of these agents (570, 584). Another SmrA ABC pump conferred resistance to fluoroquinolones, tetracycline, doxorubicin, and dyes when expressed in *E. coli* (585), and an antibody developed against this pump enhanced antibiotic susceptibility in *S. maltophilia* (586). An MFS pump, EmrCAB, is involved in the extrusion of hydrophobic toxic agents but is not well expressed intrinsically (587).

ALPHAPROTEOBACTERIA: *BRUCELLA*, *BARTONELLA*, AND *RICKETTSIA*

The alphaproteobacteria contain several species that are major human and animal pathogens, such as *Brucella*, *Bartonella*, and *Rickettsia*.

Brucella spp.

Brucella spp. are facultative intracellular coccobacilli with six recognized species (*Brucella abortus*, *B. canis*, *B. melitensis*, *B. neotomae*, *B. ovis*, and *B. suis*) that display distinct host-pathogen associations. These species are of high clinical significance given their role as causative agents of zoonotic brucellosis, which is reemerging, can be transmitted to humans, and is commonly associated with laboratory-acquired infections (particularly with *B. melitensis*) (588). *Brucella* spp. show similar genomes with two circular chromosomes and contain a number of putative drug efflux transporters (589, 590). These species contain trimeric porins homologous to *E. coli* porins with comparable permeability (591, 592).

Two RND systems, BepDE and BepFG, are involved in resistance to antibiotics and other toxic agents (including doxycycline, a major choice for the treatment of brucellosis) in collaboration with the OM channel BepC (593, 594). Repressed by the BepR regulator, the expression of BepDE was induced by deoxycholate. Inactivation of BepFG produced an increased susceptibility to toxic agents such as dyes but not to conventional antibiotics (594). The inclusion of PAβN resulted in variable reductions in erythromycin and moxifloxacin MIC values (595, 596), consistent with the RND pump's contribution to resistance. A putative BicA macrolide pump is present in *B. abortus* and *B. suis* (590). Two MATE pumps, NorMI and NorMII, were identified in *B. melitensis*, with NorMI being confirmed to yield MDR when expressed in *E. coli* (16). However, they were not involved in clinical isolates resistant to fluoroquinolones and rifampin (597).

Bartonella spp.

Bartonella spp. exist in mammalian host reservoirs and are linked to several human infections, such as cat scratch disease. While *Bartonella* species are susceptible to most conventional antibiotic classes, the major resistance mechanism characterized to date is related to target modifications for resistance to fluoroquinolones, macrolides, and rifamycins (598). A VceA MDR pump was identified in *Bartonella australis* (GenBank accession number YP_007461659). OM porins and efflux components, including a TolC homolog as well as RND systems, were identified (599–601); nevertheless, none of the efflux pumps have been characterized for their role in resistance.

Rickettsia spp.

Rickettsia spp. are obligately intracellular bacteria, including those causing spotted fevers. *Rickettsia* spp. are often resistant to β-lactams, aminoglycoside, and sulfonamide-trimethoprim but show variable susceptibilities to macrolides (602). Genome comparison of *Rickettsia* species shows the presence of a number of drug efflux pump genes, such as 6 genes encoding RND pumps and 20 genes encoding ABC transporters in *Rickettsia conorii* as well as an SMR pump in *Rickettsia bellii* (602, 603).

BETAPROTEOBACTERIA: *ACHROMOBACTER*, *BURKHOLDERIA*, AND *NEISSERIA*

Often found in natural environmental samples, the betaproteobacteria *Achromobacter*, *Burkholderia*, and *Neisseria* consist of many species (aerobic or facultative) that interact intimately with plants or animals. Nonpathogenic betaproteobacteria were also found to contribute to the evolution of class 1 integrons and resistance in the pathogens of other species (604).

Achromobacter spp.

The nonfermentative *Achromobacter* bacilli include an emerging opportunistic pathogen in cystic fibrosis patients, *Achromobacter xylosoxidans*, whose genome contains a range of resistance genes encoding drug-modifying enzymes and efflux pumps (605). The latter include 4 RND systems, additional tripartite efflux systems, and an ABC-type macrolide-specific MacAB transporter (605). Two RND pumps, AxyABM and AxyXY-OprZ, were identified to mediate MDR (606). Inactivation of AxyB produced up to a 20-fold reduction of the MIC values of several third-generation cephalosporins, with no changes in susceptibility to the aminoglycosides amikacin and tobramycin (606). However, the disruption of AxyY rendered a wild-type strain highly susceptible to aminoglycosides (16- to 128-fold MIC decreases for amikacin, gentamicin, netilmicin, and tobramycin), doripenem, erythromycin, and tetracycline (all with 4-fold MIC reductions). In an aminoglycoside-resistant mutant, AxyY inactivation restored its aminoglycoside susceptibility (20- to 192-fold MIC changes), thus revealing that AxyXY pumps out antibiotics often used for treatment of pulmonary infection of cystic fibrosis patients (607).

Burkholderia spp.

The genus *Burkholderia* contains >40 species that are present in a variety of ecological niches, including soil, water, plants, and animals. MDR is an emerging feature of many isolates such as those of *Burkholderia cepacia*. A comparison of the OM proteomes of *Burkholderia mallei* and *Burkholderia pseudomallei* suggests many similarities, which include a number of porins and efflux pumps (608). The major trimeric porin (Omp38) is a distant relative of *E. coli* OmpF, but its permeability is likely lower, by 1 or 2 orders of magnitude, than that of OmpF (609). However, another study showed that the single-channel conductance of Omp38 was similar to that of OmpF (610). The genomes of *Burkholderia* contain multiple replicons such as those in the *B. cenocepacia* genome that has three chromosomes in each strain with RND pumps encoded in all chromosomes (611). A recent study assessed all 8 RND families with 471 putative RND sequences in 26 completely sequenced *Burkholderia* genomes (including the virulent, epidemic cystic fibrosis strain J2315) (612). The drug-related HAE-1 RND pumps in the *Burkholderia* genus vary very much in number, ranging from only 4 in 3 strains of *B. mallei* to 15 in *Burkholderia* sp. strain CCGE1002 and 11 to 16 in *B. cenocepacia* (611–613).

The first RND pump characterized in *Burkholderia*, CeoAB-OpcM of *B. cenocepacia* (i.e., RND-10 of the 14 RND systems) (611), mediates resistance to multiple antibiotics, including tigecycline, and is inducible by salicylate, iron starvation, and chloramphenicol (16). While *ceoB* expression appears weak, the expression of 4 RND pump genes (*rnd-3*, *-9*, *-11*, and *-13*) is readily detectable. Expression of the *rnd-2* gene is also inducible by chloramphenicol (611). RNDs 6 and 7 are twin pumps encoded by the same operon, similar to MdtBC of *E. coli* (127, 611). Inactivation of the *rnd-4* gene led to a 4- to 8-fold increase in susceptibility to aztreonam, ciprofloxacin, levofloxacin, gentamicin, tobramycin, and chloramphenicol (614, 615). With 16 RND pumps present in *B. cenocepacia* strain J2315, their differential roles in drug resistance of planktonic and biofilm cells were investigated (613). Another study showed the high prevalence of RND pump-overproducing clinical isolates (particularly RND-3 overproducers) (616). However, the data regarding the effect of PAβN on antibi-

otic susceptibility appeared contradictory. While PAβN at 40 μg/ml did not alter the antibiotic susceptibility of several strains, as also noted with erythromycin in *B. pseudomallei* (614, 617), a newer study showed that PAβN at 64 μg/ml increased the susceptibility to tigecycline (mostly 32- to 64-fold) in the *B. cepacia* complex (618). The MICs of PAβN against several strains are 30 to 640 μg/ml (614), suggesting various susceptibilities of the individual strains to PAβN. The RND system encoded by the operon containing the BCAM0925 to BCAM0927 genes is upregulated by chlorpromazine and mediates resistance to azithromycin and chlorhexidine (619).

In *B. pseudomallei*, containing a dozen RND systems, the widespread expression of 7 RND pumps in clinical strains was evident (612, 620). Three pumps, AmrAB-OprA, BpeAB-OprB, and BpeEF-OprC, were characterized (617, 621–623). While both AmrAB-OprA and BpeAB-OprB mediate intrinsic resistance to aminoglycosides and macrolides (617, 621), expression of these pumps is commonly seen in clinical isolates (620). BpeEF-OprC is responsible for the widespread trimethoprim resistance in both clinical and environmental isolates (624). Interestingly, aminoglycoside- and macrolide-hypersusceptible strains (with gentamicin and azithromycin MICs of ca. 0.5 to 2 μg/ml) were isolated from both clinical and environmental samples and contained a point mutation in *amrB* that inactivated AmrAB-OprA. The reversion of this mutation via *in vitro* exposure of the susceptible strains to gentamicin and kanamycin (40 and 30 μg/ml, respectively) was able to restore aminoglycoside and macrolide resistance (625), highlighting the clinical concerns on current gentamicin use (e.g., 4 μg/ml) in medium to isolate *B. pseudomallei* (626). In *B. thailandensis*, multidrug-resistant mutants selected by doxycycline overproduced AmrAB-OprA and BpeEF-OprC. When either of these pumps was inactivated, a third pump, BpeAB-OprB, was hyperexpressed, indicating the interplay among the three RND pumps (627), similar to those observed in *P. aeruginosa* (628) and *Salmonella* (629). Interestingly, an antagonistic effect between PAβN (at 50 and 200 μg/ml) and aminoglycosides/β-lactams was observed, and the speculation was that PAβN might have induced the overproduction of the AmrAB-OprA and BpeAB-OprB pumps (627). It remains to be determined whether an induction process exists for explaining the above-mentioned observations that PAβN at 40 μg/ml did not alter antibiotic susceptibility in *B. pseudomallei* (614, 617) and that PAβN at 20 μg/ml did not affect the efflux activity of SmeDEF toward ciprofloxacin and tetracycline in *S. maltophilia* (630).

Neisseria spp.

Neisseria spp. are commensal bacteria in many animals, with two significant human pathogens, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, which have shown resistance emergence (for a recent review, see reference 631). These species possess trimeric porins that display high permeability with anion selectivity (632), and they may render the species more susceptible to anionic penicillins. Low-level MDR is mediated by mutations in genes encoding porins or efflux pumps (15, 633). In fact, simultaneous porin mutation and RND pump overproduction are needed for gonococcal penicillin or ceftriaxone resistance, again supporting the significance of the synergistic interplay between the OM and efflux pumps (634–636). Mutations in the "multiple transferable resistance" gene (*mtrR*) had long been associated with MDR in *N. gonorrhoeae*, with an assumed alteration in OM permeability (15), but the gene was then found to control the expression of MtrCDE, the most characterized RND pump in *Neisseria* that contributes to resistance to antibiotics (including β-lactams, macrolides, and rifampin), detergents, bile salts, gonadal steroidal hormones, as well as host cationic peptides (15, 633, 635–639). Either the deletion or overexpression of MtrCDE produces a 4-fold rifampin MIC change (639). MtrCDE deficiency renders gonococci more susceptible to progesterone (640).

Overexpression of MtrCDE due to *mtrR* or other regulatory mutations (see Regulation of Multidrug Efflux Pumps, below) has widely been observed in multidrug-resistant clinical isolates (16, 641). This includes interpatient transmission of high-level ceftriaxone-resistant/multidrug-resistant *N. gonorrhoeae* with MtrCDE overproduction (636). A new study also showed the global gene transcriptional changes as a consequence of MtrCDE overproduction, and these changes included an upregulation of the gene encoding cytochrome *c* peroxidase that is associated with tolerance to peroxides (642). The newly available crystal structures of MtrD and MtrE (643, 644) also suggest that the transport mechanisms of AcrAB-TolC discussed above (see Biochemistry and Genetics of Multidrug Efflux Pumps) are applicable to the MtrCDE pump. Another RND pump, FarAB-MtrE, of both *N. gonorrhoeae* and *N. meningitidis* mediates resistance to antibacterial fatty acids and cationic peptides that are likely present on mucosal surfaces of the host (16, 645). Since many substrates of MtrCDE and FarAB-MtrE are present in the host, these efflux pumps are also involved in bacterial pathogenesis such that *in vivo* fitness or survival is enhanced by these pumps (640, 641). The fatty acids and bile salts in the gut may likely facilitate Mtr-mediated resistance, as observed for isolates from rectal infections often possessing the MDR phenotype (646). Also, MATE and ABC pumps were also identified with additional tetracycline-specific exporters (16). The clinical importance of three MDR pumps (i.e., MtrCDE, MacAB, and NorM) was recently demonstrated by their impact on extensive or multiple drug resistance of clinical isolates in comparison with clinical susceptibility breakpoints (647). For example, in an extensively resistant isolate, the inactivation of MtrCDE changed the susceptibility status for azithromycin, penicillin, and tetracycline from R to S or I. MacAB inactivation alone also rendered the resistant isolate S, providing an important clinical example of the role of ABC transporters in bacterial drug resistance. The NorM deficiency was also able to make an R-to-I status change for tetracycline (8-fold MIC decrease) and to yield a >32-fold MIC reduction for solithromycin, a fluoroketolide for which the susceptibility breakpoints are as yet unavailable (647). Lastly, the gene encoding an MFS transporter, the Mef macrolide pump, on conjugative plasmids is present mainly in Gram-positive bacteria (15) but was also found in *N. gonorrhoeae* and *Acinetobacter junii*. These plasmids were readily transferred *in vitro* to several Gram-positive and Gram-negative bacteria, including *Neisseria* (648).

EPSILONPROTEOBACTERIA: *CAMPYLOBACTER* AND *HELICOBACTER*

Many of the epsilonproteobacterial species are host associated, such as *Campylobacter* and *Helicobacter*, and inhabit a wide variety of ecological niches ranging from gastrointestinal tracts of animals to water/marine reservoirs (649).

Campylobacter spp.

The *Campylobacter* group contains the two very important human enteropathogens *Campylobacter jejuni* and *Campylobacter coli*, which are among the top pathogens responsible for foodborne illnesses worldwide. There is an increasing emergence of resistance to clinically relevant antibiotics such as fluoroquinolones and macrolides, where drug efflux makes a major contribution (16).

Campylobacters possess a major porin, major outer membrane protein (MOMP), which appears to exist in a monomeric form as well as in a weakly associated trimeric form (650, 651) and likely yields OM permeability channels smaller than those of *E. coli* porins (652, 653). The marked susceptibility of wild-type campylobacter strains to large, hydrophobic antibiotics such as macrolides may suggest the presence of an unusually permeable bilayer domain in the OM.

The genome of *C. jejuni* indicates the presence of a large number of drug exporters (16). The CmeABC system is the most characterized RND pump in campylobacters and is responsible for both intrinsic and acquired MDR, including resistance to bile salts (654, 655). It is distributed in diverse isolates of animals and humans that are resistant to macrolides, fluoroquinolones, and tetracyclines (16, 656–661). Inactivation of CmeABC reduces significantly the *in vitro* emergence of fluoroquinolone-resistant mutants, while its overproduction facilitates such selection (659). Although the contribution of another RND pump, CmeDEF, to intrinsic resistance is likely masked by CmeABC, the two pumps interplay synergistically in providing resistance (662). CmeABC also interplays with 23S rRNA target alterations or ribosomal protein modifications in increasing macrolide resistance levels (658). The contribution of CmeABC to MDR was further observed with the synergistic interplay between anti-CmeA and anti-CmeB peptide nucleic acids to sensitize *C. jejuni* to multiple antimicrobials, thus also suggesting a potential novel approach to combat efflux-mediated MDR by using the pump-component-specific antisense peptide nucleic acids (663). The combinational use of anti-CmeA and anti-CmeB peptide nucleic acids, each at 1 to 4 μM, decreased the MIC values of ciprofloxacin and erythromycin against a wild-type strain 4- to >32-fold, while their separate use alone yielded merely a 2-fold MIC reduction. Although these peptide nucleic acids were effective in reducing the MIC of ciprofloxacin against a *gyrA* mutant 16-fold, they were able to produce only a 4-fold reduction of the erythromycin MIC for a high-level erythromycin-resistant mutant with a 23S rRNA mutation. An optimization study of these antisense peptide nucleic acids showed the importance of the ribosome-binding sites as the target (664). However, the entry of antibacterial peptides, including peptide nucleic acids, may require importers such as the SbmA/BacA IM proteins that were identified in several Gram-negative bacteria but remain unknown in campylobacters (665).

Tolerance of campylobacters to trisodium phosphate, a highly alkaline agent used to reduce pathogen prevalence on meat, is mediated by the NhaA1/NhaA2 cation/proton transporter and can be hindered by PAβN. This phenomenon was proposed to be attributable to the contribution of RND pumps (666). However, the effect of PAβN on OM permeability could also provide an alternative explanation, since the PAβN concentration used, 64 μg/ml, was quite high.

Campylobacters are widely distributed in food-producing animals, particularly in poultry. Thus, resistance in campylobacters of poultry origin has drawn much attention. Macrolides and fluoroquinolones are the drugs of choice to treat human campylobacteriosis. The exposure of chickens to either a fluoroquinolone (enrofloxacin) in drinking water or a macrolide (tylosin) in animal feed readily resulted in the isolation of CmeABC-overproducing mutants with an MDR phenotype (656, 667). *In vitro* stepwise exposures of *C. jejuni* to escalating levels of erythromycin or tylosin have generated a variety of macrolide-resistant mutants (668). The mutations in ribosome proteins L4 and L22 occurred early during this selection (low resistance level, with an erythromycin MIC of 8 to 16 μg/ml), followed by CmeABC overproduction (intermediate level, with an erythromycin MIC of 32 to 256 μg/ml), and finally accompanied by mutations in the 23S rRNA genes (high level, with an erythromycin MIC of ≥256 μg/ml), highlighting that efflux mechanisms likely facilitate the emergence of high-level macrolide resistance, similar to that of high-level fluoroquinolone resistance (659). CmeABC overexpression was partially a result of single-residue changes in the C terminus of the repressor CmeR (668). It should be noted that the multiple-stepwise-selection approach could yield pleiotropic mutations, thus underestimating the importance of the CmeABC-overproducing mutants that also possessed ~200 up- or downregulated genes (including the upregulation of genes encoding an SMR pump [Cj1173] and two MFS pumps [CmeG and Cj0035c]), and some of these changes may affect physiology and metabolism (668). The latter offers an explanation regarding the growth burdens and fitness cost of macrolide-resistant campylobacters reported by the groups of Zhang and Yuan (669–671).

Additional pumps that are independent of CmeABC and CmeEFG also likely mediate MDR (16) but remain to be further studied. Similar to the above-mentioned gene expression changes in erythromycin-resistant mutants (668), the transcriptional response of *C. jejuni* to an inhibitory concentration of erythromycin (4 μg/ml; 16× MIC) involved more than a hundred up- or downregulated genes, including two upregulated putative drug efflux operons (Cj0309c-Cj0310c and Cj1173-Cj1174), each encoding a paired SMR transporter. Inactivation of these operons impaired cell growth under conditions of high oxygen levels and colonization in chickens but did not alter the drug susceptibility toward 14 agents tested (including erythromycin) (672). The only MFS drug pump characterized in this species to date is CmeG, and its inactivation renders *C. jejuni* more susceptible to erythromycin, ciprofloxacin, and H_2O_2 (673). Finally, a novel ABC transporter, ArsP, was recently identified to mediate resistance to nitarsone and roxarsone, the organic arsenic agents used in poultry production (674).

Helicobacter spp.

Helicobacter species, including *Helicobacter pylori*, are associated with several important human and animal illnesses. As a dominant species of the human gastric microbiota, *H. pylori* causes a persistent inflammatory response and is linked to the development of ulcers and gastric cancers (675). While *H. pylori* infections need to be treated with antibiotics (676), acquired resistance to fluoroquinolones, macrolides, and metronidazole, agents used for the treatment of *H. pylori* infections, has emerged and is associated with antibiotic consumption (677, 678).

H. pylori displays intrinsic resistance to multiple antimicrobials, including glycopeptides, nalidixic acid, polymyxins, sul-

fonamides, and trimethoprim (676), suggesting that limited access to drug targets may constitute part of the mechanisms of resistance. Despite its relatively small genome size (1.7 Mb), *H. pylori* has genes encoding a large number of OM proteins (679), with several porins, including one that forms a large nonspecific channel (680). Nevertheless, the exquisite susceptibility of *H. pylori* to macrolides (MIC_{90} of <0.1 μg/ml [676]) suggests, as with campylobacter, that many drugs may permeate the bilayer regions of the OM. Of several RND systems (681), HefABC contributes to MDR (682, 683), and HefDEF (CznBAC) is a metal exporter for cadmium, nickel, and zinc resistance and gastric colonization (684). Four TolC homologs (HefA, HefD, HefG, and HP1489) were identified, and only HefA inactivation rendered the mutants more susceptible to deoxycholate and novobiocin. The simultaneous disruption of HefA and HefD increased metronidazole susceptibility (685), and laboratory-selected resistant mutants overexpressed HefA (686). The expression of HefA was increased following exposure to metronidazole in clinical isolates (687). In an interesting study, *H. pylori* cultured in the presence of cholesterol became resistant to bile salts and their analogs, and this phenotype required the HefC pump (688). Intriguingly, *hefC* missense mutations were among the several gene mutations in *in vitro*-selected isolates with high-level resistance to amoxicillin, and the introduction of a mutated allele to the wild type increased resistance (689). Through examining the whole-genome sequences of clarithromycin-resistant isolates, a new study revealed the gene clusters of TolC homologs involved in clarithromycin susceptibility profiles in individual isolates (690). Given the extremely acidic environments where *H. pylori* colonizes, the effect of this environment on secondary transporters in *in vivo* antimicrobial resistance remains to be determined. In this regard, there are a number of putative ABC transporters in *H. pylori* (679). Inactivation of the ABC-type transporter MsbA rendered the strain more susceptible to erythromycin and glutaraldehyde. MsbA also interplays synergistically with the glutaraldehyde-resistant protein Ost/Imp to enhance hydrophobic drug resistance and LPS biogenesis (691). Various types of putative drug transporters (e.g., 2 RND pumps, 1 MFS pump, 2 MATE pumps, 4 SMR pumps, and 1 ABC pump) were also identified in *Helicobacter cinaedi*, a pathogen increasingly known for infections in immunocompromised patients (692).

BACTEROIDACEAE AND *PREVOTELLACEAE*

The anaerobic families *Bacteroidaceae* and *Prevotellaceae* constitute a dominant part of the mammalian gut microbiota and play an important role in maintaining human health. However, they may cause anaerobic infections (e.g., blood and intra-abdominal infections such as those associated with *Bacteroides fragilis*) and exhibit intrinsic resistance to a number of antimicrobials (for a review, see reference 693). Increasing MDR in *Bacteroides* spp. has been observed in recent years. The OM of *B. fragilis* contains porins that produce a much lower rate of diffusion of hydrophilic saccharides than those of *E. coli* porins (16), and this would make an efflux process efficient. However, the OM was shown to allow rapid passage of hydrophobic agents such as rifamycin and clindamycin, likely through its bilayer domain, which was shown to be more lipophilic than that of the enteric bacteria (694). Considering the environments that *Bacteroides* inhabits, it is not surprising to see large genome sizes (e.g., 5.2 Mb with *B. fragilis* and 6.3 Mb with *Bacteroides thetaiotaomicron*) (695, 696), which also include a large number of drug efflux genes, e.g., those encoding 16 putative RND pumps in *B. fragilis* (697) and 60 predicated drug efflux components in *B. thetaiotaomicron* (696).

Many RND pump genes of *B. fragilis* were expressed under laboratory conditions, and some were demonstrated to play a role in intrinsic resistance (463, 697). Inactivation of BmeABC5 yielded a 4-fold reduction in the metronidazole MIC and also increased susceptibility to cefoperazone and SDS (698). Intrinsic resistance to fluoroquinolones is mediated by an MFS NorA pump in *B. fragilis* and a MATE BexA exporter in *B. thetaiotaomicron* (15). A macrolide resistance gene, *msr*(SA) (coding for an ABC exporter), previously found in Gram-positive bacteria, was also detected in *B. fragilis* (699). Thus, drug efflux is likely a major contributor to resistance in *Bacteroides*. Indeed, one study confirmed a wide presence of resistance efflux genes in a number of clinical isolates (700). Furthermore, a recent study of a multidrug-resistant *B. fragilis* isolate led to the identification of a unique conjugative transposon containing a hybrid mosaic of elements, including genes for 3 efflux systems (MefA and ABC exporters of Gram-positive bacteria and RND pump conserved in *Bacteroides*) (701), again showing the emergence of complex resistance gene assembly.

Prevotella spp. also constitute part of the oral and vaginal microflora and can also cause anaerobic infections. A recent study from the United Kingdom (702) suggested the presence of multiple RND pumps in 5 *Prevotella* spp. and their contribution to tetracycline resistance in clinical isolates from patients with cystic fibrosis and invasive infections. However, a relatively high PAβN concentration (80 μg/ml) was used.

DRUG EFFLUX GENES ON PLASMIDS

Plasmids have played a critical role in both the emergence and spread of resistance to most classes of antibiotics in bacteria (703–705). Their mobile nature (often linked to transposons/integrons) within or across bacterial species makes it extremely difficult to contain resistance. Moreover, plasmid-mediated mechanisms often confer high-level resistance. Genes encoding various classes of drug pumps have also been identified on numerous plasmids. The typical examples are the well-known single-drug-class efflux genes, such as various *tet* efflux genes usually found on plasmids and encoding MFS pumps (8, 15, 133). The *floR* gene, found first in an R plasmid in the florfenicol-resistant fish pathogen *Pasteurella piscicida* and then in *Salmonella* and *E. coli*, also codes for a singlet MFS pump responsible for amphenicol resistance (15). Another efflux gene, *mef*(B), coexists with the *aadA* and *sul3* resistance genes in plasmids of porcine *E. coli* isolates and mediates macrolide resistance (706). The plasmid-borne *qepA* and *qepA2* genes encode MFS pumps providing resistance specific to fluoroquinolones (206, 707). These drug-specific exporter genes are limited to encoding the singlet MFS pumps.

In contrast to many MFS pumps, RND family efflux genes generally require the contribution of three gene products in order to produce effective efflux. Most Gram-negative bacteria contain at least one set in their chromosome and in many cases contain several sets of genes for such systems. Hence, the RND-type transporter genes appear to be infrequently present on plasmids. However, the fact that several RND pump-encoding plasmids have been reported to date (Table 4) forces us to consider that such plasmids may exist more widely than previously suspected. Interestingly, most of these plasmids were already isolated decades ago

TABLE 4 Conjugative plasmids encoding RND or ABC multicomponent efflux systems of Gram-negative bacteria

Plasmid(s) (GenBank accession no.)	Isolation yr	Source, country	Host	Inc group	Size (kb)	Efflux component(s)	Pump regulator	Additional resistance gene(s) and transposon(s)	Resistance phenotype of initial host strain	References
pMG101 (AF067954)	1973	Burn ward, USA	*Salmonella enterica* serovar Typhimurium	IncHI	180	SilABC RND and SilP ABC transporters	SilRS	Unknown	Ampicillin, chloramphenicol, streptomycin, tetracycline, silver	716, 717
pMCBF1 (AY950444), pMCBF6 (EF107516)	1993	Marine, Sweden	Uncultured bacterial biofilm (*Pseudomonas putida* as recipient)	IncP-1	63, 67	MexEF-OprN RND homolog and MerF/MerT transporters	MerR	*merA*, *merB*, *merD*, *merP*; Tn*5053* or Tn*5058*	Mercury	721, 722
pOLA52 (EU370913)	1999	Swine manure, Denmark	*Escherichia coli*	IncX1	52	OqxAB RND pump	ORF68 (GntR family)	bla_{TEM}; Tn*6011*	Ampicillin, carbadox, chloramphenicol, kanamycin, nitrofurantoin, olaquindox, sulfamethoxazole, trimethoprim	708, 892, 1018, 1019
pB4 (AJ431260)	1999	Activated sludge, Germany	Uncultured bacterium (*Pseudomonas knackmussii* as recipient)	IncP-1β	79	MexCD-OprJ RND homolog	NfxB	bla_{NPS-1}, *chrABC*, *strA*, *strB*; Tn*5719*, Tn*5720*, Tn*5393c*	β-Lactams, spectinomycin, streptomycin, tetracycline, chromate	1020, 1021
pRSB101 (AJ698325)	2001	Activated sludge, Germany	Uncultured bacterium (*Pseudomonas knackmussii* as recipient)	IncQ	48	AcrA-ABC permease/ATPase, OprM and TetA MSF pump	AcrR and TetR	*aadA2*, bla_{TLA-2}, *chrA*, *dhfr1*, *mph*(A), *mphR*(A); *qacE*Δ, *sulI*, Tn*402*, and *intI*	Cephalosporins, erythromycin, nalidixic acid, norfloxacin, roxithromycin, spectinomycin, streptomycin, sulfonamides, tetracycline, trimethoprim	719, 1021
pNDM-CIT (JX182975)	2010	Patient, India/France	*Citrobacter freundii*	IncHI1	289	MexAB-CusC RND homolog	AcrR, HN-S-like protein	bla_{NDM-1}, *armA*, *mel*, *mph*; Tn*1548*	β-Lactams, aminoglycoside, fluoroquinolones, macrolides, nitrofurantoin, tetracycline, tigecycline	243, 724

and derived from environments with possibly strong antimicrobial selection pressures (Table 4). Of these plasmids, the *oqxAB* genes that code for such a pump and a periplasmic adaptor protein were found in a plasmid in an *E. coli* strain resistant to olaquindox (708), a quinoxaline-di-*N*-oxide agent once widely used in pigs in Europe. The OqxB transporter shows only 40% identity with AcrB but is more closely related to MexF of *P. aeruginosa*. Dependent on the host TolC protein, OqxAB has a wide substrate specificity, making *E. coli* also more resistant to fluoroquinolones, nalidixic acid, chloramphenicol, trimethoprim, benzalkonium chloride, and SDS (709). The *oqxAB* genes have since increasingly been found on R plasmids from *E. coli* (710–714) and several other species of *Enterobacteriaceae*, such as *Salmonella* and *K. pneumoniae* (235, 236, 303, 715). The coexistence of the *oqxAB* and *floR* or CTX-M gene on the same plasmids was also observed (713, 715). Chromosomal *oqxAB* was found in clinical isolates of *K. pneumoniae* and considered a possible source of plasmid-borne *oqxAB* (302).

A silver resistance megaplasmid, pMG101, the earliest isolated plasmid containing genes for an RND transporter, was obtained from multidrug-resistant *Salmonella* in a hospital burn ward where silver sulfadiazine was widely used in the 1970s (Table 4) (716, 717). (Chromosomally encoded efflux and porin loss have also been reported for *E. coli* [718].) Another tripartite exporter (AcrA-ABC pump-OprM) was encoded by a large plasmid (pRSB101) isolated from a sewage treatment plant (719). In a study of mixed plasmid DNAs from a similar wastewater treatment plant (720), homologs of *E. coli acrB* and *acrD* as well as those of *P. aeruginosa mexB*, *mexD*, and *mexY* were found. If these genes are truly derived from plasmids, this result may show us that putting RND pumps on plasmids is not so difficult and may serve as a cautionary note for the future development of MDR in Gram-negative bacteria. Indeed, this worrisome speculation is supported by a recent study that showed the inclusion of the complete *P. aeruginosa mexEF-oprN*-like genes (ca. 23%, 35%, and 17% identities with MexE, MexF, and OprN, respectively, at the protein level [X.-Z. Li, unpublished data]) into two conjugative broad-host-range plasmids from a marine microbial biofilm (721). Initially isolated as mercury resistance plasmids from marine samples collected in 1993 (722) and maintained in *Pseudomonas putida*, these two plasmids from an unknown host are largely identical and evolved through homologous recombination, and each plasmid contains transposons and various gene clusters (*trb* for mating pair function, *tra* for conjugative gene transfer, *mer* for mercury resistance, and *mexEF-oprN*-like genes for the RND pump) (Table 4) (721, 723). When tested for susceptibility to chloramphenicol, nalidixic acid, and trimethoprim, an *E. coli* isolate carrying one of these two plasmids did not show altered drug susceptibility. However, a *Pseudomonas* host with a deficiency in endogenous RND pumps and a greater variety of agents could have been used for defining the function of the plasmid-borne RND pump. Lastly, a plasmid from an extremely drug-resistant *C. freundii* isolate from a patient returning from India (724) contains genes encoding the carbapenemase NDM-1, the ArmA 16S RNA transferase, and an RND system (homologous to AcrR-MexAB-CusC) (243). The RND gene region belongs to part of the CP4-like prophage sequence, and whether this RND pump is functional in providing MDR remains unknown. Nevertheless, these RND pump-encoding plasmids (Table 4) have no doubt provided evidence for sophisticated resistance evolution with possible adverse clinical implications.

REGULATION OF MUTLIDRUG EFFLUX PUMPS

The regulation of drug efflux pump expression involves a variety of complex pathways that typically require the participation of numerous local and global transcriptional regulators or modulators as well as the two-component regulatory systems. Mutational changes of these regulators can lead to altered expression levels of the pumps. Certain pumps are also inducible by various compounds, including antibiotics. The versatile nature of pump regulation is consistent with the widespread presence of numerous differentially expressed drug pumps and their role in resistance and other physiological functions in order to adapt to diverse environments. Below are examples that demonstrate the multilevel regulation of drug exporters. An early review on the regulation of drug transporters was available (725), but since then, major advances have been made.

E. coli Efflux Pumps

AcrAB-TolC. AcrAB is constitutively expressed at a significant level in *E. coli*, and a similar situation seems to prevail for AcrAB homologs in other organisms. Although the *acrAB* and *tolC* genes are not genetically clustered, their expressions are often regulated by common regulators at multiple levels (Fig. 4). First, the local repressor AcrR, encoded by the *acrR* gene that is divergently transcribed from *acrAB*, represses directly both *acrAB* expression and its own expression (726). A member of the TetR repressor family (727), AcrR functions as a dimeric two-domain molecule, and each monomer contains a smaller N-terminal domain and a larger C-terminal domain. The latter constitutes the ligand-binding domain with a multientrance pocket to accommodate a number of ligands or inducers (e.g., rhodamine 6G), followed by the conformational change of AcrR that cooperatively affects the N-terminal DNA-binding domain (728–730). However, AcrR does not tightly inhibit AcrAB expression, and this allows the constitutive production of AcrAB, conferring intrinsic MDR. Second, other regulators, the AcrS repressor of the AcrEF pump (731), the histone-like nucleoid structuring protein (H-NS) (732), and the quorum-sensing receptor SdiA (733, 734), also participate in the regulation of *acrAB*. SdiA likely acts as a minor activator since its genetic deletion results in a small (2- to 3-fold) decrease in the MICs of fluoroquinolones (733). Third, as described below, three global regulators, MarA, SoxS, and Rob, play major roles by positively controlling the expression of *acrAB*, *tolC*, and *micF*. The *micF* transcript inhibits the translation of OmpF porin mRNA.

The multiple antibiotic resistance (*mar*) locus is a hot spot for mutation (735) and includes two divergent transcriptional units, *marRAB* and *marC*, which are transcribed from a common operator/promoter region (*marO*) (201). (However, *marC*, with unknown function, is not involved in resistance [736].) The prototypical member of the MarR family regulators, the MarR repressor, is a dimer, with each monomer containing a winged-helix DNA-binding motif. It negatively regulates the expression of *marRAB* and is critical in determining the expression of the MarA activator (737). MarA is an AraC family transcriptional activator (738) and possesses two similar helix-turn-helix DNA-binding subdomains (739). MarA not only positively controls the expression of *marRAB* (by binding to *marO* in a region that differs from where MarR binds) (201) but also is involved in the regulation of

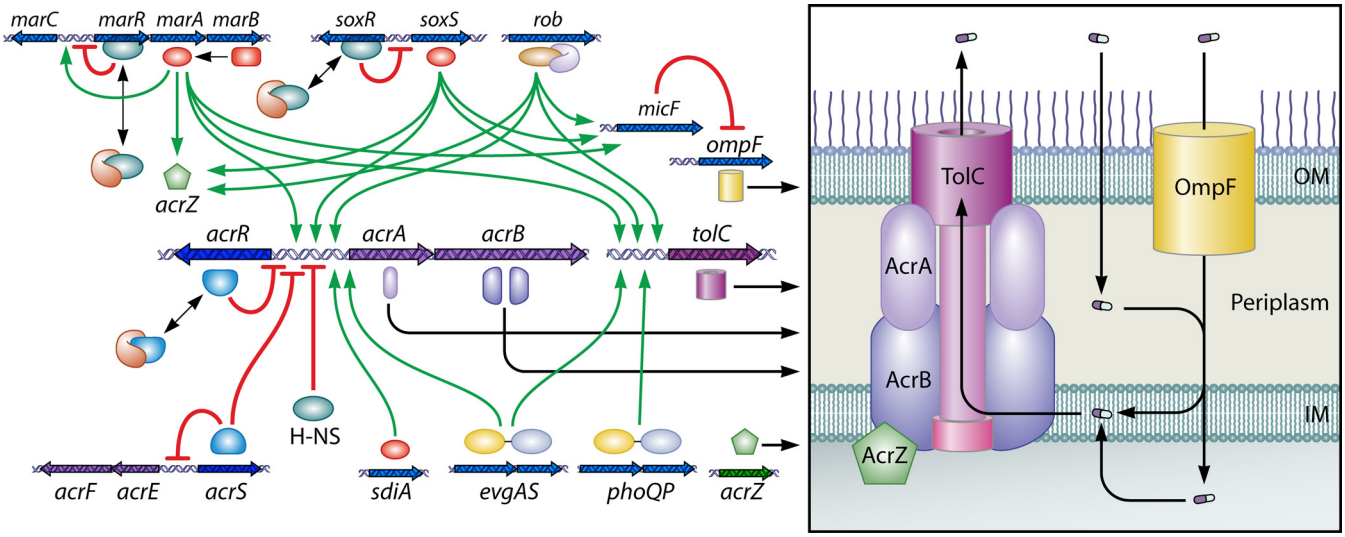

FIG 4 Regulation of expression of the AcrAB-TolC efflux system of *E. coli*. Transcription of the *acrAB* and *tolC* genes is not genetically clustered but is often regulated by common regulators at multiple levels. The local repressor AcrR represses *acrAB* expression directly. Other regulators include the AcrS repressor of the AcrEF system, histone-like nucleoid structuring protein (H-NS), and the SdiA global regulator. Three global regulators, MarA, SoxS, and Rob, positively control the expression of *acrAB*, *tolC*, and *micF*. The *micF* transcript inhibits the translation of OmpF porin mRNA. The two-component regulatory systems EvgAS and/or PhoQP can enhance *acrAB* and *tolC* expression. The red lines show the repression of the transcription of the relevant gene by the repressors AcrR, AcrS, H-NS, MarR, and SoxR. The green arrowed lines reveal the activation of relevant gene expression by the activators MarA, SoxS, SdiA, Rob, EvgS, and PhoP (SoxS and Rob can also stimulate MarA expression). MarB modulates MarA expression. Several regulators can bind with certain ligands (such as antimicrobial agents and metabolites) or be induced by oxidative stress and thus become inactivated (in the case of AcrR, MarR, and SoxR) or activated (in the case of Rob when binding with bile salts or fatty acids). Mutational changes can lead to the inactivation of AcrR, AcrS, MarR, and SoxR. The crystal structures of AcrR, MarA, MarR, SoxR, and Rob are available with identified ligand-binding domains and conformational changes for regulation. Regulation of AcrAB by noncoding RNAs has also been identified (see the text). Overall, under various conditions, these multiple regulation mechanisms can together produce MDR by allowing simultaneously decreased influx (via OmpF porin) and increased efflux (via AcrAB-TolC) of antimicrobial agents, which can be captured by the pump complex from the outer and/or inner leaflets of the IM and the periplasm (but not directly from the cytosol). Expression of *acrZ* (whose product can be copurified with AcrB) is coregulated with that of *acrAB* via MarA, SoxS, and Rob.

>60 genes (740). Promoter discrimination of MarA regulon promoters is mediated by Glu89 of MarA (741). AcrZ, a recently identified small accessory protein of AcrB (112, 123), is also positively regulated transcriptionally by MarA, SoxS, and Rob (112). The level of MarA (and SoxS) is also controlled by proteolytic degradation by the Lon protease (742). The function of MarB remained unknown until 2011 with a phenotypic examination of the *E. coli* mutant library (Table 1) (30), which revealed that MarB is a fine-tuning player inhibiting MarA and that this inhibition likely occurs via a posttranscriptional process. Two additional global activators, SoxS (the effector of the oxidative stress *soxRS* regulon) and Rob (743), also belong to the AraC family and can bind to *marO* and stimulate the gene expressions of the *mar* regulon, including *acrAB* and *tolC* (201). Because MarA and SoxS are very small proteins, they work solely by changing the level of their expression. Their upregulation may occur through the mutational inactivation of their cognate repressors MarR and SoxR, respectively; in clinical isolates showing high levels of fluoroquinolone resistance, mutations inactivating MarR are common (198). In other studies often involving other species of *Enterobacteriaceae*, fluoroquinolone-resistant mutants also had mutations in *soxRS* or *acrR*, as described above in the section dealing with *Enterobacteriaceae*. Alternatively, these repressors may become inactive through interactions with small molecules, e.g., salicylate, 2,4-dinitrophenol, or plumbagin (15, 744) binding to MarR, or oxidative inactivation of FeS-containing SoxR by superoxide (745). In contrast, Rob is much larger, and the binding of ligands such as dipyridyl (15) or fatty acids and bile salts (746) increases its binding affinity for the promoter region of *acrAB*, resulting in the upregulation of AcrB. Also, unlike with MarA, only one of Rob's two helix-turn-helix DNA motifs engages the binding site (747).

A two-component system, EvgAS, is also involved in the transcriptional regulation of not only *acrAB* and *tolC* but also *emrKY*, *mdtEF*, and *mdfA* (748, 749). Still, *tolC* expression is dependent on both EvgAS and another two-component system, PhoPQ. These systems constitute a signal transduction cascade and are connected via a small IM protein named B1500 (65 amino acids), whose expression is directly regulated by EvgSA (750). The common regulation of *acrAB-tolC* by many regulators is further elucidated by the transcriptional activation of two *tolC* promoters using one binding site (*mar* box) by MarA, SoxS, and Rob, whereas a different promoter of *tolC* is activated by EvgAS and PhoPQ (751). Interestingly, a small RNA, RyeB, expressed during the stationary phase, can inhibit TolC expression (752). This type of regulation occurs at the posttranscriptional level through binding of the small RNA to the 5′ untranslated region of mRNA targets, and it requires a small chaperone protein, Hfq. An Hfq mutant is susceptible to several antibiotics and other toxic agents, and this phenotype is dependent on a functional AcrAB, suggesting the involvement of Hfq in AcrAB production (753). Hfq mutants have reduced abilities in fitness, virulence, and biofilm formation, likely due to the effect of Hfq on the regulation of the stationary-phase sigma factor RpoS and the envelope stress response sigma factor RpoE (754). However, the clinical significance of small RNA regulation of MDR efflux systems requires further investigation.

Overall, under diverse conditions, these multiple regulatory mechanisms can together produce MDR by allowing simultaneously decreased influx (via decreased OmpF porin) and increased efflux (via AcrAB-TolC) of various agents (Fig. 4). Indeed, one *in vitro*-selected high-level ceftazidime-resistant mutant (128-fold MIC increase) had OmpF loss and increased expression levels of *acrB*, *acrD*, and *acrF* with multiple mutations in *acrR*, *marR*, and the gene for penicillin-binding protein 3 and overexpressed *sdiA*, while another mutant with low-level ceftazidime resistance (4-fold MIC increase) showed only increased *acrB* expression levels due to an *acrR* mutation (755).

Regulation of other pumps. The MdtABC and MdtEF RND pumps are regulated by the two-component systems BaeSR and EvgSA, respectively (128, 756). Expressed from the genes downstream of *mdtABCD*, BaeSR not only activates the expression of *mdtABCD* (128) but also controls the expression of >60 genes (including *acrD*) that are part of the BaeSR regulon and involved in signal transduction, the chemotactic response, flagellum biosynthesis, metal homeostasis, and sugar or drug transport (757), providing a major pathway related to the bacterial cell envelope response (758). (BaeSR is in fact required for envelope stress-induced CRISPR [clustered regularly interspaced short palindromic repeat] RNA-mediated DNA silencing that constitutes part of a defense mechanism in *E. coli* [759].) MdtBC production is increased strongly with subinhibitory concentrations of ciprofloxacin (760), but clinically relevant conditions for its overexpression are not obvious. Functioning through the BaeSR and CpxAR pathways, indole induces the expression of MdtABC and AcrD (756). Tannins, secondary metabolites of plants, can also induce the expression of MdtABC via BaeSR (761). Of the BaeSR regulon, there is a *spy* gene encoding a periplasmic chaperone (Spy), which shows increased levels in TolC or pump mutants and requires BaeSR and CpxAR systems for full activation (762). The expression of MdtEF is positively regulated by two AraC family activators, YdeO and GadX (763–765), and is also stimulated by *N*-acetyl-D-glucosamine through catabolite activation (763) and overexpression of a small (85-nucleotide) noncoding DsrA RNA (766). The latter RNA functions as an antisilencer of the H-NS-silenced genes (767). H-NS represses the expression of the efflux operons *mdtEF*, *acrEF*, and *emrKY* (732). Under anaerobic conditions, *mdtEF* expression involves regulation by the ArcBA system, which antagonizes the effect of the H-NS (768). One study also suggested that the expression of *acrAB*, *emrAB*, *emrD*, *emrE*, *emrKY*, *mdfA*, and *ydgFE* is relatively stable during the various phases of growth, but *mdtEF* has the highest expression level at the late stationary phase, and this is mediated by RpoS (769).

Salmonella Efflux Pumps

Similar to the regulation of its *E. coli* homolog, the *Salmonella* AcrAB-TolC system is controlled through several regulatory pathways, such as AcrR, MarA, and SoxS (16, 770–773). Paraquat can induce AcrAB production, and this induction is dependent on SoxS (774). A recent study also showed that the expression level of the *acrB*, *acrD*, and/or *acrF* gene was increased when one or multiple *acr* genes were deleted (629), and this observation was similar to the situation found among the Mex pump genes of *P. aeruginosa* (628). However, the compensatory *acr* expression level changes appeared to have only a minimal impact on the drug susceptibility phenotype (except some aminoglycosides) (629).

Another gene locus of *ramRA* that is widespread in *Enterobacteriaceae* except *E. coli* also significantly influences the expression of not only AcrAB but also AcrEF and MdtABC in *Salmonella* (16, 229, 773, 775, 776). *ramRA* are transcribed divergently, with RamR repressing *ramA* expression (775–779). Induced by a variety of environmental signals, including bile salts, indole, and phenothiazines, RamA serves as a small activator protein to contribute to increased AcrAB production (776, 780–782). Bile binds to RamR (778) and in this way is thought to increase the expression of AcrAB. Although an earlier study (780) claimed that cholate and indole bound directly to RamA, the binding of an effector ligand to such a small regulator (only 129 amino acids) is highly unusual. Indole induces AcrAB production by also increasing RamA expression (780). RamA expression can be also increased by the inactivation of AcrAB and is regulated by the ATP-dependent Lon protease (782, 783). (The *ramA* sequence was present, but escaped notice, within the *E. cloacae* genomic fragment that was found to downregulate *E. coli* OmpF in 1990 [784] and was correctly identified in *K. pneumoniae* later [785].) The TetR-type RamR repressor negatively controls the expression of *ramA* and *ramR* itself by binding to the intergenic *ramA-ramR* promoter region (778), and mutations in *ramR* lead to increased production of RamA (777). RamR forms complexes with multiple agents (e.g., berberine, crystal violet, dequalinium, ethidium bromide, and rhodamine 6G), and this interaction occurs in a common residue of Phe115 and reduces the DNA-binding affinity of RamR (779). A recent review also highlighted the role of RamRA in the regulation of AcrAB (36). Additionally, the expression of the ABC-type MacAB pump is negatively controlled by PhoPQ (222).

K. pneumoniae Efflux Pumps

Similar to that in *E. coli* and *Salmonella*, AcrAB expression in *K. pneumoniae*, as shown in various clinical isolates or laboratory mutants, involves both negative control by AcrR and positive control by RamA and SoxS (292, 299, 501, 786). RamA and SoxS expressions are affected by RamR and SoxR regulators, respectively (299, 787). RamA can also autoregulate its own expression through binding to two *ramA* promoter sites (788). *ramR* is located upstream of the *romA* and *ramA* genes and is transcribed divergently (787), and RamR binds to two sites of the *romA-ram* locus (788). Mutations in *ramR* can yield hyperproduction of RamA and AcrAB-TolC (300, 787, 788).

Another AraC regulator, RarA, which is encoded by a gene upstream of the chromosomal *oqxAB* pump genes (chromosomal *oqxAB* genes are found only in *K. pneumoniae* to date), also positively controls the expression of AcrAB and OqxAB. RarA is present not only in *K. pneumoniae* but also in *Enterobacter* and *Serratia* (789, 790), and it functions in a regulon that affects the expression of 66 genes (791). The expression of OqxAB is additionally downregulated by a GntR-type regulator, OqxR, encoded by a gene located downstream of *oqxAB* (790). A point mutation in *oqxR* was linked to the hyperexpression of *rarA* and *oqxB* (792). Intriguingly, the genetic arrangement of *rarA-oqxAB-oqxR* is observed both in chromosomes and on a plasmid (790). Nearly all tested clinical tigecycline-nonsusceptible isolates (25 out of 26) from China contained mutations in *ramR* and/or *acrR*, with about one-third of the isolates carrying the simultaneous overexpression of *ramA-acrB* and *rarA-oqxB* (301). The expression of *eefABC* appears to be induced by an acidic or hyperosmolar environment but not by bile salts (793). The CpxAR system and the LysR-type

OxyR regulator are also involved in the positive control of the expression of the *acrB*, *acrD*, and/or *eefB* efflux gene (794, 795).

P. aeruginosa Efflux Pumps

MexAB-OprM. Despite rather stable expression under standard laboratory conditions, the *mexAB-oprM* operon is subject to complex and finely tuned regulation. Multiple gene products have been identified to influence *mexAB-oprM* activity, and these include the regulators MexR, NalD, and ArmR (due to NalC alteration) and a two-component system, RocS2-RocA2 (Fig. 5). MexR, a MarR family repressor, is determined by a self-regulated gene (*mexR* [also called *nalB*]) adjacent to *mexAB-oprM* on the chromosome (403). The binding of MexR as a dimer to the intergenic DNA region carrying the divergent overlapping promoters of *mexR* and *mexAB-oprM* (P_I) results in the balanced transcription of both *mexR* and *mexAB-oprM*, which provides *P. aeruginosa* a protective baseline level of wide-spectrum efflux activity (405, 796). Despite the presence of another more proximal promoter (P_{II}), P_I drives most of the expression of the operon in wild-type cells (796, 797). Interestingly, some *in vitro* data support the notion that MexR dimerization through the formation of intermonomer disulfide bonds between redox-active cysteines prevents MexR from interacting with its cognate DNA-binding sites and from exerting its repressor activity (798, 799). This redox modulation of MexR was proposed to occur *in vivo* when bacteria are stressed by oxidative agents (cumene hydroperoxide) or antibiotics (meropenem and nalidixic acid) (798). However, this assumption still needs to be substantiated, as H_2O_2 (800, 801) and antibiotics such as colistin (802) and tobramycin (803, 804) apparently do not induce *mexAB-oprM* transcription significantly in planktonic and/or sessile cells.

The expression of *mexAB-oprM* can be modulated positively although indirectly by ArmR, a 53-residue peptide encoded by the second gene of a two-gene operon, PA3720-PA3719 (406). Isothermal titration calorimetry studies demonstrated that ArmR can sequester MexR via an allosteric polypeptide-protein interaction of high affinity, thus alleviating the repressor activity of MexR toward *mexAB-oprM* (805). Indeed, the conformation of MexR in complex with ArmR is incompatible with DNA binding (806); thus, ArmR may be considered an antirepressor. Unless mutations disrupt the *nalC* (PA3721) gene, which encodes a TetR family regulator (727) that strongly represses the adjacent PA3720-PA3719 operon, baseline amounts of ArmR are not expected to influence MexAB-OprM production in wild-type cells (406). However, chemostat experiments with strain PAO1 showed that various chlorinated phenols, including the environmental contaminant pentachlorophenol, can induce the expression of the PA3720-PA3719 and *mexAB-oprM* operons through reversible, noncovalent binding to the NalC protein (807, 808). Recent data, however, showed that pentachlorophenol stimulates MexAB-OprM production, surprisingly, in an ArmR-independent (although MexR-dependent) manner (809). Possibly, the regulatory pathway here involves *in vivo*-generated catabolite effectors mimicking more specific antimicrobial phenolic compounds than pentachlorophenol that *P. aeruginosa* may encounter in its natural environment (809). The physiological conditions under which ArmR ultimately activates *mexAB-oprM* thus remain unclear and require further studies.

The third known regulator of *mexAB-oprM* is NalD, another TetR-type repressor encoded by the PA3574 gene, which binds to a proximal promoter, P_{II}, upstream of the efflux operon (408, 810). Its DNA binding abolishes P_{II} activity in wild-type bacteria, resulting in *mexAB-oprM* being expressed essentially from the distal promoter P_I (810). In contrast to its homolog TtgR from *P. putida*, which negatively controls the expression of the TtgABC pump, no ligand that is able to relieve NalD from its operator site has been reported so far (811). Whether NalD-dependent induced expression of *mexAB-oprM* occurs when *P. aeruginosa* is challenged with some natural or semisynthetic antimicrobials remains to be elucidated. Moreover, experimentally adding polyethylene glycol to NalD *in vitro* during a structural study resulted in the contraction of NalD intraprotein chains (812), yet its physiological significance is unknown. Note that MexAB-OprM overproduction with combinational mutations in *mexR*, *nalC*, and *nalD* has been observed in clinical isolates, including epidemic strains (380, 382, 413, 425).

In addition to MexR, ArmR, and NalD, somewhat more complex regulatory circuits control MexAB-OprM expression. This is not really surprising *per se* in view of the major protective function of the transporter. Thus, *mexAB-oprM* expression has been reported to be growth phase regulated and to reach a maximum level at the onset of the stationary phase, independently of MexR and of LasR, a transcriptional regulator controlling the production of the quorum-sensing cell-to-cell signal *N*-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C_{12}-HSL) (16, 797, 813). Despite its structural relationship with 3-oxo-C_{12}-HSL, the Rhl quorum-sensing signal *N*-butanoyl-L-homoserine lactone (C_4-HSL) is not a substrate for MexAB-OprM (398, 399). The length of the acyl side chain seems to be an important factor in the binding affinity of acyl-HSL for MexB, with C_8 to C_{14} compounds being better substrates for the pump than C_4 to C_7 molecules (399, 400). While 3-oxo-C_{12}-HSL does not significantly impact *mexAB-oprM* transcript levels when added exogenously to *P. aeruginosa* (400, 814), C_4-HSL can induce operon expression quite strongly. Consequently, it was inferred that the C_4-HSL molecule might play a role in the growth-phase dependent regulation of MexAB-OprM (814), with MexR not being required for this control (815). In this regard, the local activator MexT of the MexEF-OprN system also displays an inhibitory effect on MexAB-OprM expression in MexEF-OprN-overexpressed *nfxC* mutants through as-yet-uncharacterized mechanisms (814). A macrolide (azithromycin) can negatively affect MexAB-OprM expression through its impact on the quorum-sensing system (16). However, the interplay between the pump and quorum sensing in *P. aeruginosa* is far from clear if one considers that the deletion of the *mexAB-oprM* operon does not result in increased production of elastase in strain PAO1 (398). This result is surprising since 3-oxo-C_{12}-HSL, which signals elastase production, would be expected to reach higher intracellular levels if it is no longer effluxed out of the cells. Moreover, a global transcriptional regulator of the LysR family, AmpR, which also controls the expression of AmpC β-lactamase, is reported to repress *mexR* expression and thus to increase the level of MexAB-OprM production (816).

Another still open issue relates to the role that MexAB-OprM potentially plays in the increased intrinsic resistance of *P. aeruginosa* biofilms to antibiotics. In some experiments, the expression levels of *mexAB-oprM* appeared to have no or a limited influence on the resilience (log reduction of CFU) of *in vitro*-developed biofilms exposed to ofloxacin, ciprofloxacin, or tetracycline (817). Supporting the notion of a weak impact of the export system on

biofilm resistance, *mexAB-oprM* expression was even found to be repressed by RocA2, a response regulator activated by two distinct sensor kinases (RocS1 and RocS2) that are themselves known to positively control the pilus assembly machinery cluster *cupC* involved in adherence/microcolony formation (814, 818). Interestingly, BrlR, a biofilm-specific MerR-type regulator, was required to sustain *mexAB-oprM* expression during an early stage of biofilm development through its binding to the promoter region of the operon (819). Compared to wild-type PAO1 biofilms, PAO1Δ*brlR* biofilms were susceptible (as evaluated by the log reduction in the number of viable cells after drug exposure) to norfloxacin, tetracycline, trimethoprim, and aminoglycosides, and they also expressed 4-fold-lower levels of the MexA protein (819, 820), supporting that MexAB-OprM indeed has a role in biofilm antibiotic resistance. (Still, plasmid-borne *brlR* overexpression produces 4- to 6-fold MIC increases for chloramphenicol, norfloxacin, tetracycline, tobramycin, and trimethoprim in planktonic cells [820]. This is attributed to the regulatory activation of *mexAB-oprM* and *mexEF-oprN* by BrlR's binding to the promoter regions of these efflux operons [819].) Both BrlR production and BrlR-DNA binding are stimulated by the secondary messenger cyclic di-GMP (c-di-GMP) (821). Since MexAB-OprM does not accommodate aminoglycosides unless bacteria are cultured in low-ionic-strength medium (164), the reported hypersusceptibility of PAO1Δ*brlR* biofilms to tobramycin and kanamycin might be interpreted as the result of OprM being in limiting amounts to form a functional MexXY/OprM system able to extrude these molecules (434). Levels of c-di-GMP are increased by the histidine kinase SagS, a two-component hybrid that is expressed from the early developmental stage of biofilms and that also affects BrlR production (822). Considering that SagS indirectly activates *brlR* expression at the irreversible attachment step of biofilm development (823), *mexAB-oprM* expression thus appears to be under the control of at least two distinct and opposite signal-transducing systems (i.e., SagS-BrlR and RocS1/RocS2-RocA2). An additional level of complexity in the biofilm-associated regulation of *mexAB-oprM* was highlighted by Pamp et al. (824). By using a p*mexA-gfp* reporter fusion and a dead-cell fluorescence probe, those authors noted an increased expression level of *mexA* in the metabolically active parts of mature biofilms challenged with colistin. More puzzling were their findings suggesting that MexAB-OprM might contribute to the tolerance of flow chamber-grown biofilms to colistin, as polymyxins (colistin and polymyxin B) do not appear to be transported by the pump (440). Similar conclusions were reached regarding the implication of two other RND pumps, MexCD-OprJ and MuxABC-OpmB, in biofilm recalcitrance to colistin (477). That MexAB-OprM might play a role in tolerance to colistin independent of its drug export activity implies that the pump has broader physiological functions than xenobiotic transport.

MexXY. As with MexAB-OprM, the MexXY/OprM(OprA) system is also subject to complex, multilevel regulation implying both local and more general regulators (Fig. 5). The very low basal expression level of *mexXY*(*oprA*) in wild-type bacteria results from the direct binding of the dimerized, strong repressor MexZ to a 20-bp palindromic sequence encompassing the overlapping promoters of *mexXY*(*oprA*) and the adjacent, divergently transcribed gene *mexZ* (458, 825, 826). Unlike other TetR-type regulators (727), MexZ's DNA binding is not relieved by antibiotics through a direct ligand-regulator interaction but seemingly via indirect protein-protein sequestration, a process relying on the product of the PA5471 gene, named ArmZ, for the antirepressor of *mexZ* (825–827). It was demonstrated that the induction of *mexXY* expression in response to protein synthesis inhibitors is totally dependent upon ArmZ and that the expression of *armZ* itself is induced by ribosome-targeting agents through a sophisticated mechanism of transcriptional attenuation involving a short 13-amino-acid leader peptide, PA5471.1 (828). When the bacteria grow in drug-free medium, the transcribed PA5471.1 sequence is predicted to form a stem-loop structure with adjacent regions of the leader mRNA ahead of PA5471; downstream, another terminator-like stem-loop is allowed to form, which attenuates transcription of the PA5471-PA5470 operon by RNA polymerase (828). Antibiotic interference with the ribosomal machinery and translation of the PA5471.1 gene would thus prevent the formation of these secondary mRNA structures and result in increased expression levels of PA5471 with subsequent *mexXY*(*oprA*) activation through ArmZ (828). Additionally, the *rplU-rpmA* operon encodes the ribosomal proteins L21 and L27. Mutations in its promoter region, as observed in pan-aminoglycoside-resistant mutants, led to the reduced expression of this operon, which was linked to ArmZ-dependent increased MexXY production. Thus, the ribosome-perturbing mutations act in a way reminiscent of *mexXY* induction mediated by ribosome-targeting antibiotics (461). However, effectors other than ArmZ seem to be required for full MexZ-dependent drug induction of the efflux operon, as the latter still remains inducible in *mexZ* and *mexZ*-PA5471 knockout mutants (441, 827). Furthermore, it was demonstrated that exposure of *P. aeruginosa* to reactive oxygen species such as H_2O_2 also results in ArmZ-dependent *mexXY*(*oprA*) derepression (454). The physiological implications of this induction remain unclear, as the pump itself does not contribute to resistance to reactive oxygen species. Lastly, activation of *mexXY* expression also occurs through a newly described pathway where the AmgRS two-component system regulates *mexXY* expression likely via its positive effect on the expression of the *htpX* and PA5528 genes (which encode an IM-associated protease and a modulator of the FtsH protease, respectively) (829–831).

In addition to ArmZ-MexZ, which functionally links the activation of the pump to ribosome dysfunction, at least two signal transduction regulatory systems interconnect MexXY/OprM (OprA) with other cellular processes. Disruption of the PA2572 gene (which encodes a noncanonical response regulator) or the PA2573 gene (the determinant of a probable methyl-accepting chemotaxis protein) strongly increased *mexXY*(*oprA*) expression (832). This led to increased resistance to aminoglycosides but, intriguingly, not to ciprofloxacin, yet another pump substrate (832). As demonstrated by gene inactivation, the putative histidine kinase sensor (PA2571) of both regulators does not seem to initiate the cascade that eventually controls pump expression, suggesting that PA2572 and PA2573 respond to so-far-unknown signals, perhaps through other chemoreceptors. More is known about the regulation of MexXY by the response regulator ParR and the membrane sensor ParS. When activated by mutations or bacterial exposure to subinhibitory concentrations of polycationic agents such as polymyxins (833), this two-component system downregulates *oprD* expression with a concomitant upregulation of the transcript levels of *mexXY*(*oprA*) and the LPS modification operon *arnBCADTEF-ugd* (444). This coordinated response results in an MDR phenotype due to the complementary

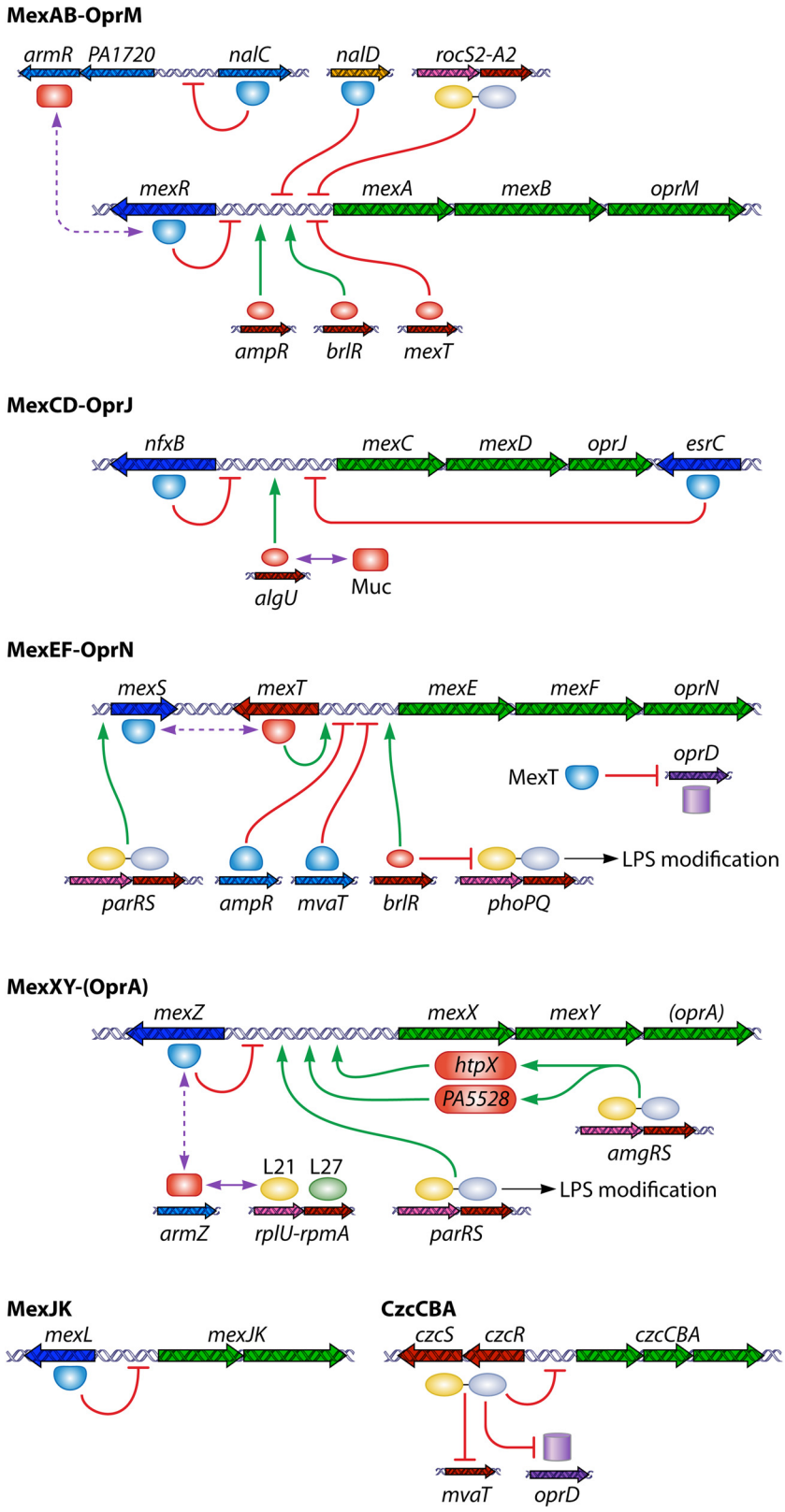

FIG 5 Regulation of expression of RND multidrug efflux systems of *P. aeruginosa*. Of 12 RND pump operons identified in this organism, half of them (presented in green, with the arrows showing their transcriptional directions) are regulated under a local regulator (mostly by a repressor [MexR, NfxB, EsrC, MexZ, or MexL] or by an activator [MexT] encoded by a gene adjacent to the efflux operons) or a two-component system of CzcRS for the *czcCBA* operon. (RND pump operons with no identified local regulatory genes are not included.) The red lines show the repression of the transcription of the relevant gene by repressors, while the green arrows reveal positive regulation by the regulators. Local repressors are controlled by antirepressor proteins (ArmR and ArmZ) and can also bind to

effects of drug efflux and reduced OM permeability (LPS modification and OprD loss) (444, 452).

Other Mex pumps. Other Mex pumps, including MexCD-OprJ and MexEF-OprN, are not typically expressed in wild-type strains. Some of these pumps are regulated by local regulators (Fig. 5), such as the NfxB and EsrC repressors of MexCD-OprJ (465, 834, 835), the MexT activator of MexEF-OprN (836), and the MexL repressor of MexJK (837). With the MexCD-OprJ system, NfxB functions as a multimer with C termini required for multimerization and N termini implicated in DNA binding (834). EsrC is a newly identified second regulator of MexCD-OprJ, and its encoding gene (PA4596) is located downstream of *mexCD-oprJ*. EsrC exerts its inhibitory impact on *mexCD-oprJ* expression only when cells are under envelope stress and is dependent on NfxB (835). *nfxB* mutations in wild-type or mutator strains or as a result of the use of mutagens can occur over the entire *nfxB* gene (838). Elevated frequencies of *nfxB* mutations are also evident with the inactivation of the DNA oxidative repair system (839). Expression of *mexCD-oprJ* can be induced by a number of biocides (e.g., chlorhexidine and benzalkonium chloride), dyes (ethidium bromide), and other membrane-damaging agents (detergents, solvents, polymyxin B, and antimicrobial peptides) (466, 467). Indeed, *mexCD-oprJ* shows the greatest transcriptomic response among the genes after exposure of *P. aeruginosa* to chlorhexidine (840). These membrane-damaging agents apparently act to disrupt the cell membranes and result in the production of membrane lipid derivatives that stimulate the membrane-associated Muc proteins and eventually activate the stress response sigma factor AlgU for enhancing MexCD-OprJ production. *nfxB* mutation-related *mexCD-oprJ* hyperexpression is also dependent on AlgU (467). However, AlgU is negatively regulated by the global regulator AmpR (816, 818).

The expression of MexEF-OprN is controlled by several regulators (Fig. 5). The LysR-type regulator MexT is involved in controlling the expression of multiple genes in *nfxC* mutants, including *mexEF-oprN*, *oprD*, and genes for virulence factors (836, 841–844). *mexT* variations have been observed in wild-type strains and MexEF-OprN-hyperproducing *nfxC* mutants, which possess inactive and active forms of MexT, respectively (845). One gene of the MexT regulon, *mexS* (encoding an oxidoreductase of unknown function [836]), is of particular importance, as its alteration in *nfxC* mutants induces *mexEF-oprN* expression with concomitant development of MDR (846). MexT may interact with MexS, whose mutations enhance MexT-dependent *mexEF-oprN* expression through the intracellular accumulation of possibly toxic metabolites recognized by MexT as coinducers (846). The consequent production of MexEF-OprN would allow the extrusion of these noxious intermediates. Recent data support this hypothesis, since the exposure of *P. aeruginosa* to nitrosative stressors such as *S*-nitrosoglutathione activates *mexEF-oprN* transcription through MexT (847). The possible MexT-MexS interaction also affects the type III secretion system (848) and the response to disulfide stress [elicited by diazenedicarboxylic acid bis(*N*,*N*′-dimethylamide)] that perturbs the thiol-disulfide balance in the cytoplasm (849), thus further confirming the linked regulation among the drug efflux pump, virulence factor production, and the redox stress response. MexEF-OprN contributes to intrinsic resistance to this disulfide stress elicitor only in the presence of MexT (849). However, the functional link between MexS and MexEF-OprN might be more complex than anticipated, as data suggest that overexpressed *mexS* would also lead to MexT-dependent *mexEF-oprN* overexpression, at least in certain genetic backgrounds (850). Moreover, mutations in the ParSR system, which is involved in the regulation of MexXY, downregulate the expression of both *mexS* and *mexEF-oprN* (387). Interestingly, concomitant upregulation of MexS and MexEF-OprN was seen when *P. aeruginosa* was exposed to airway epithelial cells releasing unknown efflux-inducing signals (851). An H-NS family protein, MvaT, is a global regulator affecting the expression of hundreds of genes, including *mexEF-oprN* and others involved in biofilm formation, quorum sensing, and virulence (16, 852–854). The inactivation or mutation of *mvaT* is linked to MexEF-OprN hyperexpression and a reduction of the OprD level, and this impact on MexEF-OprN is independent of *mexT* or *mexS* (855). An *mvaT* mutant also shows decreased expression of the two-component regulator gene (PA2570) located immediately downstream of the *czcABC* efflux operon (853). Hyperproduction of MexEF-OprN with mutations in *mexT*, *mexS*, and *mvaT* was confirmed in clinical isolates (431). Genetic inactivation of AmpR yields an increased production of MexEF-OprN with an MDR phenotype (816), and preexposure of *P. aeruginosa* to subinhibitory concentrations of antibiotics (e.g., imipenem at a concentration as low as 3 ng/ml) induces AmpR production (856). The above-mentioned regulator BrlR is also an activator for MexEF-OprN expression (819).

The CzcCBA metal exporter is upregulated by at least 2 two-component systems, CzcRS (CzrRS) (492) and CopRS (857). Subinhibitory concentrations of zinc or copper salts can induce the expression of *czcCBA*, *czcRS*, and *copRS*. CzcRS and CopRS are also involved in the downregulation of OprD expression with concomitant resistance to carbapenems (16, 493). CzcR further affects various genes involved in virulence, including gene expression of quorum-sensing 3-oxo-C_{12}-HSL and $_{C4}$-HSL autoinducers (386).

A. baumannii Efflux Pumps

The AdeABC, AdeFGH, and AdeIJK RND pumps are regulated by the AdeRS system (858), AdeL repressor (547), and AdeN repressor (543), respectively. While *adeRS* and *adeL* are located upstream of the relevant operons, *adeN* is found ca. 800 kbp upstream of *adeIJK* (543). Mutations in AdeS and AdeR (including

ligands (e.g., antimicrobial agents or metabolites, including quorum-sensing molecules) or be induced under various conditions (oxidative or nitrosative stress or the presence of different agents). The ribosomal proteins L21 and L27, encoded by *rplU-rpmA*, indirectly upregulate ArmZ and negatively control *mexXY* expression. Activation of *mexXY* expression also occurs through positive control by the AmgRS two-component system via the HtpX and PA5528 proteins. Additional regulators encoded by the genes not genetically clustered with the efflux operons also participate in regulation. Mutational changes can also lead to an inactivation of regulators (ArmR, ArmZ, MexR, NalC, NalD, MexS, and MexZ). The crystal structures of MexR and MexZ are available. AlgU is a sigma factor required for the oxidative stress response, and its activity is controlled by Muc, the inner membrane-associated proteins involved in the production of alginate exopolysaccharide and with their encoding genes clustered with *algU*. Some regulators, including AmpR, BrlR, MvaT, ParRS, and RocS2-RocA2, are involved in the regulation of expression of other genes, e.g., downregulation of OprD by MexT and CzcR (in carbapenem resistance) or LPS modification by BrlR and ParR (affecting polymyxin susceptibility). Certain gene products (e.g., BrlR) are involved in gene regulation in biofilm cells. See the text for detail.

AdeS truncated by an IS*Aba1* insertion) are related to AdeABC overproduction (538, 858–860). Functional mutations were found in the conserved domains of AdeRS in AdeABC-hyperexpressing isolates with two mutational hot spots, one in AdeS near His149 and another in the DNA-binding domain of AdeR (861). Another study also identified diverse mutations in AdeRS in extensively drug-resistant isolates with the shared residue substitutions of Gly186Val in AdeS and Ala136Val in AdeR (860).

Inactivation of *adeN* led to 5-fold *adeJ* overexpression with resistance to aztreonam, ertapenem, meropenem, minocycline, and tigecycline, and mutants carrying *adeN* mutations in the region required for dimerization of TetR-type proteins displayed elevated levels of AdeIJK production (543), suggesting that remotely encoded AdeN negatively controls AdeIJK expression. The BaeSR regulatory system was found to positively influence the expression of the AdeA-AdeA2-AdeB system (548). Transcriptional upregulation of the MFS-type transporters MdfA and Tet(A) was observed among >200 up- or downregulated genes in a multidrug-resistant mutant after its exposure to a subinhibitory concentration of tigecycline (862).

S. maltophilia Efflux Pumps

Of 8 RND Sme pumps, two two-component system regulators are linked to SmeABC and SmeYZ, while three TetR family repressors are found for SmeDEF, SmeGH, and SmeOP. SmeABC is positively controlled by SmeSR, as is evident with the reduced MDR due to the *smeR* deletion (571). Located upstream of the *smeDEF* operon, *smeT* encodes the most studied pump repressor SmeT in *S. maltophilia* (863, 864). The promoter regions of *smeDEF* and *smeT* are overlapping, and SmeT provides negative control to SmeDEF and its own expression. A Leu166Gln substitution in SmeT results in higher expression levels of both *smeDEF* and *smeT* (863). SmeT functions as a dimer, like other TetR proteins (727), but has two extensions at the N and C termini, with a small binding pocket observed in the TetR family repressors (864). SmeDEF overproduction was also found in an isolate with an IS*1246*-like element in the *smeDEF* promoter where SmeT likely binds, suggesting that an IS element may also influence *smeDEF* expression (576). Triclosan not only selects SmeDEF-overproducing multidrug-resistant isolates (577) but also induces SmeDEF production via its binding to SmeT and subsequent release from the *smeT* promoter (865). Similarly, natural flavonoids bind to SmeT and thus influence SmeDEF expression (866). While fusaric acid-inducible FuaABC is positively controlled by its local regulator, FuaR (583), another ABC-type MacABC pump was not experimentally proven to be affected by the MacRS two-component system encoded by genes divergently transcribed from *macABC* (584). The MFS EmrCAB pump is downregulated by the EmrR repressor (587).

Neisseria Efflux Pumps

Expression of the MtrCDE pump is regulated both positively and negatively by several *cis*- and *trans*-acting elements (631). The MtrR repressor binds to the *mtrR-mtrC* intergenic region and downregulates *mtrCDE* expression (15, 867). MtrR also inhibits the production of FarR, a regulator of the FarAB pump (16). (A genome-wide microarray analysis revealed ~70 genes whose expression can be repressed or activated by MtrR. One of the repressed genes is *rpoH*, encoding a stress response sigma factor, and as such, inducible MtrR production also increases gonococcal susceptibility to H_2O_2 [868].) Two AraC family regulators, the MtrA activator and MpeR repressor, also influence MtrCDE expression either directly or indirectly (637, 869). Several membrane-damaging agents can induce MtrCDE expression through their interaction with MtrA (637). This induction is also dependent on an envelope protein, MtrF, whose expression is negatively controlled by MtrR and MpeR (16, 869). A single-base-pair change located 120 bp upstream of the *mtrC* start codon generates a second promoter for *mtrCDE* expression and is sufficient not only to increase *mtrCDE* expression but also to render such expression independent of the control by MtrR or MtrA (870).

In *N. meningitidis*, *mtrCDE* expression appears to be independent of either MtrR or MtrA and is modulated by the Correia repeat enclosed element (CREE) that is inserted in the regulatory region for *mtrCDE* (871). This modulation involves the posttranscriptional regulation of the *mtrCDE* transcript by cleavage in the inverted repeat of the CREE (871). The CREEs are repetitive sequences identified in *Neisseria* spp. (commonly 153 to 157 bp or 104 to 108 bp) with an inverted repeat and a characteristic core (such as the integration host factor) that are involved in gene regulation (872). Intriguingly, CREE deletions do not show an effect on susceptibility to ciprofloxacin, erythromycin, and rifampin (873). However, whether these deletions could affect *mtrCDE* expression remains unknown.

C. jejuni Efflux Pumps

Located upstream of the *cmeABC* efflux operon, *cmeR* encodes the TetR-type repressor CmeR that binds directly to the promoter region of *cmeABC* (874). Bile salts, one type of the CmeABC substrate (655), can induce *cmeABC* expression through their interaction with CmeR (875). CmeR is a pleiotropic regulator, and its dimeric structure is unlike that of other TetR-type regulators since it has a large center-to-center distance between two N termini of the dimer and a large flexible multiligand-binding pocket in the C-terminal domain (876). The crystal structure of CmeR with bound bile acids taurocholate and cholate was obtained (877). *cmeR* mutations yield elevated expression levels of CmeABC with an MDR phenotype (878). One study also identified the negative regulation of CmeABC by another regulator, CosR, which is involved in the regulation of ~90 genes, including an element of oxidative stress defense, the catalase-encoding *katA* gene (879). Induction of *cmeABC* expression by salicylate via its binding to CmeR was demonstrated. Salicylate at 100 μg/ml enabled better growth and survival of *C. jejuni* in the presence of inhibitory levels of ciprofloxacin, erythromycin, novobiocin, or tetracycline and also enhanced the emergence of fluoroquinolone resistance under antibiotic selection pressure (880). This observation is consistent with the effect of salicylate on the induction of MDR phenotypes in a number of bacteria, including *B. cenocepacia*, *B. fragilis*, *E. cloacae*, *E. coli*, *K. pneumoniae*, *S. enterica* serovar Typhimurium, *S. marcescens*, *S. maltophilia*, *S. aureus*, and *Mycobacterium tuberculosis*, often through an induction of efflux pump expression (16).

ROLE OF EFFLUX PUMPS IN BIOFILM FORMATION AND RESISTANCE

Bacterial cells can adhere to each other or to an animate or inanimate surface (including that of medical devices); this function is involved in various processes in infections and poses a major challenge to antimicrobial therapy (881). This is because biofilm cells

display significantly higher levels of resistance to antimicrobials than do planktonic cells. Mechanisms of biofilm resistance may involve, for example, a low growth rate, altered metabolism and physiology, persister cells, an extracellular biofilm matrix, and an upregulated stress response (881, 882). Since antibiotic efflux pumps are involved in resistance and other functions, their roles in relation to biofilm formation and resistance have been investigated.

Biofilm formation occurs in response to numerous environmental signals and requires specific genes and regulatory circuits. This process involves three stages: initial attachment, maturation, and detachment (883). The impact of drug exporters on biofilm formation varies in different species, so *P. aeruginosa* mutants deficient in or overproducing RND pumps can still form biofilms (16, 884), while the loss or inhibition of any of 9 MDR pumps (AcrAB, AcrD, AcrEF, MdtABC, MdsABC, EmrAB, MdfA, MdtK, and MacAB) or the TolC OM protein in *Salmonella* impairs biofilm formation with reduced production of curli (885, 886). Interestingly, the biocide triclosan induces *acrAB* and *marA* expression in *Salmonella* biofilm cells (887). Similarly, *E. coli* mutants with a genetic deletion of one of the RND (*acrAB*, *acrD*, *acrEF*, *mdtABC*, and *mdtEF*), MFS (*emrAB*, *emrD*, and *emrKY*), SMR (*emrE*), and ABC (*macAB*) pump genes resulted in reduced biofilm formation (888). Inactivation of *macABC* in *S. maltophilia* led to a 50% reduction in biofilm formation (584). During biofilm growth, two uropathogenic *E. coli* strains showed upregulated expression of 20 transport genes, and the inclusion of an EPI, PAβN or thioridazine, in the medium reduced biofilm formation in *P. putida* and *S. aureus* (889). PAβN in combination with an iron chelator showed synergistic activity against *P. aeruginosa* biofilm development (890). Similar to the role of TolC, a plasmid encoding a TolC homolog, AatA, promoted aggregation and biofilm formation of an enteroaggregative *E. coli* isolate (891). A large conjugative plasmid encoding the OqxAB pump and type 3 fimbriae enabled *E. coli* to form a biofilm (892). In *P. putida*, inactivation of an extracytoplasmic function sigma factor, ECF-10, led to enhanced biofilm formation and the upregulation of the TtgABC pump with increased MDR (893). Upregulation of multiple proteins, including an uncharacterized RND pump and the OmpA and CarO OM proteins, also occurs in *A. baumannii* biofilms (894). However, despite the overall positive impact of drug exporters on biofilm formation, the inactivation of two RND pumps (i.e., RND-4, RND-9, or both) in a *B. cenocepacia* strain enhanced biofilm formation (615). These observations of the efflux pump's impact on biofilm formation may partially be attributable to the altered levels of certain signaling molecules in the mutant strains.

Efflux pumps, as one of the major means of conventional antibiotic resistance in planktonic cells, certainly contribute to the survival of biofilm cells in the presence of antibiotics. For instance, in *P. aeruginosa*, although still having relatively high levels of biofilm-derived resistance, MexAB-OprM-deficient biofilm cells display lower MDR than do cells of the wild-type strain (884). Biofilm resistance to ofloxacin (but not to ciprofloxacin) is dependent on MexAB-OprM expression at a low ofloxacin concentration range, and MexCD-OprJ provides a biofilm-specific mechanism for azithromycin resistance (16). Biofilm cells include distinct subpopulations, and resistance to colistin in metabolically active cells is attributable to the presence of MexAB-OprM and the *pmr*-mediated LPS modification (824). The survival of these active subpopulations after exposure to membrane-targeting agents (colistin and chlorhexidine) is also linked to not only MexAB-OprM but also MexCD-OprJ and MuxABC-OpmB pumps (477). In *E. coli*, the plasmid-borne TetA pump and TEM-1 β-lactamase interplay in the presence of subinhibitory levels of antibiotics to induce biofilm resistance to multiple antibiotics, and this is partly due to the induction of the chromosomal pump EmrKY and its regulator EvgAS (140). However, several early studies were unable to establish an additional contribution of AcrAB or MexAB-OprM to biofilm resistance to ciprofloxacin (16).

Recent studies with *P. aeruginosa* have shown the requirements of SagS and BrlR regulatory proteins for antibiotic resistance in biofilm cells (820, 822) and the linkage of these proteins to the level of the second messenger c-di-GMP (823), which was identified previously as being involved in aminoglycoside-induced biofilm formation and resistance (895). SagS is needed with at least 3 other two-component regulatory systems, BfiRS (biofilm initiation), BfmRS (biofilm maturation), and MifRS (microcolony formation), for the coordination of biofilm formation (822). BrlR, which is specifically expressed in biofilm cells (820), acts as a transcriptional activator for the expression of *mexAB-oprM* and *mexEF-oprN* (819). Unlike the MerR-type regulators (e.g., BltR and BmrR) that function in the regulation of MDR pumps of Gram-positive bacteria (896), *brlR* expression is not induced by the pump substrates. The inactivation of SagS correlates with a reduced level of c-di-GMP, and thus, c-di-GMP is positively regulated by SagS (823). c-di-GMP further positively affects the production of BrlR and also enhances the binding of BrlR to the promoters of BrlR target genes (821, 823). Together, SagS, BrlR, and c-di-GMP form an important signaling network related to the susceptibility-resistance switch and are all required for elevated expression in biofilm cells of the MexAB-OprM and MexEF-OprN pumps, which contribute to resistance in both biofilm and planktonic cells. It should be noted that similar to those in biofilm cells, increased levels of c-di-GMP in planktonic cells also confer elevated resistance to antibiotics to the cells, and this phenotype correlates well with the hyperexpression of *brlR*, *mexA*, and *mexE* (823). Additionally, a PA1875-PA1877-encoded efflux system in *P. aeruginosa* is composed of an OM protein (OpmL), an ABC exporter, and a membrane fusion protein (HlyD homolog) and contributes to biofilm-specific resistance to ciprofloxacin, gentamicin, and tobramycin (897). In a uropathogenic *E. coli* strain, inactivation of the *rapA* gene, encoding a helicase-like protein, does not alter biofilm formation but increases susceptibility to penicillin G in biofilm cells. In biofilm cells of the *rapA* mutant, the expression levels of 22 genes are reduced, including those encoding a putative MDR pump (YhcQ), a putative carbohydrate transport and metabolism protein (YeeZ), and a transcriptional regulator (SdiA) (898). *B. fragilis* cells show elevated RND pump expression upon induction by bile salts and also increased possibility of biofilm formation (899).

INVOLVEMENT OF MUTLIDRUG EFFLUX PUMPS IN OTHER FUNCTIONS

The wide distribution and overlapping functions of MDR efflux pumps in bacteria suggest a physiological role of these pumps beyond drug resistance (16, 34, 52, 54, 900). Indeed, there is an increasing understanding of the roles of multidrug pumps in bacterial cell communication, the stress response, fitness, colonization, intracellular survival, and virulence, as discussed below. However, interpretation of these results, based mostly on the

properties of deletion mutants of *acrB* and its homologs, needs some caution because such deletions are known to result in changes in the expressions of the other remaining pumps. For instance, the deletion of *acrB* in *S. enterica* serovar Typhimurium results in the overproduction of AcrD and AcrF (629, 771). The mechanism of this regulatory response is still unclear, but it may be related to the function of AcrB (and possibly other constitutively expressed RND pumps in other species) in removing toxic intermediates of metabolism, first proposed by Helling et al. (901) and more recently developed by Rosner and Martin (762, 902).

Bacterial Stress Responses

Increasing evidence has shown that antibiotic exporters also make a great contribution to bacterial stress responses, where these pumps can function as either a preexisting mechanism or an activated resource in response to numerous cellular stresses, and this can be regarded as part of the physiological functions of drug pumps (16, 53, 54). Antibiotics and other chemical substances themselves can cause stress to the microbes and frequently induce the expression of MDR pumps, which in turn increase the capacity to resist the stress. These inducers are also often the substrates of the relevant pumps. Thus, the response to bacterial stress is complex, as demonstrated by phenomic profiling of the *E. coli* mutants from the Keio collection that showed thousands of phenotypes in response to >100 antibiotic or other drug challenges with the involvement of a large number of transporter genes (Table 1) (30).

The expression of the predominant AcrAB pump can be stimulated in response to the stress posed by bile salts/fatty acids, ethanol, and high salt concentrations, and this elevated level of AcrAB production enables the survival of enteric bacterial cells against bile salt stress in the intestinal tract (16, 55, 903, 904). The MdtEF and EmrAB pumps are also involved in the efflux of free fatty acids (55). A singlet MFS pump, MdtM, protects *E. coli* from bile salt stress and functions with AcrAB-TolC in a synergistic manner (905). The MATE NorM provides protection against H_2O_2 killing by possibly extruding compounds that oxidize the guanine of DNA and nucleotides as well as susceptible proteins (906). The macrolide-specific ABC exporter MacAB in *Salmonella* is induced upon exposure to H_2O_2 and is essential for survival of *S. enterica* serovar Typhimurium against oxidative stress. Reactive oxygen species-mediated killing in macrophages is alleviated by MacAB (907). Both SmeIJK and MacABC of *S. maltophilia* also contribute to tolerance to oxidative and envelope stresses (582, 584). In addition to the role of efflux pumps themselves in the stress response, the levels of several pump or OM protein regulators (e.g., MarA, SoxS, Rob, OmpR, and EnvZ) were elevated in the presence of antibiotic or chemical stress (908). *E. coli* cells with stress caused by iron starvation showed increased expression levels of MdtF and decreased expression levels of AcrD (16). Reduced resistance to bile, hyperosmotic, oxidative, or nitrosative stress is evident in a *K. pneumoniae* mutant with an inactivated OxyR regulator and reduced AcrB expression (795). An OM lipoprotein, NlpE, is involved in the envelope stress response mediated by the CpxRA signal transduction pathway, such as in response to misfolded cell envelope proteins (909), and also positively impacts the expression of the AcrD and MdtABC pumps for providing increased resistance (910). Similarly, a reciprocal regulation of RND pumps and the Cpx system occurs in *V. cholerae*, where Cpx system activation induces the expression of VexAB-TolC and VexGH-TolC, while the inactivation of these pumps stimulates the activation of the Cpx response (345). The KpnEF pump in *K. pneumoniae* also belongs to the Cpx cell envelope stress regulon involved in the response to bile salt and osmotic stresses (163). BaeSR responds to cell envelope stress (758) and regulates the expression of several RND pumps, as discussed above (see Regulation of Multidrug Efflux Pumps).

Several Mex pumps of *P. aeruginosa* (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY) clearly function in stress responses, as described above (see Regulation of Multidrug Efflux Pumps) (Fig. 5). Their regulators (e.g., MexR, EsrC, ArmZ, and MexT) can respond to various stresses caused by, for example, membrane-damaging or ribosome-disrupting agents, reactive oxygen species, and/or nitrosative stress and subsequently cause increased activities of these pumps against the stresses (441, 454, 461, 467, 798, 799, 827, 835, 911). Consistently, *P. putida* RND pump mutants are categorized into 4 functionally distinct subgroups, and one of them is more susceptible to oxidation-inducing agents (912). In a situation mimicking chronic cystic fibrosis infections with antibiotic therapy, oxidative stress and antibiotic exposure result in the hyperexpression of AmrAB-OprM of *Burkholderia vietnamiensis* (913).

In *Neisseria*, the expression of the general stress response sigma factor RpoH is inhibited by the repressor MtrR, and this subsequently impacts the expression of at least 69 RpoH-regulated genes and the levels of gonococcal susceptibility to H_2O_2 (868). In *C. jejuni*, an OmpR-type oxidative stress regulator, CosR, provides overall negative regulation of oxidative stress defense proteins, and its expression is significantly decreased by a superoxide generator, paraquat (914). Reduced production of CosR is linked to CmeABC hyperexpression, consistent with the role of CmeABC in responding to oxidative stress (879). The CmeG exporter from *C. jejuni* also mediates oxidative defense (673).

Fitness, Colonization, and Virulence

Bacterial fitness, colonization, and virulence can be affected by changes in the status of drug pumps and OM proteins. While some resistant mutations may come with a substantial cost in these measurements, others have enhanced abilities. Despite certain exceptions, it is frequently seen that either inactivation or overproduction of an antibiotic efflux pump can add a fitness cost or reduce virulence, suggesting that the native expression status of the pumps may have been optimized for fitness and virulence. However, this observation with efflux-deficient strains may be due to the absent role of efflux pumps in responding to antibiotic killing or stress and may not be directly linked to fitness or virulence *per se*.

Enteric bacteria. AcrB or TolC mutants of *S. enterica* serovar Typhimurium colonize and persist poorly in the intestine of chickens (although this is likely caused by direct inhibition by bile salts, the primary substrates of the pump) but also fail to invade macrophages *in vitro* (915). MacAB-TolC of *E. coli* is involved in exporting an extracellular peptide toxin (enterotoxin II) produced by enterotoxigenic *E. coli* (916). A *Salmonella* isolate with an inactivated MacAB pump is less virulent, and a mutant deficient in all drug efflux pumps is avirulent in mice (222). In *E. cloacae*, inactivation of either *acrA* or *tolC* led to reduced fitness (both *in vitro* and *in vivo*) as well as reduced virulence in a mouse model of systemic infection (264). A deficiency in AcrAB and OqxAB in *K. pneumoniae* also caused reduced virulence (792). Similarly, a mutant of *V. cholerae* deficient in RND pumps pro-

duced significantly less cholera toxin and fewer toxin-coregulated pili (341, 344), and TolC mutants were deficient in intestinal colonization in mice (917) (but see the comment above on *S. enterica* serovar Typhimurium). Genetic deletion of one of the five MFS pump genes also produced a colonization defect of *V. cholerae* in mice (352). Inactivation of RND pumps in *V. parahaemolyticus* resulted in reduced virulence in a rabbit model (348). In *H. pylori*, the CznABC metal pump is also essential for urease modulation and gastric colonization (684). Administration of PAβN also decreased the colonization of *C. jejuni* in chickens (918). Of 9 *Salmonella* drug transporters assessed for their role in virulence, the MdtABC, MdsABC, and MacAB pumps were required for virulence (222). *acrAB* and *acrEF* mutants had an impaired ability to cause mortality of mice by the oral route of infection (again, see the comment above on the primary substrates of AcrAB), and a strain deleted for all 9 pump genes did not cause mortality in mice (222). In contrast, exposure of *B. fragilis* to bile salts increased, in addition to efflux, bacterial coaggregation and adhesion to intestinal epithelial cells (899). Together, these results suggest the significance of the functional presence of efflux pumps for bacterial fitness and virulence. Nevertheless, the establishment of a link between efflux pumps and virulence needs to be made prudently, since the above-described results can be due to the fact that the major function of RND pumps in enteric bacteria is protection of bacteria against bile salts.

In contrast, it is likely easier to see the relation between fitness or virulence changes and pump overproduction. Tigecycline-resistant *E. coli* strains with likely AcrAB pump overproduction or LPS alterations have reduced fitness (919). *In vitro*-selected ceftriaxone-resistant mutants of *S. enterica* serovar Typhimurium exhibited overexpression of *acrAB-tolC* and reduced expression of the *invA* virulence gene with decreased invasion of cultured epithelial cells (920). Quinolone-resistant strains of *E. coli* (and *P. aeruginosa*) that accumulated multiple mutations (including gyrase mutations and RND pump overexpression) had reduced fitness, and subsequently, fitness-compensatory mutations affecting DNA supercoiling were acquired for bacterial survival (16).

P. aeruginosa. The high prevalence of MexAB-OprM overproducers in the clinic tends to indicate that the upregulated system does not strongly impair the pathogenicity of *P. aeruginosa*. Consistent with the notion that such mutants still retain some degree of virulence, *mexAB-oprM*-overexpressing strains were isolated from patients with severe infections (385, 422, 429). A cystic fibrosis epidemic strain overproduced MexAB-OprM (and MexXY) and displayed enhanced virulence (921). Strains coisolated from chronically colonized airways of cystic fibrosis patients contained frequent mutations in *mexR* that may have resulted in MexAB-OprM overproduction (450). Decreased virulence is associated with MuxABC-OpmB inactivation (490). However, the outcome of *P. aeruginosa* infections is known to depend primarily upon the patient's conditions, and the contribution of bacterial virulence is sometimes not very clear. Results of several *in vitro* and *in vivo* experiments support the idea that *nalB* mutants might have lower fitness and lower virulence than wild-type bacteria (398, 922, 923). In agreement with this notion, it was noticed that some epidemic *nalB* isolates apparently had reverted to a basal *mexAB-oprM* expression level via mutations in the P_1 promoter or the putative ribosome-binding site ahead of the operon (see Regulation of Multidrug Efflux Pumps, above) (412). On the other hand, two observations tend to suggest that MexAB-OprM could play an essential role in baseline virulence under certain conditions through the export of still unidentified factors. First, the inactivation of *mexA* impaired the fast killing of the worm *Caenorhabditis elegans* and reduced the mortality rates of infected mice, compared with wild-type bacteria (924). Second, deletion of the whole *mexAB-oprM* operon appeared to compromise the invasiveness of *P. aeruginosa*, as judged by its capacity to transmigrate across Madin-Darby canine kidney epithelial cell monolayers and to cause lethal septicemia in mice (925). Concordant results were obtained in this cellular monolayer model with the MexAB-OprM-specific EPI D13-9001 (926). In line with this, it was reported that during the course of chronic lung colonization, cystic fibrosis strains tend to accumulate mutations in *mexA* and *mexB* resulting in a functionally deficient MexAB-OprM pump (447, 927). This evolution is parallel to the loss of multiple virulence factors and their regulators (e.g., LasR) over time by cystic fibrosis strains (928). Such MexAB-OprM-defective mutants that also occur in patients with chronic obstructive pulmonary disease are absent from acute infections, suggesting that the pump may indeed contribute to pathogenicity by as-yet-uncharacterized mechanisms.

While a rat model of acute pneumonia failed to demonstrate lower mortality rates in animals infected with MexCD-OprJ-upregulated mutants than in those challenged with a wild-type or *nalB* strain (929), other results suggest a negative impact of *nfxB* loss-of-function mutations on bacterial fitness, all forms of motility (swarming, swimming, and twitching), and the production of virulence factors (pyocyanin, caseinase, elastase, and type III secretion system-dependent cytotoxicity) (479, 922, 930). However, the effects of *nfxB* mutations on virulence factor production are highly variable and clearly strain dependent in clinical isolates (474). From very few clinical observations, it appears that the emergence of *nfxB* isolates *in vivo* occurs mainly under long-term treatment with fluoroquinolones (474), which correlates with the ability of these agents to readily select such mutants *in vitro* at concentrations around the MIC (433, 931). In the *in vitro* biofilm mode of growth, *nfxB* mutants arise very easily under ciprofloxacin exposure (932). The notion that the moderate resistance to fluoroquinolones conferred by the pump may lead to therapeutic failures is not always supported by animal models of infection (sepsis and neutropenic mouse thigh models) and pharmacokinetic/pharmacodynamic drug parameters (933). To the best of our knowledge, no fatal cases of infection attributable to MexCD-OprJ-overproducing mutants were reported in the literature. Furthermore, these mutants seem to be unable to cause bloodstream infections (385, 429, 474), likely because of their high susceptibility to serum complement (934), slow growth (474), and probably more general defects (479). The specific conditions that exist in the cystic fibrosis lung environment might be more favorable to the persistence of *nfxB* subpopulations, as the production of virulence factors and serum resistance are no longer required at the stage of chronic colonization (928). Therefore, the debate about the pathogenicity of these bacteria and why their prevalence in the clinical setting is so low remains open.

Overproduction of MexEF-OprN may also have a fitness cost and contribute to decreased survival and virulence, including impaired type III or VI secretion systems, which deliver toxins to the cytoplasm of the host cells (935). *In vitro*-selected MexEF-OprN-overproducing *nfxC* mutants are strongly deficient in the production of major quorum sensing-dependent virulence factors such

as pyocyanin, elastase, and rhamnolipids and thereby appear to be avirulent in various infection models (483, 929). This correlates with the decreased production and secretion of the Rhl signaling molecule C_4-homoserine lactone and the suppression of rhamnolipid-dependent swarming mobility (936, 937). Köhler et al. demonstrated that the decrease in virulence factor production was due solely to MexEF-OprM overproduction in an *nfxC*-type mutant and not to pleiotropic effects induced by MexT (936). Moreover, MexT can also downregulate type III secretion, pyocyanin production, and early attachment to a solid surface (polystyrene) independently of MexEF-OprN (842). Transcriptomic analysis revealed 17 genes positively controlled by MexT apart from *mexEF-oprN* (843). Lastly, c-di-GMP levels negatively affect the expression of the OprD channel, and this explains the improved survival of high-c-di-GMP strains in the presence of imipenem (938).

Other bacteria. The insertional disruption of a 4-gene operon encoding a putative toluene exporter reduces the lung persistence of *A. baumannii* (939). A deficiency in the BpeAB-OprB pump of *B. pseudomallei* is linked to attenuated invasiveness and cytotoxicity toward human lung epithelial and macrophage cells (622). BesABC of *Borrelia burgdorferi* is essential for virulence in mouse infection (16). The MtrCDE pump enhances gonococcal genital tract infection in female mice (640). Although *acrAB* deletion did not significantly impact the tissue colonization of *Yersinia pestis* in mouse pneumonic and septicemic plague models (333), the inactivation of either of the two MarA regulatory homologs decreased lung colonization by 10-fold in a mouse model (940). A heterodimeric ABC transporter, MrtAB, provides resistance to ethidium bromide and is also required for *Yersinia pseudotuberculosis* to colonize the mesenteric lymph nodes (941). However, overexpressed pumps can also have a biological cost such that *S. maltophilia* with SmeDEF overproduction displays reduced virulence (16). Additionally, TolC proteins constitute a major component of multicomponent pumps and are required for virulence of a large number of bacteria, including *Aggregatibacter actinomycetemcomitans* (an oral commensal associated with periodontitis), *Brucella suis*, and *Francisella tularensis* (16). Finally, cell polarity in the model species *Caulobacter crescentus* was linked to efflux-based resistance, where the TipN polarity landmark protein (for directing flagellum placement to the new cell pole) is also essential for induced AcrAB expression (942).

MULTIDRUG EFFLUX PUMPS AS A CHALLENGE IN DRUG DEVELOPMENT

The broad substrate profile of the major multidrug exporters, the need for the drug to traverse the IM, and the OM permeability barrier clearly indicate key challenges in antibiotic development for Gram-negative bacteria (15, 16, 943–945). The second requirement usually means that the drug must be made somewhat lipophilic, and this makes the drug susceptible to multidrug efflux. On top of this, OM penetration requires that neither the size nor the lipophilicity of the drug can be excessive. Nevertheless, the kinetics of AcrB (see "RND Transporters," above) show that the affinity and the maximal rate of transport by multidrug transporters cover a very wide range, so there is hope for producing drugs that are only poorly pumped out by prevalent RND pumps under clinically relevant conditions. In principle, small, hydrophilic drugs would act as nonsubstrates for multidrug efflux pumps. However, such molecules cannot easily cross the bilayers of the IM and usually must depend on specific (inward) transporters for access to cytosolic targets; in this case, mutant bacterial populations lacking such transporters are likely to develop as resistant clones. These considerations likely explain the lack of new antibiotic classes against Gram-negative bacilli for several decades (946, 947). New antibiotic pipelines are often available only for Gram-positive species and those Gram-negative species lacking significant drug efflux activity and OM barriers (944, 948, 949), and there is a particular need for drugs against multidrug-resistant *A. baumannii* and *P. aeruginosa*.

Ribosome-targeting omadacyclines (e.g., amadacycline), as new broad-spectrum aminomethylcyclines, possess activity against tetracycline-specific efflux and ribosome protection mechanisms (950, 951) but are still rendered inactive by the AcrAB-TolC and MexAB-OprM pumps (944). Similarly, the broad-spectrum agent eravacycline (a new fluorocycline) also lacks activity against *A. baumannii*, *B. cenocepacia*, and *P. aeruginosa* (949, 952). Plazomicin is a new aminoglycoside derivative of sisomicin with significant activity against a range of Gram-positive and Gram-negative bacteria (including multidrug-resistant isolates) (953, 954). However, its activity is adversely affected by increased efflux in *A. baumannii* and *P. aeruginosa* (955). Moreover, several new cephalosporin–β-lactamase–inhibitor combinational products in clinical trials (e.g., ceftazidime-avibactam, ceftaroline-avibactam, and ceftolozane-tazobactam) (944) are still likely to be the substrates of RND pumps, as are other β-lactams and β-lactamase inhibitors (13, 390, 439, 545), because avibactam cannot reverse efflux-mediated ceftazidime resistance (956), and both ceftaroline and ceftolozane (a new antipseudomonal cephalosporin) are still affected by efflux pump- and/or porin-related resistance mechanisms (although ceftolozane, containing multiple charged groups, appears less impacted by Mex pumps than many other β-lactams and did not select *in vitro* for pump overproducers in *P. aeruginosa*, unlike other agents) (417, 957–959). These observations could also illustrate their reduced or lack of synergistic activity against multidrug-resistant *A. baumannii* and *P. aeruginosa* (960–962). Additionally, a target-based inhibitor of LpxC (a metalloamidase involved in LPS biosynthesis), CHIR-090, is also a substrate for MexAB-OprM, MexCD-OprJ, and MexEF-OprN (963).

In spite of their synthetic nature, (fluoro)quinolones are often the typical substrates of MDR pumps. The activity spectrum profiles of several newer quinolones in clinical trials (e.g., the fluoroquinolones delafloxacin, finafloxacin, and JNJ-Q2; the nonfluorinated quinolone nemonoxacin; and an isothiazoloquinolone, ACH-702) (944, 964–968) suggest that these agents are substrates of RND pumps. Nevertheless, although still a substrate of the *A. baumannii* AdeABC and AdeM pumps, a new broad-spectrum fluoroquinolone, DS-8587, was found to have better activity against AdeABC- or AdeM-overproducing mutants than ciprofloxacin and levofloxacin (969), further supported by its efficacy in an animal model (970).

Newer ketolides (e.g., cethromycin and solithromycin) and oxazolidinones (posizolid, radezolid, and tedizolid) are still the agents mainly against Gram-positive bacteria (944), and the lack of potency for Gram-negative bacilli is likely attributable to drug efflux and the OM permeability barrier, as shown with earlier members of their classes (85, 242, 266, 971). For example, solithromycin is clearly a strong substrate for three pumps, MtrCDE, MacAB, and NorM, of different transporter families (647). Again,

these characteristics continue to show the need to specifically develop small-molecule inhibitors of RND pumps. Indeed, RND pump inhibition rendered an FtsZ-directed antistaphylococcal prodrug (TXY436) active *in vitro* against *E. coli*, *K. pneumoniae*, and *A. baumannii* (972).

Additionally, it should be emphasized that the increasing understanding of multidrug transporters certainly facilitates antibiotic discovery and development (16, 36). Hypersusceptible mutants deficient in major RND pumps and/or LPS (9, 22, 27, 164, 177, 390) can often be best used as model organisms for screening potential agents against Gram-positive and Gram-negative organisms.

EFFLUX PUMP INHIBITORS

Active drug efflux, especially that caused by the RND pumps, plays a major role in intrinsic resistance and elevated resistance (in pump overproducers) in Gram-negative bacteria. Thus, if such pumps can now be antagonized by an inhibitor, this may open up a wide possibility of adjuvant therapy. Practically all antibiotics are susceptible to active efflux, and for many, their utility has become limited because of the overproduction of pumps in pathogens. Such inhibitors can make these "old" antibiotics effective again. Furthermore, when we consider that a wide range of antibacterial agents (including lipophilic penicillins, many macrolides, glycopeptides, oxazolidinones, lipoglycopeptides, and the lipopeptide daptomycin) are useful only for treating Gram-positive pathogens and that their poor activity against Gram-negative organisms is caused at least partially by efflux, EPIs could broaden dramatically the spectrum of these agents. Finally, it is important to note that in *Salmonella* and in other organisms, EPIs were shown to inhibit biofilm formation, which is integral in pathogenesis and requires the presence of RND pumps (886, 889); this gives even more incentive for the development of clinically useful EPIs.

EPIs have been used widely in order to determine if drug resistance in clinical isolates is caused by efflux. However, in some cases, the inhibitors were used under inappropriate conditions (most often too-high concentrations), so interpretation of the results becomes difficult (see also Methodological Considerations, below).

PAβN

Reserpine and verapamil, known inhibitors of P-glycoprotein, were examined in 1991 as potential inhibitors of the Gram-positive pump NorA of *S. aureus* and were indeed found to inhibit this bacterial transporter (973). Thus, there was a precedent for an EPI. Nevertheless, as detailed by Neyfakh for the NorA homolog Bmr of *Bacillus subtilis* (59), these Gram-positive MFS transporters pump out mostly fluoroquinolones (which contain a positively charged piperazine moiety) and other cationic compounds, such as doxorubicin, puromycin, cationic dyes, and even the polyamine spermidine. Therefore, it was possible to imagine that the inhibitors, also containing cationic groups, would bind to more or less similar binding sites as the substrates. In contrast, Gram-negative RND pumps, such as MexB and AcrB, have a much wider substrate specificity (Table 2) and transport not only the substrates mentioned above but also neutral compounds (chloramphenicol and solvents) and acidic ones (β-lactams). Many researchers thought that it would not be possible to find inhibitors of such pumps, and in this context, the discovery of PAβN (MC-207,110; 446 Da; reported in 2001 by scientists at Microcide [60]) (Fig. 6) was quite remarkable. This compound was found to be a broad-spectrum inhibitor of three major RND pumps in *P. aeruginosa*, MexAB-OprM, MexCD-OprJ, and MexEF-OprN, for the efflux of fluoroquinolones and was also found to inhibit the *E. coli* AcrAB-TolC pump. At 20 μg/ml, it strongly decreased the MICs of a wide range of antibiotics in a MexAB-OprM-overproducing *P. aeruginosa* strain: those for chloramphenicol, sparfloxacin, erythromycin, and levofloxacin decreased 128-, 128-, 32-, and 32-fold, respectively. On the other hand, MICs of carbenicillin, tetracycline, and ethidium bromide showed only a marginal decrease (<8-fold), although the genetic inactivation of MexAB-OprM caused a huge decrease of the MIC for them (between 32- and 512-fold). Thus, the effectiveness of this inhibitor depends strongly on the nature of the antibiotic, an observation that suggests, for example, that the drugs and the inhibitor may or may not share the same subsite within the binding pocket of the transporter (60). Recently, some substrates of AcrB, such as chloramphenicol, benzene, and cyclohexane, were shown to stimulate the efflux of nitrocefin and cefamandole (94), rather than the expected competitive inhibition, and this observation may also be related to the presence of subsites and to the complexity of the drug extrusion process in the giant AcrB transporter.

Lomovskaya and colleagues (60) also showed that PAβN is a substrate of these pumps and therefore that their inhibitory action is likely the result of competition in the transport process. PAβN is not a proton conductor. Additionally, as expected for lipophilic peptides containing more than one cationic site, PAβN does permeate the *P. aeruginosa* OM at high concentrations, especially in strains with defective efflux. However, half-maximal permeation required a concentration of 70 μg/ml even in these cases, and this activity was completely abolished in the presence of 1 mM Mg^{2+} in the medium (60). The latter points are worth emphasizing because there are recent publications suggesting that the OM-permeating function explains most or even all of the antibiotic-sensitizing activity of PAβN. Matsumoto et al. (974) found that OM permeability, assayed by nitrocefin hydrolysis in Δ*tolC* cells of *E. coli*, became increased by PAβN, with the half-maximal increase occurring at ~16 μg/ml. The assay was conducted in the absence of Mg^{2+}, and the cells may already have been damaged because they contained a high-copy-number plasmid and were grown in the presence of 100 μg/ml ampicillin for the maintenance of the plasmid. Those authors also found that the cells became stained more by Sytox green dye at high concentrations of PAβN and took this as evidence of the permeabilization of the OM. In any case, there seems to be a huge leap from these results to the conclusion that the effect of PAβN is caused mainly by membrane permeabilization. More recently, Lamers et al. (975) showed that a *P. aeruginosa* mutant derepressed for the production of endogenous β-lactamase leaked out more enzyme into medium when relatively high concentrations (25 to 50 μg/ml) of PAβN were added. As shown by Lomovskaya et al. (60), Mg^{2+} prevented the permeabilization of the OM; however, this was observed at low concentrations but not at high concentrations of PAβN. Lamers et al. examined the entry of 8-anilino-1-naphthalenesulfonic acid as a marker of OM permeability and found that it was increased in the efflux-deficient mutant only with 50 μg/ml PAβN. Finally, those authors showed that the fluorescence of a cyanine dye was increased in the presence of 25 and 50 μg/ml PAβN and concluded that the IM became depolarized (975). However, because there was no calibration, it is impossible to tell if the "depolarization"

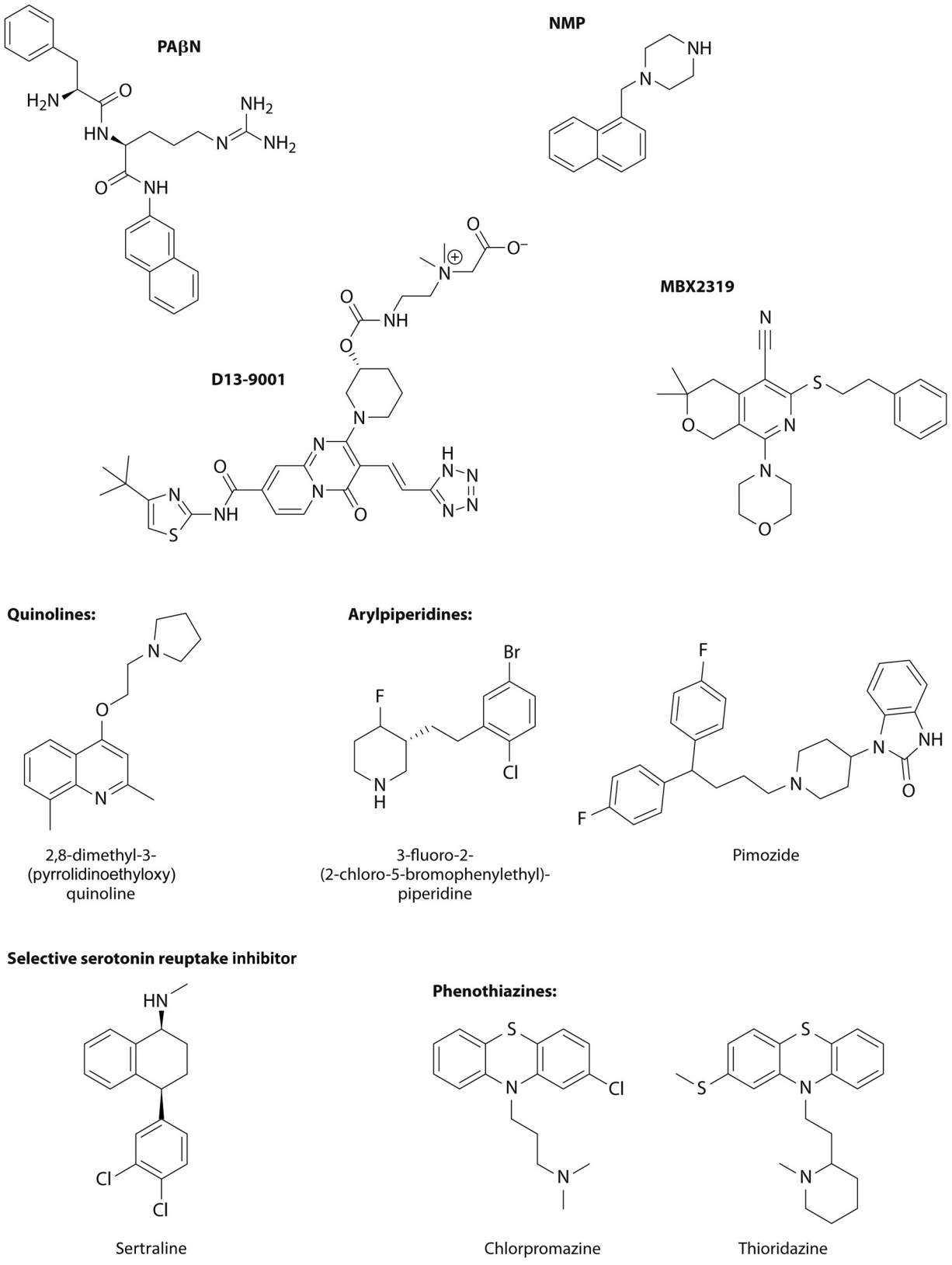

FIG 6 Chemical structures of RND pump inhibitors.

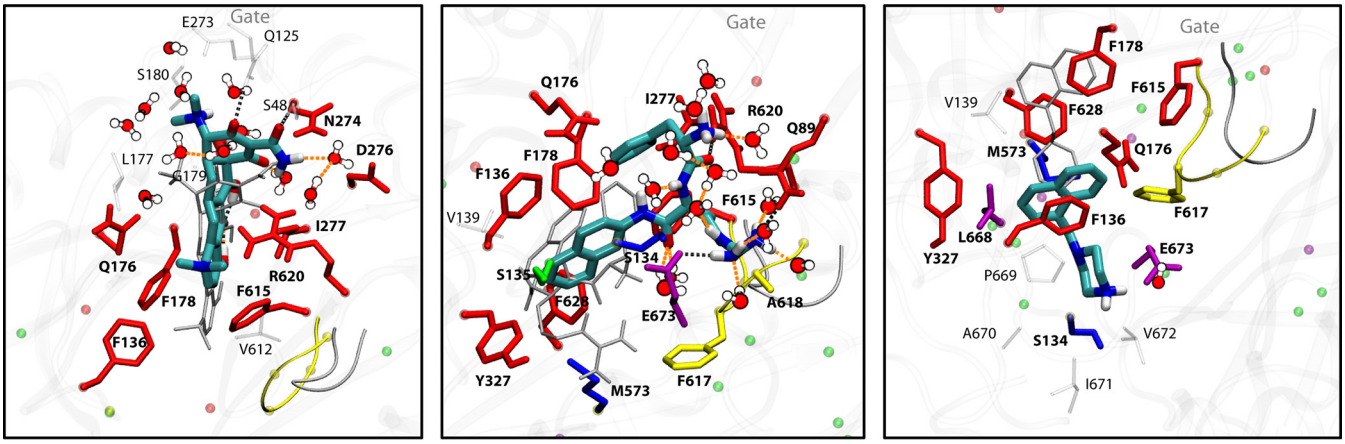

FIG 7 MD simulation-predicted binding of the inhibitors PAβN and NMP, with the substrate minocycline shown as a reference. The positions of ligands initially predicted by docking (Autodock Vina) are shown as thin gray sticks, and those in the final phase of MD simulation are shown as thick blue sticks. AcrB residues within 3.5 Å of the ligand are shown in stick models (red, green, or yellow, if they belong to the distal pocket, proximal pocket, or G-loop, respectively). For NMP, two somewhat different equilibrium positions were obtained, and only one is shown here. (Modified from reference 93.)

was of a magnitude significant for bacterial physiology. These authors (975) found significant effects usually only at high concentrations, and these observations are quite consistent with the initial characterization of PAβN (60). Thus, it is important to interpret the permeabilizing effect of PAβN on the OM in the context of both PAβN concentrations and the specific bacterial strains or species (as noted above, the MIC values of PAβN for different strains of the same species and different species may vary). The molecular basis of pump inhibition by PAβN was examined by exploring its mode of binding to the binding protomer of AcrB via MD simulation (93) and is discussed together with the mode of action of NMP below. (Although ethidium influx rates at different concentrations of PAβN have been used to calculate the K_m and V_{max} [976], these values have little credibility because such simplistic calculations cannot be applied to a complex system, where spontaneous influx, decreased by the activity of the pump, is measured rather than the pump activity itself.)

One important effect of inhibitors, established with PAβN, was that their presence dramatically decreases the frequency of emergence of resistant mutants, presumably because the concentration of the drug is so far above the MIC all the time (60). Thus, when a wild-type *P. aeruginosa* strain was plated onto agar with 1 μg/ml levofloxacin (8-fold MIC), resistant mutants appeared at a frequency of 10^{-7}; if 20 μg/ml PAβN was added to the drug, the frequency of appearance of resistant mutants was decreased to only 10^{-11}.

Although the PAβN structure has been modified to decrease acute toxicity to a tolerable level, the presence of two cationic groups led to prolonged accumulation in tissues (presumably in acidic vesicles) and prevented repeated dosing (977). This structural feature was also likely to cause renal toxicity, and thus, the development of this series was abandoned (978). Again, the issue of toxicity is a complex one, and it is unfortunate that researchers now trying to develop new classes of inhibitors are often focused on acute toxicity data alone. In this regard, one may also consider the inhibitory spectrum (broad or narrow) of an EPI as well as the clinical situations where the EPI would be administered as an antibiotic adjuvant.

NMP

The second compound that has been used widely in the laboratory is NMP (226 Da) (Fig. 6), discovered by the examination of a series of aryl piperazines by Bohnert and Kern (979). Its activity is somewhat weaker than that of PAβN, requiring 50 μg/ml to decrease the levofloxacin MIC for an AcrB-overproducing *E. coli* strain. Interestingly, the activities of NMP and PAβN were quite different in their specificity (980). Thus, NMP was very effective in making *E. coli* clinical isolates more susceptible to ethidium bromide, for which PAβN had no effect. In contrast, NMP had no effect on susceptibility to rifampin and clarithromycin, for which PAβN showed a strong effect at 25 μg/ml (980). Because NMP is likely to act as a serotonin agonist, it is said to be unlikely to be developed into a clinically useful drug (981).

The molecular basis of inhibition by NMP and PAβN was investigated by MD simulation using the binding protomer of AcrB (93). We first noticed that neither PAβN nor NMP bound exceptionally tightly to the AcrB-binding site; the calculated affinity was in the range of, or even weaker than, that of the typical substrates. This is an observation consistent with our knowledge that these inhibitors can behave as substrates and are pumped out by AcrB (60, 979). As shown in Fig. 7, the typical substrate minocycline bound to the upper subsite within the binding pocket, which is full of hydrophilic residues such as S48, Q151, S180, N274, and D276, and only F178 and F615 were close to the substrate among the six Phe residues lining the pocket. In contrast, both PAβN and NMP bound to the lower part of the pocket that is rich in Phe residues. Thus, PAβN interacted with F136, F178, F615, and F628, and the only hydrophilic residues found nearby were Q176 and E673. NMP interacts with F617, F664, and F666, and no hydrophilic residue is found nearby (except perhaps G675). It may be important that although the docking predicted that both inhibitors bound to the binding site just like the substrate, MD simulation showed that they both moved out of the pocket in the direction of the proximal pocket and ended up straddling the G-loop (also called the F617 loop [78] or switch loop [77]). Since the G-loop is thought to play a critical role in allowing the movement of sub-

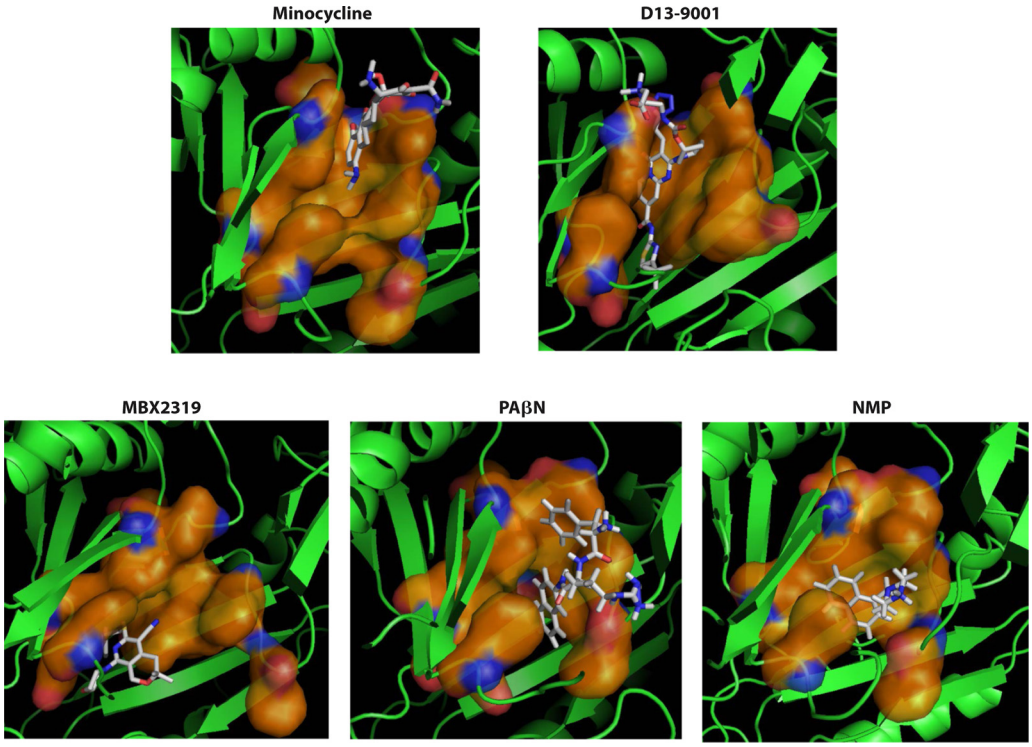

FIG 8 Binding of various inhibitors determined by MD simulation. Although the binding of PAβN and NMP was examined previously (93), the simulation process was extended to >300 ns. The orange surface shows the distal binding pocket (defined previously [91]), and the inhibitor molecules are shown in sticks with CPK colors. AcrB is shown in green cartoon models, and the part closer to the viewer was removed for clarity. This figure was drawn by using the program Pymol, on the basis of data reported previously (96).

strates from the proximal to the distal pocket, one possibility was thought to be that the straddling of the G-loop and the interference in substrate movement might explain the action of metabolites. (However, an alternative interpretation now appears more likely [see below].) A recent study using random mutagenesis of *E. coli* AcrB identified certain residues near the outer face of the distal substrate-binding pocket (such as G141, N282, A279, and G288) for efflux inhibition by NMP (982).

D13-9001

The Daiichi-Microcide collaboration that produced PAβN continued to produce MexB-specific inhibitors, and one of the final products was D13-9001, a relatively large (669-Da) (Fig. 6) pyridopyrimidine compound (983), which decreased the levofloxacin MIC 8-fold at 2 μg/ml in *P. aeruginosa*. This compound is also important because it was cocrystallized with MexB and AcrB (75). In that work, the authors showed that D13-9001 is neither a substrate of AcrB nor pumped out by the transporter. Moreover, by using purified AcrB and MexB for isothermal titration calorimetry, those authors showed that the inhibitor was bound tightly by the transporter, with a dissociation constant of ~1 μM. When the binding energy was calculated from the crystal structure, it was −11.2 kcal/mol for the inhibitor, which can be compared with the much weaker binding (−5.6 kcal/mol) for a typical substrate, minocycline. The crystal structure revealed that the lipophilic portion of this large inhibitor (*tert*-butylthiazolyl aminocarboxyl pyridopyrimidine) is bound to the bottom of the distal binding pocket and is surrounded by F136, F178, F610, F615, and F628, called a "hydrophobic trap" by those authors. In contrast, the hydrophilic parts of the inhibitor (the piperidine acetoaminoethylene ammonioacetate moiety as well as the tetrazole group) are bound to the upper, groove-like portion of the binding pocket and interact with many less-lipophilic side chains (Fig. 8). This structure then explains the molecular basis of pump inhibition by this agent. First, the bound agent prevents the binding of other substrates to the upper part of the binding pocket. Indeed, purified AcrB bound much less minocycline in the presence of D13-9001 (75). Second, even if the drugs bind to a subsite that does not overlap the inhibitor, the tight binding of the inhibitor will prevent the conformational changes needed for drug efflux through the functional rotation mechanism. Lastly, we note the fact that D13-9001 was effective in inhibiting efflux in intact cells of *P. aeruginosa* (983), although it is essentially a neutral compound and is unlikely to perturb the OM. However, the two *P. aeruginosa*-active EPIs, PAβN and D13-9001, contain multiple charged groups and may thus traverse the OM through one of the specific channels, allowing the influx of basic amino acids, peptides, and even some acidic compounds (984).

MBX2391

The pyranopyridine derivative MBX2391, recently developed by Microbiotix, is a strong inhibitor of the AcrB pump in *Enterobacteriaceae* (95). It is a relatively small (410-Da) (Fig. 6) neutral molecule and shows activity at very low concentrations. In a killing assay with *E. coli* with a minimally bactericidal concentration of ciprofloxacin, even 0.19 μM (0.08 μg/ml) the inhibitor increased killing significantly, and in the presence of 3 μM (1.2 μg/ml), 99.99% killing was achieved in 4 h, whereas ciprofloxacin

alone caused no killing at all. In a nitrocefin efflux assay using intact *E. coli* cells, this inhibitor at 0.2 μM produced a clear inhibition with a strong increase of the K_m. In contrast, much higher concentrations of PAβN could not produce clear signs of inhibition.

Some of us recently examined the binding of MBX2319 to AcrB by MD simulation (96). In this work, we also examined the binding of other inhibitors by the same method. First, we noted that D13-9001 and MBX2319 bound more tightly (−18.2 and −12.5 kcal/mol) than the typical substrate minocycline (−7.2 kcal/mol). D13-9001 bound to AcrB in exactly the same position as that shown previously by X-ray crystallography (75), confirming the reliability of our approach. MBX2319 interestingly also bound to the bottom of the distal binding pocket, or the hydrophobic trap, just like the hydrophobic portion of D13-9001. However, because MBX2319 does not have the hydrophilic side chains of D13-9001, the upper portion of the pocket, where exported drugs usually bind, is not occupied (Fig. 8).

Examination of Fig. 8 suggests furthermore that the upper "groove" portion of the binding pocket becomes closed upon the binding of MBX2319, PAβN, and NMP and occluded by the binding of the hydrophilic parts of D13-9001. Indeed, the docking of minocycline to the inhibitor-AcrB complexes showed that it is impossible for minocycline to bind to any subsite within the binding pocket (96). This observation then suggests the hypothesis most likely at present: the binding of inhibitors distorts the binding pocket so that the binding of substrates becomes difficult. Although our earlier hypothesis of interference with the G-loop cannot be totally discarded, this interpretation clearly does not apply to MBX2319, as it binds away from the G-loop, unlike PAβN or NMP (Fig. 8). Although MBX2319 does not inhibit efflux in *P. aeruginosa*, this is most likely due to its poor penetration across the OM, as it lacks any charged groups and cannot utilize common specific channels. Indeed, efflux inhibition was seen once *P. aeruginosa* OM permeability was increased by the simultaneous application of polymyxin B nonapeptide (T. J. Opperman, personal communication).

Other Compounds That Inhibit RND Pumps

Many other types of compounds have been investigated as potential EPIs and reviewed (16, 981, 985–987). Quinoline and pyridoquinoline derivatives were investigated as inhibitors of *E. aerogenes* AcrAB by the Pagès group (988). The compound showing the highest activity, 2,8-dimethyl-4-(2′-pyrrolidinoethyloxy)quinoline (Fig. 6), reversed chloramphenicol and norfloxacin resistance of clinical isolates substantially; however, it had to be used at a high concentration of 1 mM (989). The same group reported later that the derivatives of 7-chloroquinoline were more potent (1030).

Pharmacia scientists reported that arylpiperidines, such as 2-fluoro-3-(2-chloro-5-bromo-phenylethyl)piperidine (Fig. 6), were capable of inhibiting AcrAB and decreasing linezolid MICs in *E. coli* at a concentration of 100 μM (990). The Bohnert-Kern group, who discovered NMP as an inhibitor, examined other compounds with an arylpiperidine structure and found that pimozide (Fig. 6) inhibited AcrAB function strongly when Nile red efflux or ethidium influx assays were used but had little effect on the MICs of conventional antibiotics, presumably because its inhibitory action was substrate specific (991). Those researchers found that a selective serotonin reuptake inhibitor, sertraline (Fig. 6), decreased the tetracycline, clarithromycin, and linezolid MICs at 100 μM but had a much smaller effect on oxacillin MICs (992).

Another group of compounds is phenothiazines (Fig. 6), including chlorpromazine. These compounds were reported in 1997 to decrease drug resistance in *E. coli* (993), but it was unclear if the mechanism involved inhibition of the pump, because those authors showed that phenothiazine derivatives eliminated R plasmids. However, in 2003, Kaatz and others (994) demonstrated that these compounds decrease the MICs of several agents in *S. aureus*, presumably by inhibiting MFS pumps, although there was a small decrease in membrane potential. A phenothiazine, thioridazine, was found to inhibit the presumably AcrB-catalyzed efflux of ethidium in *E. coli* at a concentration of 15 μg/ml (995), and similar activity was seen with chlorpromazine (996). Recently, 40 new phenothiazine derivatives were tested with *E. coli*, and some appeared to inhibit AcrB significantly on the basis of ethidium accumulation assays (997). Still, strangely, the compounds were not effective against *S. enterica* serovar Enteritidis, and it is unclear which one of the three listed *Salmonella* strains was used for the assay. Moreover, none of the identified compounds could potentiate susceptibility of AcrAB-overproducing *E. coli* or *S. enterica* serovar Enteritidis. Additionally, certain polyamino geranic derivatives at a concentration range of 0.03 to 0.25 mM were able to decrease, by possible efflux inhibition, MIC values of chloramphenicol and nalidixic acid against *E. aerogenes* and *Salmonella* 2- to 64-fold (998). When the structures of these compounds are examined (Fig. 6), it is curious that most of the inhibitors contain a hydrophobic polycyclic core, which is likely to be bound in the hydrophobic pocket of the AcrB-binding site. Thus, all inhibitors might share a mode of binding to the transporter protein.

Martins et al. also reported a study in which verapamil (40 to 80 μg/ml) was seen to inhibit the efflux of ethidium at pH 8 in *E. coli* (999). The effect was seen to decrease in the presence of glucose, a result that led those authors to the very unlikely conclusion that ABC transporters, suggested to be MsbA without any evidence, were responsible for drug efflux at this pH. It should be noted that glucose also generates proton motive force and not only ATP.

Plant extracts often contain EPIs (mostly for pumps of Gram-positive bacteria), and the literature up to 2007 was reviewed previously (1000). Extracts of some plants were found to inhibit the AcrAB pump (1000), but this observation does not appear to have been followed up. Extracts from the genus *Berberis* are especially known to contain inhibitors of staphylococcal NorA (15). Extracts of such plants decreased ciprofloxacin MICs drastically, especially in highly ciprofloxacin-resistant strains of *E. coli* and *P. aeruginosa*, where efflux is likely to play a major role (16). Li et al. found that artesunate, a derivative of artemisinin (used against malaria), potentiated β-lactam activities against *E. coli* and increased cell accumulation of daunorubicin, possibly through its inhibition of *acrAB-tolC* expression (1001). However, artesunate concentrations required for such inhibition were quite high, in the range of 32 to 512 μg/ml. Similar to early observations (16), plant extracts containing alkaloids, flavonoids, phenols, triterpenes, and sterols potentiated antibiotic activity against multidrug-resistant *E. coli*, *E. aerogenes*, *K. pneumoniae*, and *P. aeruginosa*, with 2- to 8-fold decreases in MICs of certain antibiotics tested (but antagonistic effects were also observed with some combinations) (1002). Scientists at Microcide reported that the fermentation product of *Streptomyces* spp. contained two polycyclic compounds that showed strong potentiation of levofloxacin activity against the *P.*

TABLE 5 Methodological considerations for detection and characterization of drug efflux pumps

Method	Consideration(s)	References
Microbiological		
Antimicrobial susceptibility testing ± EPI	Can be a routine assay Used for development of novel antibacterials Requires appropriate EPI and needs to rule out nonefflux inhibitory effects of the EPI Unable to provide identity of the pumps	60, 346, 550, 553, 630, 989
Genetic and molecular		
PCR	Readily carried out and widely used Can largely screen the distribution of efflux genes Multiplex PCR can be used for identifying multiple resistance determinants Requires sequences of the pump genes	528, 1022, 1023
RT-PCR	Readily carried out (qualitative and quantitative) and widely used Can link gene expression with resistance phenotype (without or with an EPI) Can assess the impact of factors (e.g., induction) on pump expression Requires purification of RNA and that there is no DNA contamination Requires sequences of the pump genes Requires appropriate controls (e.g., a housekeeping gene) for comparison	199, 200, 380, 381, 383, 385, 413, 417, 420–422, 451, 502, 531, 572, 573, 628, 668, 1024–1027
Cloning and expression in native and/or exogenous host and mutational analysis of efflux components	Can be used for determining the function and substrate specificity (including identification of important residues of pump components) Drug efflux pump-deficient hypersusceptible *E. coli* can often be used as a host Requires appropriate expression vector and host Overexpression of a pump may be toxic to the host	82, 306, 489, 560, 623
Genetic inactivation	Can be used to assess the role of a specific pump in intrinsic and acquired resistance when combined with susceptibility testing Can be used to assess the role of pumps beyond drug resistance (e.g., biofilm formation, stress response, fitness, and virulence) Can be used to study pump regulation Requires appropriate methods to construct mutants	10, 13, 27, 28, 30, 164, 560, 574, 623
Genomic/proteomic analysis including a microarray assay	Used to determine the distribution of various classes of pumps, including putative drug pumps and other resistance determinants Microarray assay may compare a large no. of efflux pump genes and nonefflux genes May not reveal a function and needs experimental approaches for confirmation Requires certain instrument facilities	496, 500, 534, 570, 615
Biochemical		
Cell-based drug accumulation or uptake assay	Can be readily carried out Can be developed for high-throughput screening methods for searching for novel antimicrobials and EPIs May be used to measure steady-state drug levels May be used for transport kinetic studies Requires the substrates to be traceable, such as radiolabeled or fluorescent substrates An ionophore proton conductor, CCCP, has often been used	9, 11–13, 60–63, 465, 563
Membrane vesicles	Can be used to demonstrate the efflux process Require delicate experimental conditions (e.g., French cell press and radiolabeled substrates) Not widely used and mostly demonstrated in *E. coli* with certain pumps	133, 1028
Liposome reconstitution transport	Can be used to demonstrate the efflux process Requires expression and purification of efflux protein components	23, 57, 142, 1028
Immunoblot assay	Confirms the presence of pumps Quantifies pump expression Used to study pump component interactions Requires pump component-specific antibodies	483, 628, 1027
Structural studies	Determines molecular and biochemical basis of efflux pumps and drug-pump interactions Used to search for novel antimicrobials and EPIs Requires delicate biochemical experimental conditions for studying crystal structures Computer simulations can also be used	66–68, 70, 74, 75, 91, 93, 114, 166

aeruginosa MexAB-OprM system (reviewed in reference 15). Recently, a gallotannin, 1,2,6-tri-*O*-galloyl-β-D-glucopyranose from *Terminalia chebula* fruit, was revealed to potentiate antibiotic activity against multidrug-resistant uropathogenic *E. coli*, at least partly attributed to its inhibitory effect on efflux pumps (1003). Two compounds derived from human serum increased the accumulation of ethidium bromide and minocycline in *A. baumannii* cells and potentiated activity of several antibiotics against *A. baumannii* and *P. aeruginosa* (1004).

Finally, it should be mentioned that proton conductors such as CCCP are sometimes loosely called inhibitors in the literature. However, they simply abolish the energy source, the proton motive force, for the pump function and do nothing to the pump *per se*. Furthermore, they are so extremely toxic that there is no chance that they could become drugs. Thus, such compounds have no place in the discussion of pump inhibitors.

METHODOLOGICAL CONSIDERATIONS

The availability of a large amount of bacterial genome data and the rapid development of technology in biochemistry and molecular

biology have facilitated research for understanding the significance of drug transporters in antibiotic resistance. To date, various molecular and biochemical approaches have been used to detect and characterize the contribution of efflux pumps to resistance. Table 5 provides a summary of the methodological considerations for these methods. Some approaches have been discussed in different sections above. Here we discuss the application of antimicrobial susceptibility testing (AST) for studying efflux-mediated resistance.

From a clinical microbiology perspective, routine AST conducted with intact cells in the presence and absence of a known EPI may provide a good indication regarding the likely involvement of efflux pumps in resistance. Indeed, given the simplicity of AST, numerous such studies have been undertaken with clinical isolates (Table 5). However, it is of critical importance to predetermine the appropriate concentrations of an EPI and other testing conditions in order to rule out the contribution of nonefflux processes, since even a subinhibitory concentration of an EPI may exert its impact on bacterial growth.

An example for carefully assessing EPIs and establishing criteria for EPIs is the characterization of PAβN (60). This should bring much attention because PAβN has frequently been used as a classical EPI of RND pumps, yet the OM-permeabilizing effect of this agent at high levels may have often been ignored. Moreover, regardless of its mode of action, the potential antibacterial activity of an EPI itself should not be underestimated in combinational AST studies. Either membrane-permeabilizing agents or EPIs could increase antibiotic susceptibility. The MIC values of PAβN against *E. coli* and *P. aeruginosa* of different RND pump backgrounds vary, with a range of 32 to 512 μg/ml (60, 974). Thus, studies with carelessly chosen concentrations may inappropriately estimate the role of efflux mechanisms in clinical isolates. Consistent with its nature as a dipeptide amide with two positive charges at physiological pH, PAβN has been noted since its discovery for its membrane-permeabilizing effect (particularly in the absence of a functional MexAB-OprM pump) at a level of 16 μg/ml, especially in Mg^{2+}-poor media (60, 1005). In combinational studies with antibiotics, PAβN has often been used at concentrations of >20 μg/ml (Table 5) (60, 550, 1005). Several more recent studies have also highlighted such OM-permeabilizing effects (974, 975, 1006, 1007), although their conclusions need to be examined carefully, as discussed above (see Efflux Pump Inhibitors). In *Burkholderia* spp., PAβN at a concentration of up to 200 μg/ml was considered most effective as an EPI (627, 1005). With this high level, its impact on OM permeability should be investigated. Moreover, PAβN at 200 μg/ml induced the expression of AmrAB-OprA and BpeAB-OprB of *B. thailandensis* (627), and this requires further study regarding the interplay between RND pumps and PAβN.

The literature is unfortunately full of examples of indiscriminate uses of EPIs for assessing the efflux status of both Gram-positive and Gram-negative bacteria. To date, there are more potential EPIs identified for Gram-positive bacteria than for Gram-negative bacteria (16). However, the results obtained with a typical EPI for Gram-positive bacteria such as reserpine for inhibiting RND pumps of Gram-negative bacteria remain to be further assessed (1008, 1009). Hence, caution should be taken in the selection of appropriate EPIs. In this regard, guidance documents for conducting AST or analyzing resistance mechanisms in clinical laboratory settings are available, for example, for β-lactamase identification. However, despite numerous studies using AST in the presence and absence of EPIs with clinical isolates, there are currently no guidelines regarding the choice of appropriate EPIs, the selection of their proper concentrations, and other testing conditions (e.g., standard isolates with known efflux status, media, and quality control). Such a standardized method can be applied together with other approaches (Table 5), particularly the RT-qPCR technique, to characterize efflux-mediated drug resistance in clinical isolates.

CONCLUSIONS

Over the past 2 decades, impressive advances in science and technology have revolutionized our understanding of the significant role that multidrug efflux pumps play in multidrug-resistant Gram-negative bacteria. These pumps have been characterized in a large number of human and animal pathogens, as described in this review. Through numerous studies targeting RND-type AcrAB-TolC and Mex pumps, we have obtained an in-depth understanding of the structural and biochemical basis of both transport mechanisms and substrate profiles of MDR pumps as well as their role in and beyond antibiotic resistance. A better understanding of pump regulation as well as synergistic interactions between these pumps and other resistance mechanisms could provide promising targets for drug discovery. However, even with our appreciation of efflux pumps in MDR, we are still facing challenges in developing novel antibiotics that can bypass the effects of MDR pumps and clinically useful EPIs. Meanwhile, for clinical microbiologists, standardized methods that can readily identify both genotypic and phenotypic contributions of these pumps to MDR in clinical isolates should be established and validated. Furthermore, the broad substrate specificity of these pumps and the rapid selection of pump-overproducing isolates during clinical therapy illustrate the importance of antibiotic stewardship by optimizing antibiotic use and reducing antibiotic overuse.

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The views in this review do not necessarily reflect those of Health Canada.

We have no disclosable conflicts of interest.

ADDENDUM IN PROOF

A new family of drug efflux transporters was recently reported (K. A. Hassan, Q. Liu, P. J. F. Henderson, and I. T. Paulsen, mBio 6:e01982-14, 2015, http://dx.doi.org/10.1128/mBio.01982-14). Also, a recent report describes the synthesis of derivatives of MBX2319, some of which are 30 times more potent than the original inhibitor, based on the potentiation of levofloxacin and piperacillin (S. T. Nguyen et al., Bioorg Med Chem, in press). Finally, Blair et al. discovered that AcrB protein of *Salmonella* Typhimurium from a patient treated with ciprofloxacin had a single amino acid substitution at the binding site, which made the pump more effective for fluoroquinolones and less effective for other antibiotics (J. M. A. Blair et al., Proc Natl Acad Sci U S A, in press, http://dx.doi.org/10.1073/pnas.1419939112).

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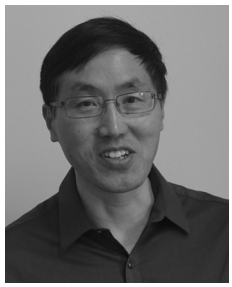

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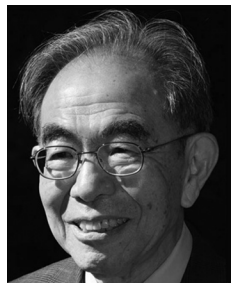

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