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MINIREVIEW

Outer Membrane Barrier as a Mechanism of Antimicrobial Resistance

HIROSHI NIKAIDO

Department of Molecular and Cell Biology, University of California, Berkeley, California 94720

Gram-negative bacteria are surrounded by a permeability barrier, the outer membrane (OM). Hydrophilic solutes most often cross the OM through water-filled channels of porins. For example, in *Escherichia coli* all β-lactams in a study were shown to utilize predominantly the porin pathway (59). Since porin channels are totally nonspecific, it is expected that other small, hydrophilic agents, such as tetracyclines, aminoglycosides, and chloramphenicol, would also utilize this pathway. In exceptional cases, however, some antibiotics can mimic natural ligands for specific pathways (see below). Hydrophobic quinolones may also diffuse through the bilayer by first disorganizing it through the chelation of divalent cations (9). Furthermore, in some organisms the diffusion of aminoglycosides through the perturbation of the highly negatively charged bilayer is significant (41).

Porin channels can, however, be very narrow and can act as effective barriers for the penetration of large or hydrophobic compounds. The OM barrier thus creates various degrees of intrinsic resistance to antimicrobial agents, even in wild-type bacteria. Furthermore, the level of resistance can be increased by genetic or physiological alterations that lower the permeability of this membrane. Antibiotic penetration through the OM was discussed extensively in a recent review (40).

QUANTITATIVE ESTIMATION OF THE BARRIER EFFECT

It would be easier if solutes could be classified as being able or unable to permeate across the OM. However, such a simplistic and qualitative view can be quite misleading, and the quantitative evaluation of the rates of solute permeation becomes absolutely necessary for an understanding of the role of the OM barrier in antibiotic resistance. Unfortunately, such rates are usually expressed as permeability coefficients, and this makes it difficult to get an intuitive feel for the magnitude of these values. In Fig. 1, therefore, these values have also been converted into half-equilibration times for permeation across the OM.

If the half-equilibration time values in Fig. 1 are very large and in the range of generation times for these bacteria, then the extremely slow influx of the agents across the OM can be counterbalanced by the dilution resulting from the expansion, owing to growth, of the periplasmic space. Thus, the agents can be thought of as being, for all practical purposes, not able to penetrate. However, for any agents that penetrate faster, the periplasmic concentration approaches that in the external medium unless an inactivation process occurs inside the cell (Fig. 2a and b). Thus, for most agents the decisive factor is not the absolute rate of OM penetration but the balance between the penetration rate and the subsequent inactivation rate.

This relationship has been best studied with β-lactams, which are degraded by the nearly ubiquitous periplasmic β-lactamase once they penetrate the OM. Sawai et al. (53) and Zimmermann and Rosselet (60) proposed a model which assumes that the influx across the OM occurs by simple diffusion and that the hydrolysis in the periplasmic space follows Michaelis-Menten kinetics.

Normark and I recently tested the validity of this model (42). We calculated the external concentration of the drug which produces a periplasmic concentration that just inhibits the target for the β-lactams, the penicillin-binding proteins. This was done by using the values of permeability coefficients determined mostly in the reconstituted liposome system (59). In 44 cases among 65 attempts, the predicted MICs were indeed close to the observed ones. More recently, an improved procedure for the assessment of parameters was proposed, and with this method MICs were correctly predicted in 61 of 65 attempts (14a). (Although it was suggested that the model did not apply to one strain, this was apparently owing to the presence of β-lactamases on the outer surface of the OM [23].)

An important consequence of this model is that the OM barrier and the β-lactamase barrier are strongly synergistic (40). Thus, decreases in OM permeability do not necessarily produce large increases in MIC if the agent is not inactivated (Fig. 2a and b) or if it is only slowly inactivated (Fig. 2e and f). In other words, the effect of decreased permeability becomes amplified by the presence of an effective enzymatic inactivation barrier (Fig. 2c and d).

What is shown semiquantitatively in Fig. 2 can be analyzed rigorously by calculating the numerical index that predicts the success with which given β-lactam molecules reach the target after passing through the OM barrier and the β-lactamase barrier, an index that reflects the "balance" between the two barriers. Such an index, the target access index (TAI), was defined recently (42). The value of the TAI is proportional to the permeability coefficient of the drug and inversely proportional to the rate of inactivation of the drug in the periplasm.

The TAI is useful because it has a simple relationship with the predicted MIC:

$$\mathrm{MIC} = C_{\mathrm{inh}} (\mathrm{TAI}^{-1} + 1) \quad (1)$$

where C_{inh} is the drug concentration needed to inhibit the target and is therefore constant for any given drug for any given species. These theoretical considerations permit an understanding of the effect of decreased OM permeability on the MIC. A 10-fold decrease in permeability, for example, results in a 10-fold decrease in the TAI. However, a 10-fold decrease in the TAI does not result in a 10-fold increase in the MIC, owing to the form of equation 1. When the initial TAI is much larger than 1, for example 100, then a 10-fold decrease in the TAI increases the term within the parentheses of equation 1 from 1.01 to only 1.1, producing no detectable change in the MIC. The MIC shows significant increases following a decrease in OM permeability only

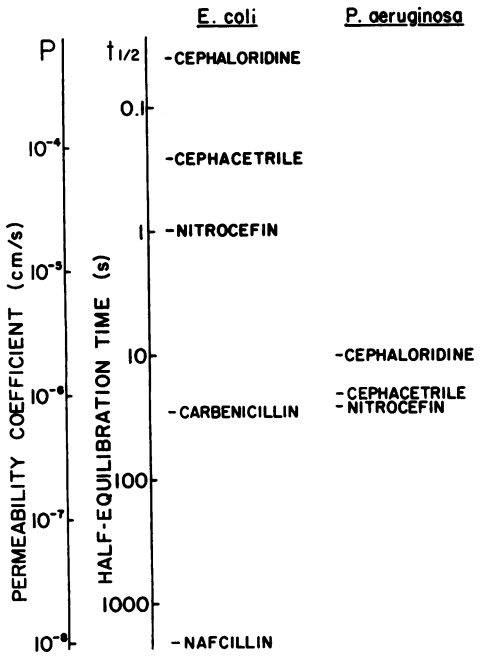

FIG. 1. Permeability coefficients (P) and half-equilibration times ($t_{1/2}$) of selected β-lactams. Permeability coefficients were measured in intact cells (for *P. aeruginosa* [41]) or in liposome vesicles containing *E. coli* OmpF porin and then normalized to intact-cell values (42). Half-equilibration times, which correspond to the times required for the periplasmic concentration to reach half of the external concentration, were calculated from the surface area and cell volume measured for *S. typhimurium*, assuming that there is no degradation of the drug and that the cytoplasmic membrane is completely permeable to the drug (43). The permeability coefficient for nafcillin was obtained by using intact cells of *S. typhimurium* rather than *E. coli* (42). Permeability coefficients and half-equilibration times of many other β-lactams in *E. coli* can be found in reference 39.

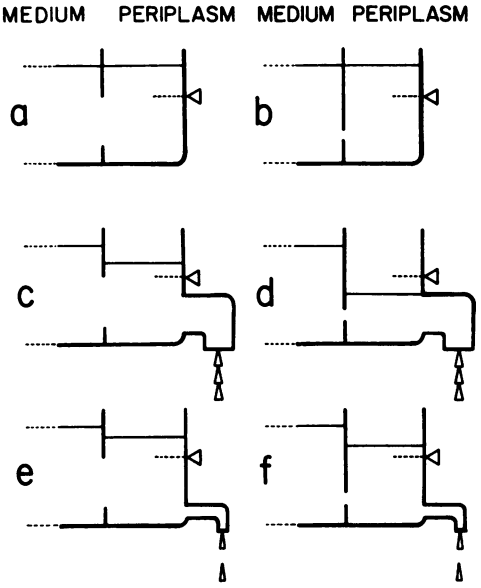

FIG. 2. Periplasmic concentrations of β-lactams determined by the balance between the rates of influx through the OM and the rates of degradation by β-lactamases. The levels of the liquid represent drug concentrations, and the OM permeability is represented by the size of the gap between the two compartments. The rate of removal by degradation is represented by the size of the faucet. Arrowheads denote the hypothetical concentrations needed to saturate the target enzymes. (From reference 39, reprinted with permission.)

when the original value of the TAI is much below 1; this corresponds to the situation in which the enzymatic inactivation rate is far greater than the influx rate, as in Fig. 2d. However, even in this case the increase in MIC tends to be lower than predicted because the bilayer regions must allow a slow penetration of solutes, and this "leakage" can become more significant for compounds with very low ability to penetrate porins (40). Thus, simplistic conclusions cannot be drawn regarding the OM permeation of a drug on the basis of the observed changes in MICs in the presumptive permeation mutants. For example, decreases in porin content in *E. coli* K-12 produced only minimal increases in the MICs of some broad-spectrum cephalosporins, and this observation led some workers to conclude that these agents do not use the porin pathway (12). However, the observation is perfectly compatible with their diffusion through the porins when the data are analyzed quantitatively by using the TAI (40).

INTRINSIC RESISTANCE IN WILD-TYPE BACTERIA

(i) Resistance to hydrophobic agents. Many gram-negative bacteria are resistant to a number of hydrophobic antibiotics and dyes (38) that are quite effective against gram-positive bacteria. These antibiotics include macrolides, novobiocin, the more hydrophobic β-lactams, rifamycin SV, and actinomycin D. Hydrophobic solutes cannot diffuse through the porin channels rapidly (43). The outer leaflet of the OM bilayer, composed of lipopolysaccharide, appears to have an unusually low permeability and does not allow the diffusion of such hydrophobic agents (44). Indeed, resistance to these agents is decreased drastically when the structure of the OM bilayer is modified by mutational alteration of (38), transient removal of (32), or attachment of polycationic molecules to (56) its lipopolysaccharide component.

(ii) Bacteria with low-permeability OM. Many gram-negative bacteria show good susceptibility to some β-lactams and also to aminoglycosides, chloramphenicol, tetracyclines, quinolones, etc., which are not too large, are rather hydrophilic, and are therefore expected to diffuse rather rapidly through porin channels. There are, however, bacteria that are generally resistant to agents of this type, primarily because they produce OMs with very poor general permeability. For example, the permeability of *Pseudomonas aeruginosa* OM to several cephalosporins is about two orders of magnitude lower than that of *E. coli* OM (41) (see also Fig. 1).

The search for proteins with porin activity in reconstituted systems led to the identification of protein F as the porin (41). It has a large channel size, but it was proposed that only a small fraction of the porin molecules form open channels (41). Recently, however, these conclusions were questioned by others, who claimed that only solutes of the size of monosaccharides or smaller can penetrate through the porin channel (see reference 41). If this is true, *P. aeruginosa* must show much higher susceptibility to exceptionally small agents; however, this does not appear to be the case.

Low OM permeability, together with inactivation by the chromosomally coded β-lactamase, certainly explains the resistance of *P. aeruginosa* to most β-lactams. However, its resistance to other agents, which are not known to be inactivated in R-plasmid-free strains, is more difficult to explain.

It seems likely that other intrinsically resistant gram-negative bacteria also produce OMs of low nonspecific permeability. In some of these organisms, porins with exceptionally narrow channels have indeed been found (40).

ANTIBIOTIC RESISTANCE OWING TO DECREASED OM PERMEABILITY

(i) Porin-deficient mutants. Porin-deficient, antibiotic-resistant mutants were first isolated from laboratory strains. Harder et al. (22) isolated spontaneous mutants of *E. coli* K-12 with moderate degrees of resistance to carbenicillin or sulbenicillin. These mutants lacked the OmpF porin, which produces slightly larger pores, but retained the OmpC porin with its narrower channel. For these mutants, MICs of agents with slower penetration rates (and presumably with lower TAI values) were significantly increased, but the increase was minimal for rapidly penetrating agents with high initial TAI values. These results are precisely as predicted from the theoretical analysis discussed above.

Similar mutants were isolated in another laboratory from *E. coli* K-12 (30). Cephaloridine and cefazolin (with large TAIs because of good abilities to penetrate) and moxalactam (with a large TAI owing to β-lactamase stability) efficiently selected *ompR* mutants that lacked both the OmpF and OmpC porins. Such an extreme deficiency in porins is needed to increase the MICs of these compounds, because a more-than-100-fold decrease in the permeability coefficient would be needed to produce a significant increase in the MIC, according to equation 1.

There are many more reports on the isolation of moderately β-lactam-resistant, porin-deficient mutants in the laboratory (8, 18–20, 31, 35, 37, 50, 52, 54, 58). There are many fewer reports on the isolation of similar mutants from clinical samples, undoubtedly owing to the fact that clinical laboratories do not usually examine OM protein composition. However, it is also possible that most OM permeability mutants are at a disadvantage in relation to the parent strains in their natural habitats.

A *Salmonella typhimurium* clinical isolate lacking a porin was thoroughly examined (36). The parent strain harbored an R plasmid coding for a TEM-type β-lactamase and showed a high degree of resistance to most cephalosporins. When the patient was treated with cephalexin, a mutant lacking the OmpC porin became predominant. Interestingly, the porin that produced a wider channel, OmpF, was still present, yet this protein was not expressed in media containing 0.5% NaCl and thus, presumably, in the tissues of the patient. The OM permeability to cephaloridine was less than 5% of that of the wild type if the strains were grown in L broth (containing 0.5% NaCl), but there was no significant difference between the strains if they were grown in low-osmolality media, such as Difco Nutrient Broth. These results suggest that, in enteric bacteria capable of producing two or three species of porins, the porin with the narrower channel is synthesized predominantly in the body of animals, an environment with high osmotic activity and high temperature (44).

An early report on the selection of porin-deficient mutants during antibiotic therapy is that of Goldstein et al. (17). A patient with *Serratia marcescens* bacteremia was treated with a combination of amikacin and cefotaxime, and this resulted in the isolation of mutants with lowered OM permeability. A 41,000-dalton OM protein was drastically decreased in the mutant. The MICs of β-lactams increased between 8- and 64-fold. Rather unexpectedly, the aminoglycoside MICs also showed 16- to 64-fold increases. Possibly, the aminoglycosides are inactivated in this strain.

Sanders and Watanakunakorn (51) found that *S. marcescens* acquired general β-lactam–aminoglycoside resistance during combination therapy with ticarcillin-cefazolin and tobramycin-gentamicin. All mutants contained decreased levels of a 42,000-dalton OM protein. The aminoglycoside resistance, however, was apparently the result of an additional, independent genetic event.

Another clinical case involved the emergence of *S. marcescens* permeability mutants in a patient treated with moxalactam (19). Again, the 41,000-dalton OM protein was decreased, and there was an 8- to 16-fold increase in the MICs of various β-lactams.

These reports suggest that permeability mutants are selected especially frequently in *S. marcescens*. This may be because *S. marcescens* cannot survive the attack by broad-spectrum cephalosporins through the overproduction of its chromosomal β-lactamase, which has a rather low affinity toward these agents and is therefore rather inefficient in hydrolyzing them.

An *E. coli* mutant with diminished levels of OmpC and OmpA proteins was isolated from a patient receiving ceftazidime (3). The mutant showed an approximately 100-fold increase in the ceftazidime MIC. Other than a fourfold increase in the MIC of ceftriaxone, there was no change in MICs of other β-lactams or chloramphenicol. The decrease of OmpA suggests an involvement of a pleiotropic regulatory mutation. Interestingly, clavulanate induced the production of both OmpC and OmpA in this mutant.

Burns and Smith (6) found that some *Haemophilus influenzae* isolates resistant to β-lactams lack the 40,000-dalton major porin. The same workers also described chloramphenicol-resistant clinical isolates of *H. influenzae* that lacked the same protein (4). The observed increase in the MIC (more than 100-fold) cannot be explained unless chloramphenicol becomes inactivated after penetration across the OM. The loss of porin might be only a part of extensive, pleiotropic alterations (see below).

Some cefoxitin-resistant clinical isolates of *Bacteroides* were shown to lack the 49,000- to 50,000-dalton OM protein (47), but the observation is difficult to interpret since the isolates differed from type strains in other properties.

(ii) Pleiotropic mutations. If an agent is not inactivated at a significant rate, then it has a very high TAI regardless of its ability to penetrate the OM, and thus decreases in OM permeability do not cause much of an increase in the MIC. In fact, the porin-deficient mutants of *E. coli* K-12 show only marginal increases in MICs of aminoglycosides, tetracyclines, and chloramphenicol (40).

Since no inactivation mechanism for quinolones is known, one would expect little effect of porin deficiency on susceptibility to quinolones. Indeed, MICs of several quinolones for a porin-deficient mutant of *E. coli* K-12 were nearly unaltered (46). Although others (24) reported increased MICs of quinolones for an *ompF* mutant of *E. coli*, the increases were only two- to fourfold.

Mutants isolated on the basis of their resistance to quinolones obviously include the ones with altered targets, i.e., *gyrA* (*nfxA*, *norA*). They also include other classes showing cross-resistance to other agents, such as β-lactams, chloramphenicol, and trimethoprim (20, 51); and at least some of them appear to lack porin (20, 51). In some cases, the missing protein was identified as the OmpF porin (25, 28). This at first sight suggests a causal relationship between the loss of OmpF and resistance to quinolones. However, the situation is more complicated. Mutations called *norB* or *cfxB* are alleles of *marA* (25, 27), a gene that produces pleiotropic resistance to several antibiotics owing to the extrusion of these antibiotics from the cytoplasm (15). Norfloxacin is also known to be extruded from the cytoplasm of *E. coli* (10). It seems more likely, therefore, that the quinolone resistance of these mutants is caused by increased extrusion across the

cytoplasmic membrane rather than by decreased OM permeability. Some norfloxacin-resistant mutants of *P. aeruginosa* apparently overproduce an OM protein of 54,000 daltons (26). The appearance of this protein could be one manifestation of a pleiotropic alteration similar to those seen in *E. coli*.

The results described above suggest the existence of a regulatory mutation(s) that produces antibiotic resistance by causing several pleiotropic alterations, including decreased expression of the wider channel porin, OmpF, in *E. coli*. Thus, even when a decrease in porin content and increased antibiotic resistance are found as a result of a single mutation, one cannot presume the existence of a causal relationship between the two events. Indeed, such regulatory mutations are not limited to *marA*. *E. coli* strains containing mutations in *nfxB* produce reduced levels of OmpF, yet the mutation is located neither in the structural gene *ompF* nor in the classical porin regulatory genes *envZ-ompR* (28).

Some cases of multiple-drug resistance associated with porin deficiency can be most easily understood as examples of similar pleiotropic mutations. A mutation in *Salmonella paratyphi* A produced decreases in porins as well as large increases in resistance to β-lactams, aminoglycosides, quinolones, trimethoprim, and chloramphenicol (14, 18). The resistance to agents other than β-lactams is difficult to explain by porin deficiency alone, and the alteration of lipopolysaccharide size distribution in the mutant further suggests the involvement of a pleiotropic regulatory gene. Selection of nalidixic acid-resistant mutants of *Enterobacter cloacae* led to a strain which produced large amounts of some porins and which showed dramatic increases in resistance to chloramphenicol and trimethoprim as well (14). Some *E. cloacae* strains selected for β-lactam resistance are also much more resistant to chloramphenicol and tetracycline (52, 58). Most likely the mutants of both classes contain a pleiotropic mutation(s). A similar interpretation suggests itself with intrinsically carbenicillin-resistant strains of *P. aeruginosa*, for which MICs of chloramphenicol and nalidixic acid are usually increased (33).

Quinolone-resistant mutants with altered OM protein patterns have been isolated from clinical specimens. An *E. coli* isolate produced an altered DNA gyrase and was defective in the OmpF porin (1). A *P. aeruginosa* mutant emerged in a patient treated with enoxacin (45). It showed increased resistance to not only quinolones but also many β-lactams and produced only undetectable levels of protein F. On the other hand, the loss of a "31- to 32-kDa [kilodalton] protein," presumably different from protein F, was reported in *P. aeruginosa* isolates from patients receiving ciprofloxacin treatment, and these isolates did not show any cross-resistance to β-lactams (13). Two ciprofloxacin-resistant *Pseudomonas cepacia* isolates were shown to lack the porin, to produce an OM of lower permeability, and to be resistant also to ceftazidime (2).

There are antibiotic-resistant strains with unknown mechanisms. Some of these mutants may have pleiotropic alterations in various functions, similar to the cases discussed above. For example, the quinolone–β-lactam resistance mutation of *P. aeruginosa* studied by Rella and Haas (49) is difficult to explain on the basis of lowered OM permeability alone. Burns et al. (5) cloned the chloramphenicol resistance gene from transposon Tn*1696*. This gene is expressed in *E. coli* and decreases the amount of a minor 50,000-dalton OM protein, as well as the intracellular accumulation of chloramphenicol without inactivating it enzymatically. Since chloramphenicol is likely to penetrate mainly through the major porin channel in *E. coli*, the effect on this OM protein may be only a part of pleiotropic alterations of various functions.

(iii) Mutations involving specific pathways. Gram-negative bacteria often produce specific diffusion pathways for nutrients that diffuse with insufficient rates through the nonspecific porin channel. For example, maltodextrins, vitamin B_{12}, and iron-chelator complexes are all too large for the small diameters of *E. coli* porin channels, and *E. coli* produces various specific transporters for these compounds (44). Similarly, *P. aeruginosa* produces a glucose-specific channel protein, D1 (41).

The idea of "smuggling" chemotherapeutic agents through specific transport pathways is an old one. However, it has not met with many cases of success, presumably because the microbial population can easily become resistant to such agents by mutational loss of the specific transport proteins.

Some antibiotics were intentionally designed to utilize these specific pathways, for example, cephalosporins containing catechol or similar substituents for diffusion through the iron-chelator pathways (57). The expected transporter-deficient mutants are not selected by these agents, at least in *E. coli* (57), apparently because they transverse the OM by using one of the two alternative pathways, coded for by *cir* and *fiu* genes (11; H. Nikaido and E. Y. Rosenberg, manuscript in preparation). The selection enriches for *tonB* mutants, but they may not cause difficulties in the clinical setting because they are avirulent owing to their generalized defect in siderophore transport (57).

Some other antibiotics use specific pathways because they fortuitously resemble the proper ligand of the pathway. Diffusion through such specific channels makes a major contribution in organisms such as *P. aeruginosa*, which has an OM of very low general (nonspecific) permeability. Several laboratories showed that clinical isolates of *P. aeruginosa* that are moderately resistant to imipenem but not to other β-lactams lacked an OM protein of apparent molecular weight 45,000 (7, 34, 48). The β-lactamase activity and the penicillin-binding proteins were unaltered. Although one laboratory has reported that liposomes reconstituted with the mutant OM were impermeable to imipenem, my co-workers and I could not reproduce this result. In our hands, the mutant OM was shown to exhibit lower permeability specifically to imipenem only when the test was done with low concentrations (e.g., 50 μM) of substrates, with both intact cells and liposomes containing an imipenem-hydrolyzing β-lactamase of *Pseudomonas maltophilia* (55). Imipenem is probably transported by both a specific pathway and the nonspecific porin pathway; and the penetration through the former pathway becomes saturated at low imipenem concentrations (55), whereas that through the porin continues to increase nearly infinitely in proportion to the external imipenem concentration. Thus, at high, pharmacologically irrelevant drug concentrations used in the liposome swelling assay (more than 10 mM), the penetration occurs mainly through the porin; but it occurs mainly through the putative specific pathway at low concentrations of the substrate. *P. aeruginosa* OM proteins with molecular weights of about 45,000 include proteins D1 and D2. Trias and I identified, by the use of specific antibodies, the protein missing in imipenem-resistant mutants as protein D2 (J. Trias and H. Nikaido, submitted for publication). We also reconstituted the purified D2 protein into liposomes and showed that it produces a channel favoring imipenem over all other substrates tested (Trias and Nikaido, submitted). Thus, the exceptional efficacy of imipenem against *P. aeruginosa* was the result to a large extent of its utilization of a

specific diffusion channel, D2, which apparently facilitates the uptake of basic amino acids and peptides (J. Trias and H. Nikaido, manuscript in preparation).

(iv) Altered bilayer region. If a significant portion of the antibiotic molecule penetrates the bilayer, then alterations of bilayer components may produce significant resistance. The *mtr* mutants of *Neisseria gonorrhoeae*, which are resistant to various hydrophobic antibiotics and dyes, contain a new major OM protein (21), and this possibly changes the organization and the permeability of the lipid bilayer region. In *P. aeruginosa*, much of the penetration of aminoglycoside occurs through the perturbed bilayer region (41), and mutants that show significant resistance because of resistance to aminoglycoside-induced disorganization of the bilayer have been described (see reference 41).

Godfrey et al. (16) described mutants of *P. aeruginosa* that are altered in their lipopolysaccharides and are more resistant to β-lactams. This could be a pleiotropic mutation, but in this organism even slow diffusion through the bilayer might be significant because its porin pathway has an extremely low permeability.

CONCLUSIONS

The OM barrier can produce significant levels of resistance, especially when it is combined with the enzymatic inactivation barrier. Indeed, the emergence of organisms such as *P. aeruginosa* as serious infectious agents in recent years owes much to the low permeability of their OMs. Yet acquired resistance in normally susceptible bacteria usually involves enzymatic inactivation mechanisms, rather than a lowering of OM permeability. This is understandable because organisms with intrinsically low-permeability OMs must already be equipped with such compensating mechanisms as multitudes of specific diffusion pathways, but the sudden lowering of OM permeability in organisms that are not so equipped obviously creates disadvantages. Nevertheless, some mutants that are resistant owing to lowered OM permeability have emerged in recent years. Apparently, "better" antibiotics and chemotherapeutic agents that are able to overcome most other resistance mechanisms are precisely those that are likely to select out the porin mutants. Although it seems unlikely that these mutants will become a major problem, thanks to their lowered ability for nutrient uptake, some pleiotropic regulatory mutants may be better adapted for survival. Finally, there is a possibility that decreases in porin content could be caused by some R plasmids: plasmids rRM98 (29) and R124 (49a) cause the disappearance of OmpF porin in *E. coli* strains harboring them.

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