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Role of Efflux Pump(s) in Intrinsic Resistance of *Pseudomonas aeruginosa*: Resistance to Tetracycline, Chloramphenicol, and Norfloxacin

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Most strains of *Pseudomonas aeruginosa* are significantly more resistant, even in the absence of R plasmids, to many antimicrobial agents, including β-lactams, tetracycline, chloramphenicol, and fluoroquinolones, than most other gram-negative rods. This broad-range resistance has so far been assumed to be mainly due to the low permeability of the *P. aeruginosa* outer membrane. The intrinsic-resistance phenotype becomes further enhanced in "intrinsically carbenicillin-resistant" isolates, which were often assumed to produce outer membranes of even lower permeability. It has been shown, however, that this hypothesis cannot explain the β-lactam resistance of these isolates (D. M. Livermore and K. W. M. Davy, Antimicrob. Agents Chemother. 35:916–921, 1991). In this study, we examined the uptake of tetracycline, chloramphenicol, and norfloxacin by intact cells using strains showing widely different levels of intrinsic resistance. Their accumulation and the response to the addition of a proton conductor showed that even relatively susceptible strains of *P. aeruginosa* actively pump out these compounds from the cell and that the efflux activity becomes much stronger in strains showing higher levels of intrinsic resistance. We conclude that the efflux mechanism(s) are likely to contribute significantly to the intrinsic resistance of *P. aeruginosa* isolates to tetracycline, chloramphenicol, and fluoroquinolones, as does the low permeability of the outer membrane. This conclusion is supported by the observation that the hypersusceptibility to various agents of the mutant K799/61 (W. Zimmermann, Antimicrob. Agents Chemother. 18:94–100, 1980) was apparently caused by the lack of active efflux. Although the hypersusceptibility of this mutant has hitherto been assumed to be solely due to its higher outer membrane permeability, its outer membrane was shown to have a coefficient of permeability to cephaloridine that was not significantly different from that of the parent, resistant strain K799/WT. The strains with elevated intrinsic resistance overproduced two cytoplasmic membrane proteins and one outer membrane protein; at least two of these proteins appeared different from the proteins overproduced in the recently described mutant with a derepressed multidrug efflux system, MexA-MexB-OprK (K. Poole, K. Krebes, C. McNally, and S. Neshat, J. Bacteriol. 175:7363–7372, 1993).

It is well known that most strains of *Pseudomonas aeruginosa* show significant degrees of intrinsic resistance to a wide variety of antimicrobial agents, including most β-lactams, tetracyclines, chloramphenicol, and fluoroquinolones. The outer membrane of this species shows a very low nonspecific permeability to small, hydrophilic molecules (1, 46), and this has generally been thought to be the main cause of the resistance of this organism.

Nevertheless, the low outer membrane permeability cannot be the entire explanation of the intrinsic resistance. First, theoretical analysis shows that even the low-permeability outer membrane of this species should allow a sufficiently rapid equilibration of drugs across the membrane, with half-equilibration times expected to be less than 1 min for typical cephalosporins (33). The outer membrane barrier contributes strongly to the resistance of the organism usually only when the agent is degraded or inactivated following its influx (see Fig. 2 of reference 33). Yet, although tetracyclines and chloramphenicol do not appear to be modified or degraded by *P. aeruginosa* strains not containing any R plasmids, they are still significantly resistant to these agents. It is true that the β-lactams may become degraded by the chromosomally encoded β-lactamase of this organism, but the uninduced levels of this enzyme are usually extremely low, and many of the more recently developed β-lactams do not induce the production of this enzyme (19, 21). Furthermore, in some strains the mutational loss of the β-lactamase activity still leaves the mutants quite resistant to many β-lactam antibiotics (7, 39, 40). Thus, it is difficult to explain, on the basis of available data, the intrinsic antibiotic resistance of at least some *P. aeruginosa* strains.

Second, R plasmids are not the cause of resistance in the majority of carbenicillin-resistant isolates of *P. aeruginosa* in the United Kingdom. They are altered neither in their β-lactamases nor in their penicillin-binding proteins (18, 45), and this phenotype was often called intrinsic carbenicillin resistance. Since these isolates are also much more resistant to other agents, such as tetracycline, chloramphenicol, and fluoroquinolones (20), in this paper we will call this phenotype elevated intrinsic resistance. Because of the wide range of agents the isolates are resistant to, the phenotype of these strains was often ascribed to a more effective permeability barrier. Livermore and Davy (20) compared several clinical isolates of *P. aeruginosa* showing a wide range of such intrinsic resistance. When a plasmid coding for PSE-4 β-lactamase was introduced into these strains, their resistance to β-lactams increased to a uniform level, regardless of the initial level of resistance. These results are clearly inconsistent with the model (33, 36) according to which the level of resistance to

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TABLE 1. MICs for various strains

Strain	Reference or origin	MIC (μg/ml) of:							
		Carbenicillin	Azlocillin	Cefoperazone	Norfloxacin	Ciprofloxacin	Tetracycline	Chloramphenicol	Novobiocin
Ps50SAI+	20	2	1	1	0.5	0.1	8	32	32
R93	20	2	2	2	1	0.25	8	16	16
10701	20	64	4	8	1	0.5	32	128	128
M1251	20	512	64	32	4	1	64	256	1,024
PAO1		64	4	4	0.5	0.1	8	8	512
PAO4098	This study	32	8	4	0.5	0.1	8	8	256
PAO4098E	This study	1,024	32	32	2	0.8	32	128	512
K799/WT	47	64	32	8	ND	0.8	32	32	128
K799/61	47	0.04	0.01	0.01	ND	0.05	0.5	1	1

β-lactams is determined solely by synergistic interactions between the outer membrane barrier and the enzymatic inactivation in the periplasm.

One resistance mechanism that has not been considered in the above analysis, however, is that of active efflux of agents from the cell, a mechanism that is being identified in an increasing number of cases in recent years (16, 34). When we examined the accumulation of various agents by intact cells of *P. aeruginosa*, we found that even those isolates that have very low levels of intrinsic resistance pumped out tetracycline, chloramphenicol, and norfloxacin actively and that the efflux was significantly increased in isolates with elevated intrinsic resistance. These results are described in this paper. An accompanying paper describes our analysis of the molecular basis of β-lactam resistance in the same set of isolates (17).

MATERIALS AND METHODS

Antibiotics and reagents. Azlocillin, carbenicillin, cefoperazone, chloramphenicol, tetracycline, norfloxacin, ciprofloxacin, and novobiocin were purchased from Sigma Chemical Co., St. Louis, Mo. Cefsulodin was a gift from Takeda Pharmaceutical Industries. [7-^{3}H(N)]tetracycline and [ring-3,5-^{3}H]chloramphenicol were obtained from NEN/Dupont, Boston, Mass., and [piperazine-^{14}C]norfloxacin was a generous gift from Merck, Sharpe and Dohme.

Strains and their cultivation. Most of the *P. aeruginosa* strains used (Table 1) have been described earlier (20); they are clinical isolates except for PAO1 and 10701, which are laboratory strains coming from culture collections. In addition, we used a mutant of PAO1, PAO4098 (FP$^-$ *met-9020 pro-9024 blaP9208*), which produced a low, noninducible level of β-lactamase (17), kindly provided by H. Matsumoto, Shinshu University, Matsumoto, Japan. The hypersusceptible mutant strain K799/61 and its parent, K799/WT (47), were provided by W. Zimmermann, Ciba-Geigy, Basel, Switzerland.

For comparison of protein patterns, we also used two PAO1 derivatives, K372 (parent) and K385 (multidrug-resistant mutant), known to overexpress the *mexA-mexB-oprK* operon (42), as well as a single-step PAO1 mutant, OCR1 (24), which shows a multidrug-resistant phenotype similar to that of our own PAO4098E (Table 1).

The isolation of single-step mutants was carried out by spreading approximately 10^7 to 10^8 cells, without mutagenesis, on Luria-Bertani (LB) plates containing both cefsulodin (2 μg/ml) and ciprofloxacin (0.2 μg/ml). Several colonies were usually found on each plate and were tested for their drug resistance pattern, after purification by restreaking. Many of these strains showed simultaneous resistance to β-lactams as well as to tetracycline, chloramphenicol, and fluoroquinolones (see Results).

The strains were grown either in LB broth supplemented with 5 mM $MgCl_2$ (46) or in M63 medium with glucose (0.2%) as the carbon source (46), with shaking at 37°C. Harvesting and washing were both performed by centrifugation at room temperature or at 37°C, in order to avoid the autolysis often induced by the chilling of *P. aeruginosa* cells.

MICs. MICs were determined by twofold serial broth dilution, with LB broth. The inoculum was 5×10^4 cells per tube, and the results were read after an overnight incubation at 37°C.

Assay of tetracycline accumulation in intact cells. Cells in the mid-exponential phase of growth were harvested by centrifugation when the culture density reached 0.12 to 0.2 mg (dry weight) ml^{-1}. They were washed once in a solution (prewarmed to 37°C) containing 50 mM potassium phosphate (pH 7.0), 1 mM $MgSO_4$, and 0.2% glucose and were resuspended in the same buffer solution at a density of 1 mg of protein ml^{-1}. The suspension was kept at 37°C with aeration by shaking, and the assay was started by the addition of radiolabeled drug. At appropriate times, a proton conductor, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), was added to portions of the suspensions. At various time points, 0.05 ml of the suspension was removed and diluted into 1.5 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 0.1 M LiCl (wash buffer), and the mixture was immediately filtered through a Gelman Metricel GN-6 membrane filter (0.45-μm pore size). The filter was washed with 5 ml of wash buffer, and the radioactivity retained on the filter was quantitated with a Beckman LS7000 liquid scintillation spectrometer.

Assay of chloramphenicol and norfloxacin accumulation. Preliminary studies of the accumulation of chloramphenicol and norfloxacin by the filtration procedure described above produced poorly reproducible results, presumably because a significant portion of these lipophilic compounds has leaked out from the cells during the filtration and washing. We therefore used centrifugation through a layer of silicone oil (6, 12) in order to remove the external fluid from the cells.

The cells were grown, washed, resuspended, and incubated with the radiolabeled drug as described above. Samples (0.05 ml) were removed at indicated times and placed on top of a mixture of silicone oils (0.15 ml; a 3:7 [vol/vol] mixture of Dow Corning silicone oils 510 and 550) placed in a narrow plastic centrifuge tube (0.4 ml, long style; USA/Scientific Plastics, Ocala, Fla.), and the tubes were immediately centrifuged in an Eppendorf Microfuge. The tubes were frozen in a dry ice-ethanol bath, the bottom portion of the plastic centrifuge tubes containing the cell pellet was cut off, and the radioactivity in it

was determined by liquid scintillation counting as described above.

Assay of tetracycline accumulation in inverted membrane vesicles. The tetracycline accumulation assay was carried out essentially as described by McMurry et al. (27) with the following modifications. The vesicles were used immediately without freezing. In order to improve the recovery of vesicles during the filtration step, poly-L-lysine was added to a final concentration of 0.4 mg/ml just before the filtration.

Determination of outer membrane permeability to cephaloridine. Outer membrane permeability to cephaloridine was determined by using strains to which a plasmid coding for a PSE4 β-lactamase was transferred as described elsewhere (20). The Zimmermann-Rosselet procedure (48), slightly modified (46), was used with 0.5 mM (external concentration) cephaloridine. Although we believe that some strains actively pump out β-lactams (see reference 17), this should not interfere much with this assay, because the active efflux should be negligible in comparison with the rapid influx of cephaloridine from such a high external concentration.

Analysis of membrane protein pattern. Outer and cytoplasmic membranes were prepared and their protein composition was analyzed by sodium dodecyl sulfate (SDS) slab polyacrylamide gel electrophoresis, as described elsewhere (24).

RESULTS

Description of the strains used. Livermore and Davy (20) showed that all of the strains used in that study (and also here), that is, Ps50SAI+ through M1251 in Table 1, produced only the chromosomally encoded class C β-lactamase characteristic of *P. aeruginosa* in an inducible manner and that the strains showed identical profiles of penicillin-binding proteins. Thus, the β-lactam resistance (and also the cross-resistance to other agents) of strains such as M1251 can be considered as an example of elevated intrinsic resistance. During this study, strain M1426 (20) was found to produce a β-lactamase somewhat different in its substrate specificity from the typical *P. aeruginosa* enzyme. For this reason, this strain was excluded from the comparison.

We also studied a single-step multidrug-resistant mutant derived from laboratory strain PAO4098. This strain is described below.

Isolation of multidrug-resistant mutants from PAO4098. Because Ps50SAI+, M1251, and other strains used in earlier (20) and present studies were clinical isolates and nonisogenic, we isolated a mutant with a similar phenotype from a PAO1 derivative, PAO4098. This particular strain was chosen because we initially believed it to be essentially deficient in β-lactamase activity, a feature that would be useful in the study of accumulation of β-lactam antibiotics. (However, later study showed the strain to produce a basal, noninducible level of the enzyme [17].) A single-step selection without prior mutagenesis, as described in Materials and Methods, produced several intrinsically resistant mutants, with elevated MICs of β-lactams as well as tetracycline, chloramphenicol, and fluoroquinolones (norfloxacin and ciprofloxacin). One of these single-step mutants, PAO4098E, was used for further study. Table 1 shows that in clinical isolates with elevated intrinsic resistance, as well as PAO4098E, MICs of all of the agents listed above, and also of novobiocin, were increased. Among these strains, there was little difference in levels of resistance to erythromycin and aminoglycosides (not shown). A hypersusceptible mutant, K799/61 (47), showed much lower MICs of all agents listed in Table 1.

Evidence for the efflux of tetracycline. We first examined the accumulation of tetracycline by intact cells of various strains. In wild-type strains of *Escherichia coli*, tetracycline is accumulated passively in the cytoplasm because it easily crosses the lipid bilayer regions of the cytoplasmic membrane and its final distribution is determined by the pH gradient across the membrane (37). When a proton conductor such as CCCP is added, the pH gradient is destroyed and the cytoplasmic level becomes significantly decreased (see Fig. 1A of reference 16). We could obtain similar results by using the hypersusceptible mutant strain K799/61 (Fig. 1). The cells were grown in M63 medium with glucose as the carbon source, harvested, washed, and incubated with tetracycline as described in Materials and Methods. At the indicated times, samples were withdrawn, and the amount of accumulated tetracycline was determined. After 15 min, the proton conductor CCCP was added to 250 μM to one-half of the incubation mixture. (Use of different CCCP concentrations showed that complete inhibition of the efflux system required at least 250 μM with most strains [results not shown].) The addition of CCCP lowered the level of accumulation of tetracycline (Fig. 1), as was found with the susceptible *E. coli* cells.

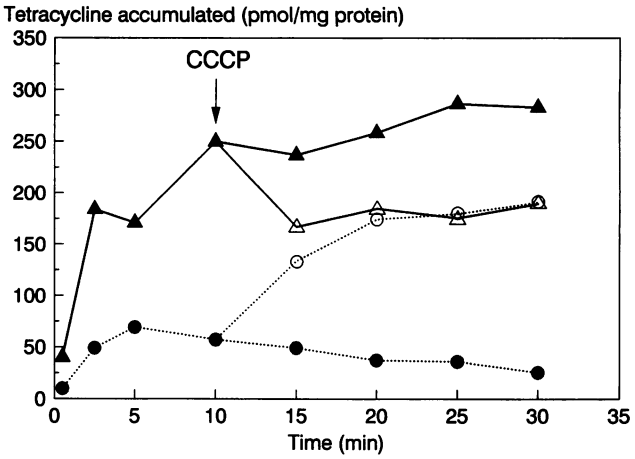

FIG. 1. Accumulation of [^{3}H]tetracycline in a hypersusceptible strain, K799/61 (▲, △), and its parent, K799/WT (●, ○). The cells were grown in M63 medium with glucose as the carbon source, harvested, washed, and resuspended in a phosphate buffer containing glucose as the energy source, as described in Materials and Methods. After 5 min at 37°C, [^{3}H]tetracycline (specific radioactivity, 0.55 Ci/mmol) was added to 5 μM, and samples were taken, filtered, and counted. At 10 min, 0.25 mM CCCP was added to one-half of each reaction mixture. Open symbols show the data from CCCP-treated samples, and closed symbols show data from those that did not receive CCCP addition. The data shown are the averages of four separate experiments.

The estimated total cellular concentration of tetracycline before deenergization was around 42 μM, if we assume that proteins make up about 50% of the cell dry weight (29) and the cell volume is around 3 μl/mg (dry weight) (28). However, a substantial portion of the drug is probably bound to ribosomes. If we assume that there are 20,000 ribosomes per cell (29) and that 1 mg (dry weight) contains 10^9 cells (29), then about 30% of the accumulated tetracycline is likely to be bound to the ribosomes. Even so, much of the accumulation of tetracycline probably represents its passive distribution along the pH gradient across the inner membrane as mentioned above. Tetracycline contains a weak acid group with a macroscopic pK_a of 7.7 (37). It most probably crosses the cytoplasmic membrane bilayer as the uncharged species (37), and equili-

bration across this membrane to equalize the concentration of this species produces a higher total concentration in the cytoplasm, as more tetracycline would exist in the dissociated form with a net negative charge in the higher pH environment of the cytoplasm (37). The addition of a proton conductor such as CCCP abolishes ΔpH and this accumulation in the cytoplasm as well (Fig. 1). The post-CCCP accumulation level corresponds to 29 μM, or about 15 μM after correction for the binding to the ribosomes. Although this level is higher than the external level of tetracycline, 5 μM, the apparent accumulation may in part be due to the concentration of tetracycline in the periplasm in response to the Donnan potential across the outer membrane (37) and the consequent elevation of the cytoplasmic concentration, which is in equilibrium with the periplasmic tetracycline.

The hypersusceptible mutant therefore did not pump out much tetracycline, and much of the high pre-CCCP level of accumulation of tetracycline observed was presumably the result of its expected passive distribution dictated by the pH gradient across the cytoplasmic membrane. When the pH gradient was abolished by the addition of CCCP, the tetracycline level decreased as expected.

The parent strain K799/WT, however, produced unexpected results (Fig. 1). Thus, the initial steady-state level of accumulation was much lower than that observed with K799/61. This suggested strongly that tetracycline was being actively pumped out of the cytoplasm of this wild-type strain. This was further supported by the observation that the addition of CCCP increased, rather than decreased, the accumulation level, because in the presence of active efflux, the major effect of the proton conductor is to inhibit the active efflux (see Fig. 1A of reference 16).

It may be argued that the accumulation was lower in K799/WT because its outer membrane permeability was lower. However, if this were the case, it would have taken longer to reach the steady state in K799/WT but the steady-state level of accumulation of tetracycline would have been the same as in K799/61. In fact, the accumulation reached a steady state in K799/WT within 5 min, and the final steady-state level was only one-fifth of the level found in K799/61. Thus, clearly, the difference in outer membrane permeability alone cannot explain these data. Although it has been reported that K799/61 has a much higher outer membrane permeability to nitrocefin than K799/WT (1, 31), reexamination by using 0.5 mM cephaloridine as described in Materials and Methods showed that there is essentially no difference in permeability between these two strains, with permeability coefficients (average of five experiments ± standard deviation) of 54 ± 19 and 71 ± 26 nm/s for K799/WT and K799/61, respectively. K799/61 has so far been thought to be hypersusceptible to a number of agents mainly because of its higher outer membrane permeability (1, 31), but in fact the main cause of its hypersusceptibility is most probably the defect(s) in active efflux, a hitherto unsuspected phenotype of this strain.

The accumulation of [^{3}H]tetracycline was monitored also in clinical isolates (Fig. 2), in a highly susceptible strain, Ps50SAI+, and in a highly resistant strain, M1251 (Table 1). Again, the level of tetracycline quickly reached a steady state in the cells of both strains. With Ps50SAI+, a small increase in the accumulation level occurred following the addition of CCCP (Fig. 2), in contrast to the apparently efflux-deficient K799/61, which showed a significant decrease upon addition of CCCP (Fig. 1). The simplest explanation of these data is that there is a significant level of active efflux even in Ps50SAI+, a very susceptible example among clinical isolates, so that CCCP affects the accumulation level more through the inhibition of efflux than through its effect on the passive distribution of tetracycline based on transmembrane ΔpH. The pre-CCCP steady-state level of accumulation in M1251, the resistant strain, was only about one-half of the level in Ps50SAI+, presumably because the efflux system was more active (Fig. 2). (This difference was consistently observed when the experiment was repeated several times.)

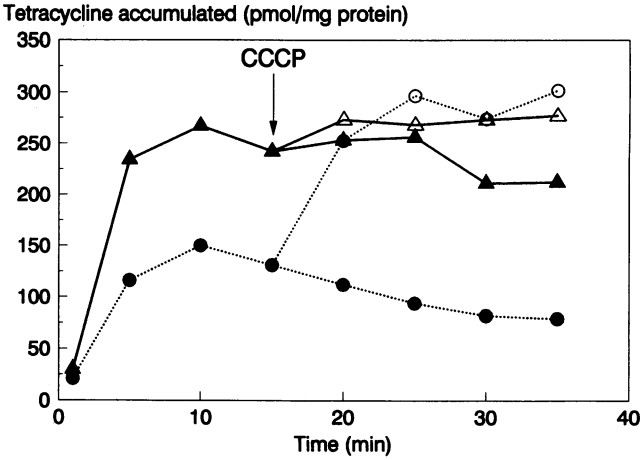

FIG. 2. Accumulation of [^{3}H]tetracycline by intact cells of Ps50SAI+ (▲, △) and M1251 (●, ○). The cells were grown in M63 medium with glucose as the carbon source, harvested, washed, and resuspended in a phosphate buffer containing glucose as the energy source, as described in Materials and Methods. After 5 min at 37°C, [^{3}H]tetracycline (specific radioactivity, 0.55 Ci/mmol) was added to 5 μM, and samples were taken, filtered, and counted. To one-half of the reaction mixture, CCCP was added at 15 min to a final concentration of 250 μM. Accumulation in CCCP-treated samples is shown with open symbols, and that in samples not receiving CCCP is shown with closed symbols. The data shown are the averages of two separate experiments.

The interpretation that the level of accumulation in M1251 was lower because of its lower outer membrane permeability can be rejected, because this will create slower attainment of the steady-state level but will not lower the final steady-state level, as argued earlier for K799/WT. Furthermore, the measurement of the actual rates of diffusion of tetracycline into CCCP-poisoned cells revealed no difference between Ps50SAI+ and M1251 (Fig. 3). Another possible explanation may be that tetracycline is accumulated actively in the cytoplasm by a specific transporter and that the activities of this transporter differ between the strains used. However, this is highly unlikely as there is no convincing evidence for such a specific uptake system for tetracyclines, despite extensive searches in the past (37).

After the addition of CCCP, the accumulation in M1251 was strongly increased, again presumably because of the inhibition of the efflux system. The final level of accumulation was similar to that in the susceptible strain Ps50SAI+ (Fig. 2), confirming that the strains behave similarly once the efflux system becomes inactive because of the deprivation of energy.

Less extensive studies were performed with other strains examined by Livermore and Davy (20). These strains generally showed intermediate levels of accumulation of tetracycline, as expected from their intermediate levels of resistance to tetracycline and other drugs (results not shown).

The isogenic pair PAO4098 and PAO4098E was also examined in a similar way (Fig. 4). The parent strain of PAO4098, PAO1, was previously shown to have higher levels of resistance

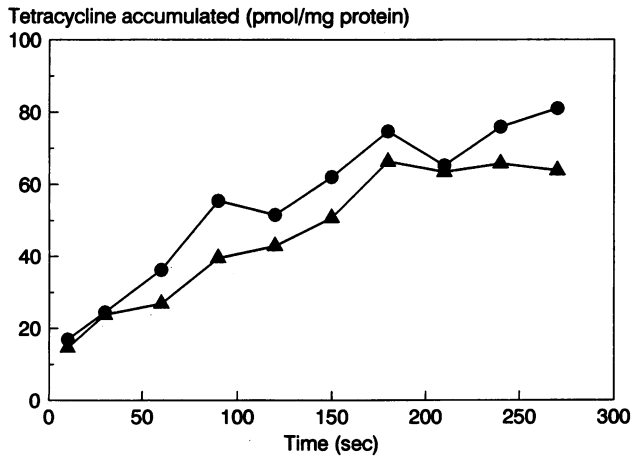

FIG. 3. Influx of [^{3}H]tetracycline into CCCP-poisoned cells of Ps50SAI+ (▲) and M1251 (●). CCCP was added to the cell suspensions to a final concentration of 150 μM, and 5 min later [^{3}H]tetracycline was added as in the experiment described in the legend to Fig. 1.

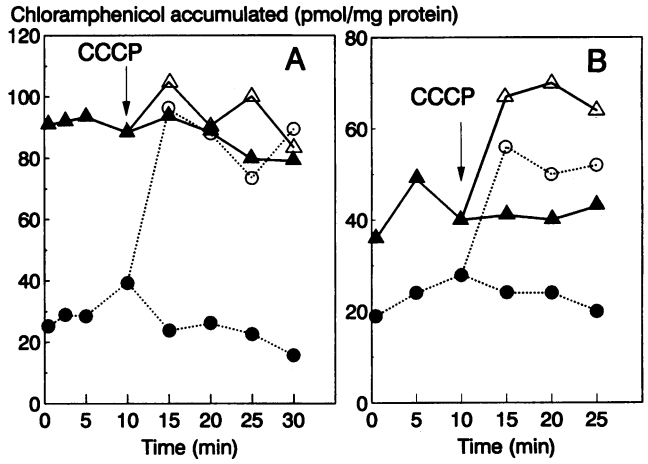

FIG. 5. Accumulation of [^{3}H]chloramphenicol in intact cells of K799/WT (●, ○) and its hypersusceptible mutant K799/61 (▲, △) (A) and in intact cells of Ps50SAI+ (▲, △) and M1251 (●, ○) (B). Cells were grown in M63 medium, harvested, and resuspended in phosphate buffer-$MgSO_4$-glucose as described in Materials and Methods. [^{3}H]chloramphenicol was diluted with the nonradioactive compound to a specific activity of 0.5 Ci/mmol and was added to the final concentration of 5 μM. At 10 min, CCCP was added to one-half of the mixture to a final concentration of 250 (A) or 500 μM (B); accumulations in these samples are shown with open symbols. The entry of the drug into the cells was assayed by centrifugation through silicone oil, as described in the text. The results shown are averages of four experiments.

to β-lactams, quinolones, tetracycline, and chloramphenicol than Ps50SAI+ (20) (see also Table 1). Consistent with this, the pre-CCCP steady-state level of tetracycline accumulation in PAO4098 was much lower than that in Ps50SAI+. (The accumulation level, however, was even lower than that observed with the more resistant strain, M1251, in Fig. 2. This unexpected result is apparently due to the use of LB broth for the growth of PAO strains, which could not be grown in defined media because of the presence of undocumented nutritional requirements. We have observed with several strains that growth in LB broth tends to lower the level of tetracycline accumulation [data not shown].) With the multidrug-resistant mutant, PAO4098E, the steady-state level of accumulation was even lower (Fig. 4), indicating an even higher level of efflux. After the addition of CCCP, both strains achieved the same levels of accumulation, as predicted under the condition of a nearly complete inhibition of the efflux activity (Fig. 4).

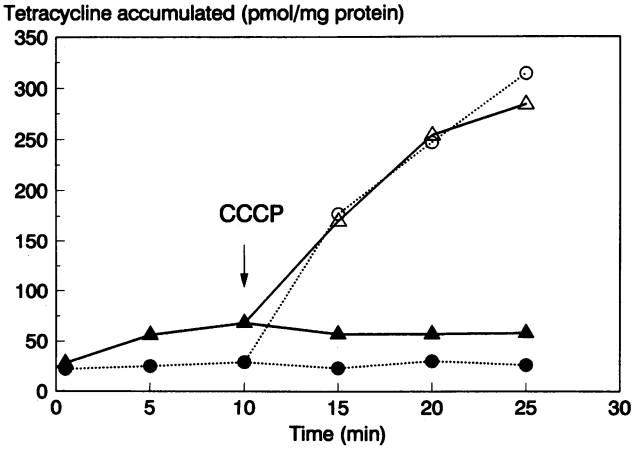

FIG. 4. Accumulation of [^{3}H]tetracycline in intact cells of PAO4098 (▲, △) and PAO4098E (●, ○). The experiment was performed similarly to that described in the legend to Fig. 1, except that the cells were grown in LB. The open symbols represent accumulation in samples that received 500 μM CCCP at 10 min. The results shown are the averages of four separate experiments.

Our conclusion that tetracycline is pumped out from the cytoplasm even of susceptible strains is based on the increase of tetracycline uptake upon the addition of CCCP (Fig. 1, 2, and 4). It may be argued that CCCP might somehow increase the permeability of the outer membrane and in this way increase the level of accumulation of tetracycline. However, such an increase in outer membrane permeability is highly unlikely, as the addition of CCCP actually decreases the accumulation level significantly with K799/61 (Fig. 1). Furthermore, we measured directly the influx rate of cephaloridine across the outer membrane, as described in Materials and Methods. The presence of up to 400 μM CCCP was found to have no detectable influence on the outer membrane permeability of both strains Ps50SAI+ and M1251 to cephaloridine (results not shown).

Accumulation of tetracycline in inverted membrane vesicles. Another way of showing the occurrence of the efflux mechanism is to demonstrate the accumulation of drugs inside everted membrane vesicles (16). When this was attempted with everted vesicles prepared from PAO4098 and PAO4098E with [^{3}H]tetracycline, differences were seen especially during the earlier time points. In one experiment, for example, vesicles from PAO4098 accumulated 400 cpm of tetracycline in 1 min inside the vesicles, energized with ascorbate and phenazine methosulfate. In contrast, a similar quantity of vesicles from the presumed high-efflux mutant PAO4098E accumulated 1,200 cpm in 1 min. These results are encouraging, but the experimental parameters need to be examined more carefully before firm conclusions can be drawn.

Efflux of chloramphenicol. K799/WT and K799/61 were also compared in terms of the accumulation of chloramphenicol (Fig. 5A). In contrast to that of tetracycline, the chloramphen-

icol level in the cell was already very close to the steady-state level in the earliest samples examined (0.5 min), a result showing that chloramphenicol diffuses through the bacterial membranes much more rapidly than tetracycline, as predicted from its much higher solvent-water partition coefficient (32). Free chloramphenicol, an uncharged molecule, should distribute equally across the bacterial membranes in the absence of any active efflux. The addition of CCCP thus should affect only the active efflux, and the passive distribution of the drug should not be affected by the alteration in pH gradient. The level of accumulation in the mutant K799/61 showed little change after the addition of CCCP, and this indicates that in this strain there was little active efflux of the drug. The steady-state accumulation level was much lower in the wild-type strain K799/WT, and the inhibition of the presumed efflux mechanism by CCCP increased the level to that seen in K799/61. Thus, even in the wild-type strain there was a strong active efflux of chloramphenicol.

The level of accumulation in K799/61, calculated in a way similar to that used for tetracycline, was about 15 μM. If corrected for ribosome binding, this gives a free chloramphenicol concentration of 5 μM, identical to the concentration in the extracellular medium. This confirms that there was no accumulation or active efflux of chloramphenicol in K799/61. The intracellular concentration in K799/WT was clearly much lower than this level but increased, upon the addition of CCCP, precisely to the value expected for passive equilibration across the membranes.

These conclusions were further supported by the comparison of Ps50SAI+ and M1251 (Fig. 5B), two clinical isolates differing greatly in their levels of intrinsic resistance. The steady-state level of accumulation in the more susceptible strain, Ps50SAI+, was lower than in the mutant K799/61, showing that even in this unusually susceptible isolate there is a significant active efflux of chloramphenicol. Indeed, CCCP addition produced a significant increase in the level of accumulation in this strain. In the isolate with elevated levels of intrinsic resistance, M1251, the efflux activity was clearly much stronger, as indicated by the lower pre-CCCP level of accumulation.

Comparison between PAO4098 and 4098E also produced similar results (not shown), the more resistant mutant 4098E showing a lower steady-state level of accumulation and the accumulation in both strains increasing to similar, high levels after the addition of CCCP.

Efflux of norfloxacin. The accumulation of norfloxacin, a fluoroquinolone, was studied by using a ^{14}C-labeled compound (Fig. 6). As seen, the resistant strain M1251 showed a much lower steady-state accumulation level than the susceptible isolate, Ps50SAI+. The accumulation level showed a dramatic increase upon the addition of CCCP in the former strain. Since the proton gradient normally present across the cytoplasmic membrane is expected to lower the cytoplasmic concentration of norfloxacin (37), the collapse of the proton gradient by CCCP by itself will increase the accumulation of this drug. However, the magnitude of increase observed was larger than expected on that basis alone (about 50% at the external pH of 6.5 and even less at pH 7.0 [37]), and the different levels of pre-CCCP accumulation in M1251 and Ps50SAI+ also strongly argue that norfloxacin was being pumped out more rapidly from the cytoplasm of M1251 before the addition of the uncoupler. (The intracellular concentration of norfloxacin was not compared with the extracellular concentration, because fluoroquinolones are known [3] to become bound nonspecifically to a number of cellular constituents.)

Detection of overproduced membrane proteins. If efflux is the mechanism responsible for drug resistance in some of these strains, the more resistant strains are expected to contain higher levels of efflux transporter proteins. Because some of the drug efflux systems in gram-negative bacteria are suspected to include outer membrane components (34), we also analyzed the outer membrane proteins. We included in the comparison *P. aeruginosa* PAO derivatives K372 (parent) and K385 (mutant), the latter known to be derepressed for the three proteins of the recently identified *mexA-mexB-oprK* efflux system (42), as well as another PAO1 derivative, OCR1 (24), which shows a multidrug resistance pattern similar to that of our strains and was shown to overproduce an outer membrane protein, OprM.

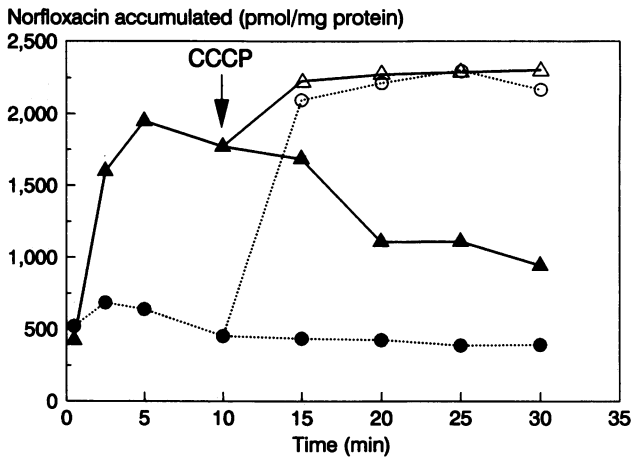

FIG. 6. Accumulation of [^{14}C]norfloxacin by intact cells. Ps50SAI+, a susceptible strain (▲, △), and M1251, a resistant strain (●, ○), were grown in M63-glucose, and the washed cells were incubated with 40 μM [^{14}C]norfloxacin (specific activity, 14.9 mCi/mmol) as described in Materials and Methods. At 10 min, 0.5 mM CCCP was added to one-half of each incubation mixture, and the accumulation in these samples is shown with open symbols. The data shown are the averages of four separate experiments. There was a rapid decrease in the accumulation levels in untreated samples of Ps50SAI+. Possibly this was caused by some energy-dependent cell damages caused by norfloxacin, which was present at a concentration more than 20 times higher than the MIC for Ps50SAI+.

The gel pattern showed that the outer membrane of both M1251 and PAO4098E contained higher levels of protein(s) with mobilities expected for proteins of about 50 kDa (Fig. 7A). However, the mobility of the protein found in PAO4098E was clearly different from that found in K385 (OprK) and was indistinguishable from that of the OprM protein in strain OCR1; the different mobilities most certainly indicate different proteins, as these strains are both derivatives of PAO1. The comparison between Ps50SAI+ and M1251 is not so simple, because these are clinical isolates. However, M1251 appears to overproduce two outer membrane proteins of this molecular-weight range, which correspond in mobility to OprM and OprK (Fig. 7A). (The protein band that is slightly slower in mobility than OprK and is absent in M1251 is apparently flagellin, because its intensity is affected greatly by the method of preparation of the membrane, it is not soluble in nonionic detergents, and all isolates lacking this band are immobile.)

When the cytoplasmic membrane proteins were analyzed by SDS-polyacrylamide gel electrophoresis without treating the samples at 100°C, the more resistant strains were seen to overproduce a protein band with the apparent mobility of around 100 kDa, which presumably corresponds to MexB (42) or its homolog (not shown). In addition, overproduction of the

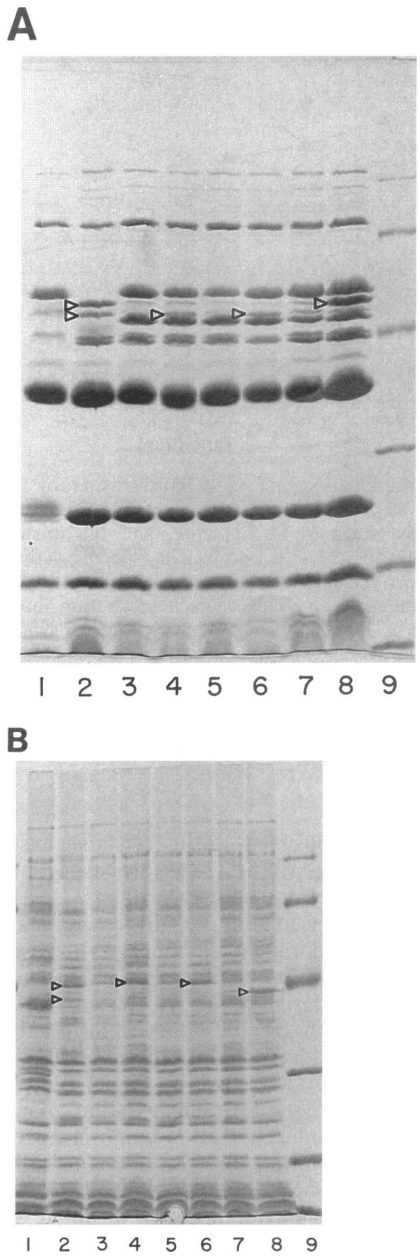

FIG. 7. Outer membrane (A) and cytoplasmic membrane (B) proteins of various strains. Membranes were prepared and analyzed by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. The proteins were stained with Coomassie blue. Arrowheads show overproduced proteins in resistant strains. Lanes: 1, Ps50SAI+; 2, M1251 (resistant); 3, PAO4098; 4, PAO4098E (resistant); 5, PAO1; 6, OCR1 (resistant); 7, K372; 8, K385 (resistant); and 9, molecular mass standards. A major protein band absent in M1251 (just above the arrowheads) is flagellin (see the text). There are several more differences between M1251 and Ps50SAI+, as they are clinical isolates of totally different origins. The 43-kDa band in the M1251 cytoplasmic membrane (lower arrow) appears to be somewhat overproduced, but this is uncertain. The molecular mass standards correspond to 97, 66, 45, 31, 21.5, and 14.5 kDa.

smaller cytoplasmic membrane proteins of 43 to 46 kDa was apparent when samples solubilized at 100°C were analyzed (Fig. 7B). Again, the protein overproduced by PAO4098E was clearly different from the MexA protein found in K385 (42) but was indistinguishable from the overproduced protein seen in the mutant OCR1 of Masuda and Ohya (24). M1251 appeared to overproduce a protein similar to the one found in OCR1 and possibly also the faster-migrating protein, but the identification is made uncertain because the mobility of various proteins is expected to be somewhat different from that in the PAO line, in this clinical isolate with a totally different genetic constitution.

DISCUSSION

Intrinsic multidrug resistance in *P. aeruginosa*. As presented in the introduction, the molecular mechanism that produces intrinsic resistance in *P. aeruginosa* has remained an enigma. Clearly the low permeability of the outer membrane contributes to the resistance. Yet even with the low permeability coefficients measured (46), one can predict that many agents would reach half-equilibration across the outer membrane in less than a minute (33), a very short period in comparison with the doubling time of the organism. Thus, in addition to the low permeability, removal of the incoming antibiotic molecules via mechanisms such as degradation or modification is needed in order to produce a significant level of resistance (33). But this second contributor to the resistance has not been identified. Tetracycline, chloramphenicol, or fluoroquinolones have not been reported to be inactivated or modified by wild-type *P. aeruginosa* strains, and the inactivation process is expected to be insignificant even for some β-lactams, as described in the introduction.

Clinical isolates of *P. aeruginosa* vary extensively in levels of intrinsic resistance (20, 45). In the so-called intrinsically carbenicillin-resistant strains, the MICs of tetracycline, chloramphenicol, fluoroquinolones, and also of many β-lactams may reach very high levels (18, 20). For example, the MIC of carbenicillin in M1251 is more than 200 times higher than that of the susceptible isolate, Ps50SAI+ (Table 1). In this study, we used this variation as a tool to study the mechanism of intrinsic resistance.

A major finding in this study was that wild-type strains of *P. aeruginosa*, including the laboratory strains of the PAO line, catalyze an efficient active efflux of tetracycline, chloramphenicol, and norfloxacin and, even more importantly, that the higher efflux activity was seen in the more resistant clinical isolates as well as a single-step laboratory mutant showing the multiple-resistance phenotype. These observations certainly identify the efflux pump as the hitherto missing factor that makes a major contribution to the general resistance of *P. aeruginosa*.

Up to now, the low outer membrane permeability of this species has been thought to be the most important factor in making it resistant to a number of agents including those studied in this paper. In order to assess, more quantitatively, the significance of the permeability barrier, we solved, by numerical integration, a differential equation of the type used by Frère et al. (8), which describes the entry by diffusion of the drug and the dilution of the intracellular drug concentration by exponential growth. With the permeability coefficient experimentally determined for tetracycline (Fig. 3), we could show that even a very rapid growth rate (generation time, 20 min) decreases the intracellular drug concentration by less than 0.2% from that expected for thermodynamic equilibration (result not shown). Thus, the outer membrane barrier by itself cannot produce significant resistance. Since agents such as tetracycline, chloramphenicol, and fluoroquinolones are not known to be inactivated by *P. aeruginosa*, it is clear that the efflux pump is the hitherto missing factor that makes a major

TABLE 2. Drug-resistant mutants of *P. aeruginosa* overproducing 50-kDa outer membrane proteins reported in the literature

Locus	Reference	Map location	Susceptibility to[a]:					
			Quinolones	β-lactams	Tetracycline	Chloramphenicol	Novobiocin	Aminoglycosides
nalB[b]	43	30′	R	R	NC	?	R	NC
nalB(cfxB)[b]	44	30′	R	R	R	R	R	?
(oprM)	24	30′	R	R	R	R	?	NC
nfxB	13	4–8′	R	S	NC	NC	R	S
nfxC	9	46′	R	S	NC	R	?	S
Unknown	11	?	R	NC	?	NC	NC	NC
Unknown	15	?	R	R	?	?	?	R

[a] Changes in susceptibility to drugs are shown as follows: R, the mutant becomes less susceptible to the drug; S, the mutant becomes more susceptible to the drug; NC, no change in susceptibility to the drug; ?, not reported.

[b] The production of 50-kDa proteins in strains carrying these mutant genes was shown in reference 2.

contribution to the general resistance of this organism. This does not mean, however, that the outer membrane barrier is not important. The slow influx through less permeable outer membranes is predicted to act synergistically with an active efflux (34), and both factors are most probably essential in the intrinsic resistance of *P. aeruginosa*.

Active efflux is already well-known as a major mechanism of antibiotic resistance (16). Furthermore, the presence of active efflux was noted even in the wild-type strains of *E. coli*. Thus, the efflux of tetracyclines was found to occur if the cells were grown in LB broth (25) or if a more favored substrate, such as minocycline, was used (16). Very recently, an active efflux of chloramphenicol was documented in *E. coli* (26). Cohen et al. (5) showed convincingly that *E. coli* has an intrinsic, saturable efflux mechanism for fluoroquinolones. (Although it has been concluded in several laboratories that wild-type strains of susceptible organisms pump out fluoroquinolones, on the basis simply of the uncoupler-induced increase in accumulation, some of these conclusions may not be valid since cytoplasmic concentrations of most fluoroquinolones are kept at low levels because of their passive distributions across the cytoplasmic membrane in accordance with the pH gradient [37; see also reference 10].) Thus, the finding of active efflux processes for several drugs in the wild-type *P. aeruginosa* is not surprising. What is worth stressing, however, is that the active efflux processes, probably in synergy with the low permeability of its outer membrane barrier, can produce high levels of intrinsic resistance in wild-type *P. aeruginosa*. In contrast, *E. coli*, with its higher-permeability outer membrane, remains much more susceptible to these agents in spite of the presence of the documented efflux pumps. (We cannot, however, exclude the alternative possibility that, in *E. coli*, the efflux pumps do not produce significant resistance because their activities are weaker.)

There may be some concern that the observed differences in accumulation levels are smaller than the differences in MICs in most cases. However, this is the case even in the well-established example of efflux-based tetracycline resistance: a 245-fold increase in MIC caused by the presence of Tet protein decreased the measured accumulation of tetracycline only by a factor of 7 (27). It is therefore not surprising that Ps50SAI+ and M1251, differing only eightfold in their MICs of tetracycline (Table 1), show accumulation levels that are different by a factor of about 2 (Fig. 2). This discrepancy between the MICs and accumulation levels presumably arises from the fact that we measure the sum of accumulation in all cellular compartments, only some of which are relevant to the action of the drug and are affected by the efflux process.

The identity of the efflux pump. There are two questions for which the uptake studies alone could not provide ready answers. First, the drug efflux pump is usually located in the cytoplasmic membrane, as exemplified by the Tet protein (16). If the drug molecules, pumped out by these proteins, find themselves in the periplasmic space, the pump will be less efficient in creating resistance because it will increase the periplasmic concentration of the drug, which will then flow back into the cytoplasm. It is likely that some efflux pumps functionally bypass the outer membrane barrier (6), but the uptake data by themselves gave no clue to the molecular mechanisms of this process. Second, a single-step mutation produced decreased susceptibility not only to a wide variety of β-lactams but also to tetracycline, chloramphenicol, and fluoroquinolones (Table 1, PAO4098E). The accumulation experiments could not show whether this multiple resistance was due to the activation or induction of a single efflux system with a wide substrate specificity (34) (for example, similar to the *Bacillus subtilis* Bmr transporter, presumably pumping out ethidium bromide, chloramphenicol, and puromycin [30], to the EmrB transporter of *E. coli*, which produces resistance to CCCP, nalidixic acid, and phenylmercury acetate [22], or to the AcrE protein of *E. coli*, which seems to pump out an even wider variety of substrates [23]) or to a regulatory change (similar to Mar [4]) possibly resulting in the activation or induction of several different efflux proteins, each with a narrower specificity.

After our accumulation studies had been completed, a paper that suggests answers to these questions appeared. Thus, Poole et al. (42) showed that a probable operon, *mexA-mexB-oprK* of *P. aeruginosa*, is likely to code for a periplasmic lipoprotein, an inner membrane efflux pump, and a 50-kDa outer membrane protein. These three proteins can conceivably form a complex, allowing the drugs to be pumped out of the cytoplasm into the external medium through a direct channel, without going through the normal periplasm-porin pathway (see Fig. 8 of reference 17). Furthermore, disruption of any of these genes makes *P. aeruginosa* extremely susceptible to tetracycline, ciprofloxacin, and chloramphenicol (42), suggesting that this presumed efflux complex may have an extraordinarily wide substrate specificity.

Interestingly, there are reports of several types of multidrug-resistant mutations in *P. aeruginosa* that result in the overproduction of an outer membrane protein of approximately 50 kDa (Table 2). The mechanism of resistance in these mutants has remained unknown. Although the resistance was sometimes thought to be due to decreased outer membrane permeability, we do not know of any mutation for which this assumption has been proven to be true. In view of our present data and of the discovery of the overproduced 50-kDa protein

OprK by Poole et al. (42), it seems likely that resistance is caused by active multidrug efflux in most of the cases listed in Table 2. The differences in the resistance phenotypes and in the locations of mutations, then, suggest that there may be more than one endogenous multidrug efflux pump in *P. aeruginosa*.

In order to find out whether the multiple-resistance phenotype of our single-step mutant, PAO4098E, and of clinical isolates is related to those that have been studied earlier, we analyzed the membrane protein patterns in various strains (Fig. 7). The results suggest the following. (i) There is indeed evidence for the overexpression of at least a system containing one approximately 50-kDa outer membrane protein and two cytoplasmic membrane proteins of about 46 and 100 kDa in our resistant strains. This suggests strongly that the increased efflux is carried out by a three-protein complex, similar to the MexA-MexB-OprK system (42; for a review, see reference 34). (ii) At least two of the proteins overproduced by PAO4098E are different in their sizes from MexA and OprK. We name, tentatively, the overproduced proteins in PAO4098E as MexC (a 46-kDa protein in the cytoplasmic membrane), MexD (an approximately 100-kDa protein in the cytoplasmic membrane), and OprM (the "50 kDa protein" identified by Masuda and Ohya [24]). (iii) Since only one set of membrane proteins was overproduced in PAO4098E, the simplest hypothesis is that this single-efflux system, composed of three subunits, carries out the efflux of all the substrates studied in this work. Of course, we cannot exclude the possibility that other efflux proteins were overproduced but escaped detection or that some efflux systems were functionally activated without increased transcription. (iv) Finally, the electrophoretic pattern of proteins from M1251 suggests that this strain may overproduce, in addition to MexC-MexD-OprM, another efflux system possibly corresponding to MexA-MexB-OprK. The identities of the overproduced proteins, however, are uncertain because proteins from clinical isolates often show altered mobilities in comparison with those from PAO derivatives.

Hypersusceptibility of mutant K799/61. K799/61 was isolated by Zimmermann (47) after several successive steps of mutagenesis. Thus, undoubtedly, several mutations contribute to the hypersusceptibility of this strain. However, since the appearance of reports that this mutant has a much higher outer membrane permeability than the parent strain (1, 31), it has been generally accepted that this increased permeability was the major cause of the hypersusceptibility of this strain to a wide range of agents. In this study, we found that the outer membrane of this mutant had about the same permeability as that of the parent strain to a hydrophilic agent, cephaloridine (see Results). In fact, this finding is consistent with a number of observations, which appeared to be in conflict with the suggested alteration in the porin-mediated permeability. Thus, neither the amount, the structure, nor the pore-forming activity of the major porin F (OprF) was altered in this mutant (1, 35, 38). Although the structure of lipopolysaccharide appeared to be altered in the mutant (14), the possibility that the lipopolysaccharide would affect the function of OprF could not be supported because proteoliposome vesicles made by reconstituting fragments of outer membrane from K799/WT showed permeability to sugars that was identical to that of the vesicles made from the outer membrane of K799/61—both of these vesicle preparations should have contained porins surrounded by large numbers of endogenous lipopolysaccharide molecules (35). All these data are consistent with our finding that the K799/61 outer membrane shows a near-normal permeability at least toward hydrophilic agents. It is still possible that the lipid bilayer domains of the mutant outer membrane show a higher permeability to such hydrophobic compounds as nitrocefin (1, 31) and some fluorescent probes. However, since the outer membrane bilayer usually has permeability about 2 orders of magnitude lower than that of the glycerophospholipid bilayers (41), it seems unlikely that permeation through the bilayer domain makes significant contributions to the susceptibility to those antibiotics examined in this study, which show at most moderate hydrophobicity (32, 37).

Our data also showed that K799/61 was essentially incapable of pumping out tetracycline and chloramphenicol (Fig. 1 and 5). Thus, the active efflux activity of this strain is much lower than that of its parent strain and other wild-type strains, and this low efflux activity appears to make this mutant strain almost as susceptible as *E. coli* to a number of antimicrobial agents (41), in spite of the essentially unaltered permeability of its outer membrane to at least one hydrophilic agent and presumably to many others. It should be added, however, that K799/61 is hypersusceptible to agents such as aminoglycosides, for which there is no evidence for active efflux; the altered structure of its outer membrane is indeed likely to be responsible for such phenotypes of this strain, which contains multiple mutations.

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