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Prevention of Drug Access to Bacterial Targets: Permeability Barriers and Active Efflux

Hiroshi Nikaido

Some species of bacteria have low-permeability membrane barriers and are thereby "intrinsically" resistant to many antibiotics; they are selected out in the multitude of antibiotics present in the hospital environment and thus cause many hospital-acquired infections. Some strains of originally antibiotic-susceptible species may also acquire resistance through decreases in the permeability of membrane barriers. Another mechanism for preventing access of drugs to targets is the membrane-associated energy-driven efflux, which plays a major role in drug resistance, especially in combination with the permeation barrier. Recent results indicate the existence of bacterial efflux systems of extremely broad substrate specificity, in many ways reminiscent of the multidrug resistance pump of mammalian cells. One such system seems to play a major role in the intrinsic resistance of *Pseudomonas aeruginosa*, a common opportunistic pathogen. As the pharmaceutical industry succeeds in producing agents that can overcome specific mechanisms of bacterial resistance, less specific resistance mechanisms such as permeability barriers and multidrug active efflux may become increasingly significant in the clinical setting.

Antibiotics have been highly effective in the treatment of infectious diseases, and the general population now expects that any bacterial infection will be cured easily by one of these agents. The emergence of resistant bacteria is changing this situation. As described by Neu (*1*), patients in major hospitals staffed by highly competent personnel are dying as a result of infections by resistant bacteria. These resistant bacteria are of two kinds. First, the constant presence of antibiotics in the hospital environment has selected out the unaltered strains of those species that may not possess strong virulence but are intrinsically resistant to a number of antibiotics. These include *Pseudomonas aeruginosa* and *Enterococcus* species, which infect debilitated patients in hospitals as "opportunistic pathogens." Second, there are those bacterial species that are well known for their pathogenicity. Many of these "professional pathogens" used to be exquisitely susceptible to antimicrobial agents. But many years of antibiotic usage have selected out drug-resistant strains, which either contain alterations in their chromosome or have acquired resistance plasmids (R plasmids) or resistance-conferring transposons from another organism.

Bacteria utilize several ingenious mechanisms to develop resistance. These include degradation of the drug, inactivation of the drug by enzymatic modification, and alteration of the drug target (*2*). These mechanisms are all quite specific for a single drug or a single class of drugs. There are, however, more general mechanisms of drug resistance, in which access of the unaltered agent to the target is prevented by the barrier and active transport functions of biological membranes. Thus, an organism can surround itself with a barrier of low permeability in order to decrease the influx of the drug into the cell and can also pump out the drug in an energy-dependent fashion. During the last few decades, the pharmaceutical industry has been successful in producing many synthetic and semisynthetic agents that are able to withstand the action of most of the enzymes that degrade or modify natural antibiotics. Because of this success, the less specific mechanisms such as the permeability barrier and the active efflux are likely to become more important in the clinical setting. Especially noteworthy is the recent observation, presented below, that some bacterial species already possess efflux transporters of very broad substrate specificity, reminiscent of the multidrug resistance (mdr) pump of mammalian cells (*3*).

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Bacterial Species Surrounded by Low-Permeability Barriers

Bacteria are unicellular organisms and their cytoplasm is separated from the external environment by the cytoplasmic membrane. The major permeability barrier in any membrane is the lipid bilayer structure, and its barrier property is inversely correlated with its fluidity (*4*). It is not possible to make the cytoplasmic membrane much less permeable, because this would require decreasing the membrane fluidity and interfering with the proper functioning of membrane proteins. Thus, some bacteria protect themselves by constructing an additional structure that surrounds the cell, outside the cytoplasmic membrane.

Most Gram-positive bacteria are surrounded by a thick peptidoglycan cell wall (Fig. 1). This structure, although mechanically strong, appears to offer little resistance to the diffusion of small molecules such as antibiotics, because its meshwork is too coarse (*5*). In contrast, Gram-negative bacteria, such as *Escherichia coli*, surround themselves with a second membrane, the outer membrane, which functions as an effective barrier (Fig. 1). The outer leaflet of the outer membrane bilayer is composed of an unusual lipid, lipopolysaccharide (LPS), rather than the usual glycerophospholipid found in most other biological membranes. Fatty acid chains present in LPS are all saturated. Because unsaturated fatty acid residues make the interior of the lipid bilayer fluid by preventing the tight

packing of the hydrocarbon chains (*4*), the absence of unsaturated fatty acids is expected to make the interior of the LPS leaflet much less fluid. In addition, an LPS molecule contains six or seven covalently linked fatty acid chains, in contrast to the glycerophospholipid that contains only two fatty acid residues. In general, a larger number of hydrocarbon chains linked to a single head group decreases the fluidity of the lipid interior (*6*). Indeed, the interior of LPS bilayer structure has been shown experimentally to have very low fluidity (or high order) (*7*), and hydrophobic probe molecules have been shown to partition poorly into the hydrophobic portion of LPS and to permeate across the outer membrane bilayer at about one-fiftieth to one-hundredth the rate through the usual phospholipid bilayers (*8*).

The vast majority of clinically important antibiotics and chemotherapeutic agents show some hydrophobicity, which allows them to diffuse across the lipid bilayers of the cytoplasmic membrane. [Prominent exceptions include fosfomycin and aminoglycosides (such as streptomycin) (*9*).] Compounds with multiple protonation sites are usually thought to exist in charged forms, but considerable amounts may exist in uncharged forms (*10*) that are able to diffuse across the bilayer. Clearly, the LPS-containing asymmetric bilayer of the bacterial outer membrane serves as an efficient barrier against rapid penetration by these lipophilic antibiotics and chemotherapeutic agents.

Bacteria surrounded by such an effective barrier, however, must develop a separate mechanism to bring in essential nutrients from the external medium. For this purpose, the outer membrane contains proteins of a special class, porins, which produce nonspecific aqueous diffusion channels across the membrane (*11*). The properties of the porin channels contribute to the exclusion of antibiotics in several ways. In *E. coli*, the most constricted portion of the channel has an opening of only 7 by 10 Å (*12*), which makes the influx of many antibiotics nearly impossible or extremely slow. Furthermore, the constriction is lined with a number of charged amino acid residues, which orient the water molecules in a fixed direction. This makes entrance of lipophilic molecules into the constriction difficult, because it disturbs this energetically favorable orientation of water (*13*).

In spite of this arrangement, relatively hydrophilic agents, such as certain semisynthetic β-lactams, can readily penetrate through the porin channels of enteric bacteria (*14*). A different strategy is adopted by another Gram-negative soil organism, *P. aeruginosa*. This organism totally lacks the "classical" high-permeability porins that are present in most other Gram-negative bacteria, and is left only with a low-efficiency porin that allows the diffusion of small molecules at about one-hundredth the rate through the classical porin channels (*15*). Thus, hydrophilic antibiotics diffuse across the outer membrane of this organism only very slowly (*15*), and the organism is thus "intrinsically resistant" (that is, resistant without any chromosomal mutation or the acquisition of R plasmids) to a wide variety of commonly used antibiotics. On the other hand, even such an organism must take up essential nutrients, and for this purpose the *P. aeruginosa* outer membrane contains a number of special channels, each facilitating the diffusion of a specific class of compounds (*16*).

Mycobacteria, Gram-positive bacteria phylogenetically remote from *P. aeruginosa*, have adopted a similar strategy of surrounding themselves with a barrier of generally low permeability. These bacteria are again intrinsically resistant to most antibiotics and pose a major public health problem when they develop resistance to the few available antimycobacterial agents, as occurred with the recent emergence of multiply drug-resistant tubercle bacillus (*17*). The mycobacterial barrier also appears to consist of a lipid bilayer of unusually high order, and thus presumably low fluidity (*18*) (Fig. 1). As with the outer membrane of Gram-negative bacteria, one of the leaflets consists of an unusual lipid. The fluidity in this case is expected to be even lower than in the LPS leaflet, because the main fatty acid in the mycobacterial cell wall is mycolic acid, which contains more than 70 carbon atoms with only a few double bonds. In LPS, six or seven fatty acids were joined to a single head group; here, hundreds of mycolic acid residues are covalently linked to a common head group, an arabinogalactan polysaccharide, which in turn is covalently linked to the underlying peptidoglycan structure (*19*). The influx of nutrients is apparently carried out by the mycobacterial porin, which is present in very small amounts and allows only very slow diffusion of small molecules through its channel (*20*). The low permeability of both the lipid matrix and of the porin channel results in the very slow penetration by antibiotics. The diffusion rate of β-lactam antibiotics, for example, is even slower, by a factor of 10 or more, than the rates found in *P. aeruginosa* (*21*).

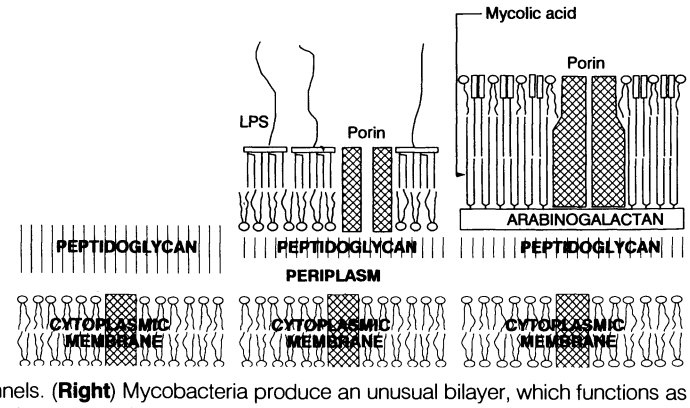

Fig. 1. Cell envelopes of bacteria. (**Left**) Most of the Gram-positive bacteria are covered by a porous peptidoglycan layer, which does not exclude most antimicrobial agents. (**Middle**) Gram-negative bacteria are surrounded by the outer membrane, which functions as an efficient permeability barrier because it contains lipopolysaccharide (LPS) and porins with narrow, restrictive channels. (**Right**) Mycobacteria produce an unusual bilayer, which functions as an exceptionally efficient barrier, outside the peptidoglycan layer. For details see text.

Resistance Caused by Decrease in Outer Membrane Permeability

Even organisms normally surrounded by a cell envelope of relatively high permeability can develop resistance by decreasing the permeability of the envelope (*22*). When an agent mainly diffuses across the barrier through a specific channel, mutational loss of the channel can be an efficient mechanism for resistance. A "nonclassical" β-lactam compound, imipenem, shows an exceptional activity against *P. aeruginosa*, mainly because this agent diffuses through a specific channel, OprD, whose physiological function appears to be that of the transport of basic amino acids (*23*). But this meant that *P. aeruginosa* could become resistant to imipenem by simply losing the OprD channel (*24*), and currently a large fraction of *P. aeruginosa* strains isolated from the hospital environment are resistant as a result of this mechanism. In a similar manner, β-lactam compounds designed to mimic iron-chelating compounds (siderophores) during their transport through the outer membrane are known to select mutants that are defective in the specific transport of these siderophores (*25*).

Mutations producing reduced expression of nonspecific porin genes can also make Gram-negative organisms significantly more resistant to certain agents. Such mutations are readily selected in the laboratory and have been reported in some clinical isolates (*22*).

Permeation Barrier Alone Rarely Produces Significant Resistance

Surprisingly, mutants with decreased expression of porins are not very resistant to certain antibiotics. For example, in *E. coli* the

minimal inhibitory concentration (MIC) for tetracycline increases by only 50% after the loss of porins (*26*), and that for cefotaxime (a β-lactam compound only slowly hydrolyzed by the β-lactamase commonly found in Gram-negative bacteria) increases by only 100%, in contrast to an 8- to 32-fold increase in the MIC for cephalothin (a β-lactam rapidly hydrolyzed by the same enzyme) (*27*). This occurs because even the most effective permeability barrier of bacteria cannot completely shut out the influx of small molecules. Experimental data show that the half-equilibration time for hydrophilic β-lactams across the outer membrane is less than 1 second in *E. coli* (*22*). Thus, decreasing the outer membrane permeability further, say to 1% of the initial value, by decreasing the porin content will still produce a half-equilibration time of a few minutes. In other words, the drug will penetrate rapidly enough to exert its action. Similarly, even the low-permeability outer membrane of *P. aeruginosa* cannot prolong the half-equilibration time of most hydrophilic antibiotics beyond several minutes (*22*).

The half-equilibration times mentioned above suggest that achievement of significant levels of drug resistance requires a second contributor in addition to a low-permeability barrier. *Escherichia coli* does not inactivate tetracycline and it hydrolyzes cefotaxime only slowly; for these agents the second contributor is essentially absent, and thus decreased outer membrane permeability has little effect on MIC. In contrast, the hydrolytic inactivation of the agents by β-lactamase, located in the periplasm (the space between the outer and cytoplasmic membranes; see Fig. 1), functions as an effective second factor for readily hydrolyzed β-lactams, such as cephalothin, in *E. coli*. The two contributors, the outer membrane and β-lactamase, work together synergistically, because under these conditions the enzyme can produce significant resistance by hydrolyzing only the small number of β-lactam molecules that slip through the outer membrane barrier (*22, 28*). When the hydrolysis occurs efficiently, the permeability barrier becomes a limiting step in the overall degradation of drugs by intact bacteria, and thus decreases in porin level have pronounced effects on MIC, as described above for cephalothin.

Active Efflux as a Mechanism of Drug Resistance

Resistance caused by the active pumping out, or transmembrane efflux, of noxious agents began to attract the attention of scientists around 1980, when S. B. Levy and co-workers showed that plasmid-coded tetracycline resistance of *E. coli* (*29*) is based on energy-dependent efflux. This was followed shortly afterward by the demonstration that the plasmid-coded cadmium resistance of *Staphylococcus aureus* was also based on an efflux mechanism (*30*).

Active drug efflux systems in bacteria (*31*) can be divided into four families (Table 1) on the basis of supramolecular assembly, mechanism, and sequence homology: (i) "major facilitator (MF) family" (*32*), which shows sequence homology to the glucose facilitators of mammalian cells and also includes drug efflux proteins of eukaryotic microbes—for example, ATR-1 of yeast (*33*); (ii) resistance-nodulation-division (RND) family (*34*), which also includes transporters that pump out cadmium, cobalt, and nickel ions (*34*); (iii) Smr or staphylococcal multidrug resistance fam-

Table 1. Examples of active efflux systems in bacteria.

Transporter	Organism	Accessory protein	Gene location	Substrate	Reference
			Major facilitator in Gram-positives		
OtrB	*Streptomyces rimosus*		Chromosome	Oxytetracycline	*67*
Tel(L)	Various cocci, *Bacillus subtilis*		Plasmid	Tetracycline	*68*
Mmr	*Streptomyces coelicolor*		Chromosome	Methylenomycin	*38*
ActII	*S. coelicolor*		Chromosome	Actinorhodin	*69*
TcmA	*Streptomyces glaucescens*		Chromosome	Tetracenomycin	*70*
NorA	*Staphylococcus aureus*		Chromosomal	Fluoroquinolones, basic dyes, puromycin, chloramphenicol, tetraphenylphosphonium	*44, 47*
QacA*	*S. aureus*		Plasmid	Quaternary ammonium compounds, basic dyes	*71*
Bmr	*B. subtilis*		Chromosome	Basic dyes, chloramphenicol, puromycin, fluoroquinolones	*47*
			Major facilitator in Gram-negatives		
TetA	*Escherichia coli*	None	Plasmid	Tetracycline	*72*
CmlA	*Pseudomonas aeruginosa*	None	Plasmid	Chloramphenicol	*41*
Bcr	*E. coli*	None	Chromosome	Bicyclomycin	*73*
EmrB	*E. coli*	EmrA	Chromosome	CCCP,† nalidixic acid, tetrachlorosalicylanilide, phenylmercury acetate	*49*
EmrD	*E. coli*	?	Chromosome	CCCP,† phenylmercury acetate	*74*
			RND family		
AcrE(AcrB)‡	*E. coli*	AcrA	Chromosome	Basic dyes, SDS,† erythromycin, novobiocin, fusidic acid, tetracycline, mitomycin C	*50, 51*
EnvD	*E. coli*	EnvC	Chromosome	Basic dyes, SDS,† erythromycin, fusidic acid, tetracycline, mitomycin C, and others§	*75*
MexB	*P. aeruginosa*	MexA	Chromosome	Tetracycline, chloramphenicol, fluoroquinolones, β-lactams, pyoverdine	*57, 58*
			Smr family		
Smr(QacC)‡	*S. aureus*	None	Plasmid	Quaternary ammonium compounds, basic dyes	*76*
QacE	*Klebsiella aerogenes*		Plasmid	Quaternary ammonium compounds, basic dyes	*77*
MvrC(EmrE)‡	*E. coli*	?	Chromosome	Methyltriphenylphosphonium, basic dyes	*66*
			ABC transporters		
MsrA	*Staphylococcus epidermidis*		Plasmid	14- and 15-membered macrolides	*39*
DrrA, DrrB	*Streptomyces peucetius*		Chromosome	Daunorubicin, doxoribucin	*78*
TlrC, TlrB(?)	*Streptomyces fradiae*		Chromosome	Tylosin	*79*

*QacB is very similar to QacA. †CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; SDS, sodium dodecylsulfate. ‡Alternate names are shown in parentheses. §A study with newly constructed null mutants suggests that the original *envC* mutant contains other, uncharacterized mutations (*54*).

ily (35), consisting of small transporters that presumably contain only four transmembrane helices; and (iv) ABC [adenosine triphosphate (ATP)–binding cassette] transporters, which are composed of two transmembrane and two ATP-binding subunits or domains (36). Each member of the first three families is a single cytoplasmic membrane protein that extrudes drugs by using proton-motive force—in other words, acting as a H^+-drug antiporter (32, 34–36). There is little sequence homology between MF and RND proteins, although both are composed of two homologous halves, each usually containing six transmembrane α helices (32, 34, 36). Efflux transporters of the MF family are different from the transporters involved in nutrient uptake, in that they contain the consensus sequence G-X-X-X-G-P-X-X-G-G (37). Furthermore, some efflux transporters of the MF family (including QacA, Mmr, EmrB, and ActII) are predicted to contain 14, rather than 12, transmembrane α helices (37).

It is increasingly recognized that active efflux plays a major role in the resistance of many organisms to many agents (31); among the three clinically relevant resistance mechanisms listed by Neu (1) as involving reduced permeability, two, and possibly all three, are mainly due to efflux. (i) Beginning with the demonstration, by Neal and Chater in 1987 (38), that *Streptomyces coelicolor* protects itself from the methylenomycin it produces, a number of antibiotic efflux genes have been identified in antibiotic-producing *Streptomyces* species. Some of them belong to the MF family and others to the ABC family. (ii) A new type of plasmid-mediated macrolide resistance in *S. aureus*, which was originally thought to be due to decreased permeability, was shown to involve active efflux (39). (iii) The commonest mechanism of chloramphenicol resistance involves the enzymatic acetylation of the drug. The "nonenzymatic mechanism of chloramphenicol resistance," again originally thought to be due to decreased influx of chloramphenicol (40), was shown to be caused by active efflux, because the *cmlA* gene from transposon Tn*1696* appears to be an active efflux transporter of MF family (41, 42). Furthermore, *Haemophilus influenzae* contains a homologous chromosomal gene (41), and thus the nonenzymatic resistance of this (and perhaps many other) species is very likely due to active efflux. (iv) Plasmid-coded resistance to quaternary ammonium antiseptics in staphylococci was shown to involve efflux, through the QacA-QacB transporter of MF type and the QacC transporter of Smr type. These proteins also pump out some basic dyes (Table 1). (v) Active efflux of norfloxacin, a fluoroquinolone, was first discovered in wild-type *E. coli* (43). A gene (*norA*) involved in a similar active efflux process was later sequenced from the chromosome of a resistant mutant of *S. aureus* and was shown to code for an efflux transporter of the MF family (44). NorA pumps out a variety of fluoroquinolones (44, 45).

Multidrug Efflux Systems in Bacteria

An exciting development in this field has been the discovery of bacterial efflux systems that can handle a wide variety of drugs (46), reminiscent of the mdr system in mammalian cells (3). Systems such as QacA, Smr, QacE, or MvrC pump out quaternary amine compounds as well as basic dyes and are often called multidrug efflux systems. However, the substrates of these systems are at least physically similar, being amphiphilic molecules with positive charges. In contrast, the Bmr transporter, found in a rhodamine-6G–resistant mutant of *Bacillus subtilis* (47), catalyzed the active efflux not only of cationic dyes such as rhodamine-6G and ethidium bromide, antibiotics puromycin (basic) and netropsin (strongly basic), and an organic cation, tetraphenylphosphonium, but also chloramphenicol (uncharged). It was later shown to pump out fluoroquinolones (47), most of which should exist as zwitterions at a neutral pH (10). NorA in *S. aureus* turned out to be a Bmr homolog and was indeed shown to pump out cationic dyes, puromycin, and chloramphenicol (48), solutes that are unrelated not only in chemical structure but also in physical properties.

Another multidrug efflux system was identified in an *E. coli* mutant resistant to an uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (49). This transporter, EmrB, pumps out in addition such unrelated compounds as phenylmercuric acetate, nalidixic acid (a weak acid), and thiolactomycin (uncharged).

The *acrA* mutation of *E. coli* K12, which had been thought to produce drug hypersusceptibility by increasing the outer membrane permeability, was shown to inactivate a multidrug efflux complex, AcrAE (50, 51). In the wild-type *acrA*$^+$ strain, the steady-state accumulation of acriflavine is extremely low (50). Because cationic dyes should be concentrated in the cytoplasm in response to the interior-negative potential across the cytoplasmic membrane, this indicates that acriflavine must be pumped out very actively. In an *acrA* mutant, the steady-state accumulation increases at least fivefold (50), indicating that the AcrAE efflux system participates in the extrusion of this dye (52). This solves a long-standing mystery, because no defect has been found in the outer membrane of *acrA* mutants despite much research (53), and their outer membrane permeability was shown to be normal at least to one probe (54). The substrate range of the AcrAE system appears to be very broad and includes hydrophilic antibiotics such as novobiocin, erythromycin (a macrolide), fusidic acid, mitomycin C, and tetracycline, as well as a detergent, sodium dodecylsulfate (SDS) (50).

An alteration at a chromosomal gene cluster of *E. coli*, *marRAB*, also produces significant resistance to a wide range of antibiotics, including fluoroquinolones, chloramphenicol, tetracycline, and β-lactams (55). (At least the resistance to fluoroquinolones and tetracycline appears to involve efflux.) MarA, however, is a regulatory protein that affects many processes (55), and the nature and number of pumps affected by this protein are presently unknown.

Most recently, the well-known intrinsic resistance of *P. aeruginosa* to a large variety of antimicrobials was shown to be due as much to an efflux system as to its low-permeability outer membrane. Clinical isolates of *P. aeruginosa*, even when they are free of R plasmids, show widely different levels of "intrinsic" resistance to antimicrobial agents. Furthermore, there is a good correlation among resistance levels to different agents, such as β-lactams, chloramphenicol, tetracycline, and fluoroquinolones (56). Different levels of intrinsic resistance were thus thought to be caused by corresponding differences in outer membrane permeability. Experiments, however, ruled out this hypothesis (56). When we tested the accumulation of various drugs, we discovered that even wild-type strains of *P. aeruginosa* pump out tetracycline, chloramphenicol, and norfloxacin very effectively, and this activity was correlated with the intrinsic resistance level of the strain (57).

The genetic identity of this efflux system was suggested by the study of K. Poole and associates (58). In their study of Fe^{3+} uptake in *P. aeruginosa*, they cloned an operon, *mexA-mexB-oprK*, which is believed to function in the export of the siderophore pyoverdine. MexB has a typical sequence for an RND family transporter. When this operon was inactivated by insertion mutagenesis, the *P. aeruginosa* strain became almost as susceptible as *E. coli* to both chloramphenicol and tetracycline (58). This suggests that this single efflux system is the major reason that this species displays the generally drug-resistant phenotype.

But how do we explain the resistance to β-lactams? *Pseudomonas aeruginosa* produces a chromosomally coded, inducible β-lactamase, and the synergy between this enzyme and the outer membrane barrier explains its resistance to some compounds that act as strong inducers of this enzyme (59). However, strains with a high intrinsic resistance are also highly resistant to compounds that do not induce much β-lactamase and are quite stable to enzymatic hydrolysis. Further, these strains are unaltered in their targets of β-lactam action or in the

levels or properties of the β-lactamase (56). Our results indeed indicate that their β-lactam resistance is also caused by active efflux (57). Unlike other hydrophobic agents, however, some β-lactams cannot cross the cytoplasmic membrane barrier (57), and their targets are on the periplasmic side of the cytoplasmic membrane. How could the efflux system help? It is useful here to recall that the mammalian mdr protein apparently intercepts its substrate during its transit through the lipid bilayer. Our observation on bilayer-impermeant β-lactams gives strong support to this idea (Fig. 2).

The apparently wide substrate specificity of the *P. aeruginosa* efflux system is somewhat surprising. We should again recall that the mammalian mdr pump extrudes not only basic compounds, such as doxorubicin, but also neutral compounds, such as taxol, and weakly acidic compounds, such as mithramycin (3). Indeed, the mammalian mdr pump is inhibited by hydrophobic cephalosporins, which presumably act as substrate analogs (3).

The Efflux System and the Outer Membrane Barrier

Efflux transporters are located in the cytoplasmic membrane, and thus in Gram-negative bacteria the agents may be assumed to be pumped out into the periplasm (see Fig. 1). If so, the efflux is unlikely to make these bacteria more resistant, because the antimicrobial agents will not be able to leave the cells easily owing to the presence of the outer membrane barrier. One way to overcome this barrier was suggested by the presence of accessory proteins that occur together with many efflux transporters of both MF and RND families in Gram-negative cells (Table 1). These proteins are thought to "bridge" the cytoplasmic transporter and an outer membrane channel so that the drugs can be extruded directly into the surrounding medium rather than into the periplasm (Fig. 2), as they are related to a group of proteins in Gram-negative bacteria, including HlyD, LktD, CyaD, AprE, and CvaA (34), which similarly act as bridges and help extrude their substrates, proteins, directly into external media (60). It is thus likely that the accessory proteins form complexes with some channel proteins in the outer membrane, for example, TolC (60). This model was supported recently by the discovery of the *mexA-mexB-oprK* system of *P. aeruginosa* (58). This presumed operon codes not only for an accessory protein MexA but also for an outer membrane protein OprK, and this gene organization suggests that the three proteins form a complex that likely forms a continuous channel opening into the external medium (Fig. 2). Indeed, OprK shows sequence homology with CyaE and PrtF (58), outer membrane proteins that are presumably involved in the formation of three component export complexes containing a transporter in the cytoplasmic protein, a periplasmic accessory protein, and an outer membrane channel, which function in the direct export of cyclolysin and proteases B and C into the medium in *Bordetella pertussis* and *Erwinia chrysanthemi*, respectively (60). Furthermore, inactivation of OprK led to hypersusceptibility to many agents, just like the inactivation of MexA (58). We do not yet know how the systems that do not contain accessory proteins extrude the agents efficiently into the medium.

As mentioned, the low permeability of the outer membrane alone is not likely to produce clinically significant levels of resistance, and a second contributor is needed for this purpose. In many systems, the active efflux system appears to be this second factor. But this does not mean that the outer membrane barrier is not important. Because the intracellular concentration of any drug is the result of a balance between influx and efflux, it is likely that the slow influx of various agents through the low-permeability outer membrane makes efflux an especially effective mechanism for resistance in *P. aeruginosa*. That is, even if organisms with a high-permeability outer membrane, such as *E. coli*, had an efflux machinery of comparable efficiency, it would not produce a significant level of resistance unless the agent has the size or structure that slows down its permeation through the outer membrane.

In this connection, it is important that *E. coli* mutants at the *marRAB* cluster produce smaller amounts (55) of OmpF porin, which produces a larger channel among the two nonspecific porins of *E. coli* and therefore plays a predominant role in the penetration of most antibiotics (14). With the influx thus decreased, active efflux can create a much higher resistance. Some plasmid-coded efflux transporters in Gram-negative bacteria may also use a similar strategy. An R plasmid repressing the synthesis of OmpF porin was reported as early as 1978 (61). The gene for nonenzymatic chloramphenicol resistance in *Haemophilus influenzae*, most likely a homolog of CmlA efflux transporter (see above), represses the synthesis of the major porin in this organism (40). Cloned CmlA gene represses porin synthesis in *E. coli* (41). A clinical isolate of *Salmonella typhi* with nonenzymatic chloramphenicol resistance, probably with a transposon containing CmlA or its homolog, was found to be severely repressed in the synthesis of OmpF (62). This ability of some plasmid- or transposon-based efflux genes to decrease outer membrane permeability has been seen so far only with resistance determinants of a narrow range. It would create a major health-care problem if such an activity were to become combined with broad substrate profile efflux transporters.

Increased expression of efflux transporters, often accompanied by the repression of OmpF porin synthesis, may occur without any genetic alteration. Thus, chloramphenicol and tetracycline increase the transcription of MarA regulatory protein (55), thereby presumably increasing the synthesis of the efflux transporter or transporters. Interestingly, oxygen stress, to which pathogenic bacteria are exposed in host tissues, is known to produce OmpF repression with increased resistance to several agents (63), possibly as a result of increased efflux. Similarly, salicylate, which is produced in plant tissues in response to the invasion by microorganisms (64), is known to repress the synthesis of OmpF porin and to make *E. coli* transiently more resistant to chloramphenicol, tetracycline, quinolones, and ampicillin (64). This range of agents is again suggestive of the involvement of an efflux system or systems.

Because specific mechanisms of antibiotic resistance were thought to be more important, efforts to produce more effective antibiotics have usually involved modification of specific groups on antibiotic molecules in order to make them inert as potential substrates for commonly occurring an-

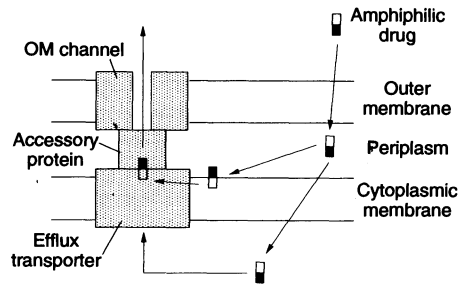

Fig. 2. Proposed structure and mechanism of action of most efflux complexes in Gram-negative bacteria. The efflux transporter is usually of the MF or RND family. This is connected to the accessory protein, such as EmrA, AcrA, EnvC, or MexA, which in turn is associated with an outer membrane channel protein, OprK in the MexAB system of *P. aeruginosa* and probably TolC in *E. coli*. Amphiphilic drugs (with its hydrophilic end shown as black rectangle) traverse the outer membrane (often via porin channels), and become partially inserted into the bilayer of the cytoplasmic membrane. The transporter captures the drug molecules in the bilayer and pumps them out, bypassing the outer membrane barrier. For agents that cross the cytoplasmic membrane rapidly, the transporter should be able to accept substrates from the cytoplasm; this may occur directly as shown, or after the insertion of the drugs into the bilayer.

tibiotic-inactivating enzymes. The presence of the more general mechanisms of resistance, discussed in this article, forces us to reevaluate this strategy. These mechanisms produce clinically significant resistance: The intrinsic resistance to a wide variety of antibiotics seen in the important opportunistic pathogen, *P. aeruginosa*, is indeed due to a combination of a multidrug efflux transporter and an effective permeability barrier, and increased expression of the efflux transporter is the most probable cause of resistance in most of the clinical isolates from the British Isles showing increased levels of carbenicillin resistance (*56*, *57*). It will be a major challenge for the pharmaceutical industry to produce compounds that are able to overcome mechanisms of this type, because some of the multidrug efflux system seems to pump out almost any amphiphilic compound (*65*). Obviously, we need to obtain more knowledge about the substrate-binding process of these transporters. Another possible approach would be to increase the spontaneous influx of drugs—for example, by making the drug sufficiently lipophilic so that efflux can be counterbalanced by rapid influx. Indeed, more lipophilic derivatives of tetracycline and fluoroquinolones are more active on resistant strains of Gram-positive bacteria that pump out these agents (*10*). In Gram-negative bacteria, however, more lipophilic agents will be slower in traversing the porin channel, and increased lipophilicity may not increase the efficacy of the agents.

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Resistance to Antibiotics Mediated by Target Alterations

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The development of resistance to antibiotics by reductions in the affinities of their enzymatic targets occurs most rapidly for antibiotics that inactivate a single target and that are not analogs of substrate. In these cases of resistance (for example, resistance to rifampicin), numerous single amino acid substitutions may provide large decreases in the affinity of the target for the antibiotic, leading to clinically significant levels of resistance. Resistance due to target alterations should occur much more slowly for those antibiotics (penicillin, for example) that inactivate multiple targets irreversibly by acting as close analogs of substrate. Resistance to penicillin because of target changes has emerged, by unexpected mechanisms, only in a limited number of species. However, inactivating enzymes commonly provide resistance to antibiotics that, like penicillin, are derived from natural products, although such enzymes have not been found for synthetic antibiotics. Thus, the ideal antibiotic would be produced by rational design, rather than by the modification of a natural product.

The widespread use and misuse of antibiotics imposes immense selective pressures for the emergence of antibiotic-resistant bacteria, and as a consequence the development of antibiotic resistance is inevitable. For those antibiotics that are derived from natural products, resistance is most commonly due to the acquisition of genes encoding enzymes that inactivate the antibiotic, modify its target, or result in active efflux of the antibiotic (*1*). These resistance genes are believed to have evolved hundreds of millions of years ago in soil bacteria, either as protection from antibiotics produced by other soil bacteria or as protection for antibiotic-producing soil bacteria against their own antibiotics (*2*). Enzymes that inactivate synthetic antibiotics such as quinolones, sulfonamides, and trimethoprim have not been found, and for these antibiotics and those natural products where inactivating or modifying enzymes have not emerged (for example, rifamycins), resistance usually arises by target modifications (*3*–*6*).

Several types of target modification are found in antibiotic-resistant clinical isolates of bacteria. Resistance to a few antibiotics occurs by the acquisition of a gene encoding a new target enzyme that has much lower affinity for the antibiotic than the normal enzyme does. Resistance to sulfonamides and to trimethoprim, which inhibit dihydropteroate synthase and dihydrofolate reductase, respectively, is usually achieved by this mechanism (*4*, *5*). Methicillin resistance in *Staphylococcus aureus* also involves this mechanism (*7*). In all of these examples, the source or sources of the antibiotic-resistant target enzymes is unclear. Production of increased amounts of normal target enzymes can also provide resistance (for example, as in a minority of trimethoprim-resistant clinical isolates of enteric bacteria) (*5*).

However, the most common mechanism of resistance is the development of altered forms of the normal targets that have increased resistance to antibiotics. Such resistance may involve the acquisition of new genes, almost invariably carried on plasmids or transposons, that result in enzymatic modification of the normal target so that it no longer binds the antibiotic [for example, resistance to macrolide antibiotics by methylation of 23*S* ribosomal RNA (rRNA)] (*8*). Alternatively, resistance may result from mutational (or recombinational) events that lead to the development of antibiotic-resistant forms of the normal targets.

Here, I will focus on this latter mechanism, and particularly on resistance due to the development of altered target enzymes that have a reduced affinity for antibiotics. I will not discuss resistance due to ribosomal mutations (*8*), not only because ribosomes are not enzymes in a strict sense, but also because this type of resistance is of doubtful clinical significance—almost all examples of resistance to antibiotics that act on the ribosome involve the acquisition of genes that either result in the protection of the ribosomes from the antibiotics or that inactivate the antibiotics (*8*). The development of enzymatic targets with reduced affinity for antibiotics is a major mechanism of resistance when inactivating or modifying enzymes are absent. This includes resistance to rifamycins and quinolones (*3*, *6*) and to β-lactam antibiotics in species where β-lactamases are absent (*7*). Most of my review here will focus on resistance to β-lactam antibiotics, for this provides a particularly instructive example of resistance that is the result of modification of enzymatic targets.

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