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Role of MexA-MexB-OprM in Antibiotic Efflux in *Pseudomonas aeruginosa*

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We have earlier described *mexA-mexB-oprK*, an operon involved in pyoverdine export in *Pseudomonas aeruginosa*, and suggested that the products of these genes also contribute to the active efflux of several antibiotics (K. Poole, K. Krebes, C. McNally, and S. Neshat, J. Bacteriol. 175:7363–7372, 1993). Recently the outer membrane component of this efflux system was shown to be OprM, rather than OprK (N. Gotoh and K. Poole, unpublished results). In the present study, the conclusion concerning the efflux activity of this system was confirmed and extended by the measurement of drug accumulation in intact cells. Thus, the steady-state accumulation levels of tetracycline and norfloxacin were increased in *mexA* and *oprM* null mutants. *mexA* and *oprM* null mutants also showed an increase in susceptibility to a wide variety of β-lactam antibiotics and an increase in the steady-state accumulation level of benzylpenicillin, indicating that the MexA-MexB-OprM pump also effluxes β-lactams. Furthermore, deenergization of the cytoplasmic membrane with a proton conductor always produced a strong increase in the accumulation level. Finally, a single-step mutant overproducing MexAB-OprM accumulated less tetracycline and chloramphenicol than the parent strain and was more resistant to a wide range of antimicrobial compounds, including β-lactams. These results support the notion that these proteins contribute to the intrinsic resistance of *P. aeruginosa* through the multidrug active efflux process.

Pseudomonas aeruginosa shows significant degrees of intrinsic resistance to a wide variety of antimicrobial agents, including most β-lactams, tetracyclines, chloramphenicol, and fluoroquinolones. Although the outer membrane of this species has a very low nonspecific permeability to small, hydrophilic molecules (1, 26), this alone is insufficient to explain the degree of resistance observed (16), and an additional resistance mechanism must be postulated. With some β-lactams, hydrolysis of the drugs by the periplasmic β-lactamase can serve as this additional mechanism (see reference 18 for a discussion of this phenomenon in *Escherichia coli*). For other compounds that are not inactivated by wild-type cells, their active efflux out of the cell may be the most likely second contributing factor to resistance (17). Recently, a putative operon, *mexA-mexB-oprK*, which codes for the export of a siderophore, pyoverdine (20), was suggested to function also as a multidrug efflux pump because overexpression of this operon increased the resistance of *P. aeruginosa* to chloramphenicol, tetracycline, nalidixic acid, ciprofloxacin, and streptonigrin, and disruption of these genes made the mutants hypersusceptible to these agents (21). (The outer membrane component of this system, previously thought to be the OprK protein seen in the multidrug-resistant strain K385 [21], was shown recently [5], however, to be identical to the previously described protein OprM [12]. Thus, the operon contains genes *mexA*, *mexB*, and *oprM* rather than *oprK*.) An independent study also showed that an energy-dependent efflux process decreased the steady-state accumulation levels of tetracycline, chloramphenicol, and norfloxacin in *P. aeruginosa* and that the efflux activity was increased in resistant clinical isolates as well as in certain laboratory mutants, although the genes involved in the efflux process were not identified (7). In the present study, we show, by the direct assay of drug accumulation, that MexA-MexB-OprM indeed pumps out various antibacterial agents in an energy-dependent manner.

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MATERIALS AND METHODS

Antibiotics and reagents. Benzylpenicillin, azlocillin, carbenicillin, chloramphenicol, tetracycline, norfloxacin, ciprofloxacin, and novobiocin were purchased from Sigma Chemical Co., St. Louis, Mo. Imipenem, aztreonam, moxalactam, carumonam, cefpirome, and cefepime were gifts from Merck Sharp & Dohme, E. R. Squibb & Sons, Eli Lilly & Co., Hoffman-La Roche, Hoechst-Roussel, and Pfizer, respectively. [7-^{3}H(N)]tetracycline and [ring-3,5-^{3}H]chloramphenicol were obtained from NEN/Dupont, Boston, Mass., and [phenyl-4(n)^{3}H]benzylpenicillin was from Amersham Corp., Arlington Heights, Ill. [Piperazine-^{14}C]norfloxacin was a generous gift from Merck Sharp & Dohme.

Strains and their cultivation. The *P. aeruginosa* strains used (Table 1) are derivatives of strain PAO1 and have been described earlier (7, 20, 21). HN854 and HN856 were made by transferring pPV22 (21), containing *oprM*::ΩHg^r, from *E. coli* S17-1 into a spontaneous streptomycin-resistant (*rpsL*) derivative of PAO4098E, HN853 (7). Since this plasmid cannot replicate in *P. aeruginosa*, only the transconjugants whose *oprM* gene was replaced by the *oprM*::ΩHg^r allele through homologous recombination are selected by $HgCl_2$. The transfer was carried out as described previously (21), except that the selection for the recombinants was carried out with Luria-Bertani plates (7) containing 1 mg of streptomycin sulfate per ml and 20 μg of $HgCl_2$ per ml.

The strains were grown either in glucose-M63 minimal medium supplemented with 0.1% Difco Casamino Acids, in Luria-Bertani broth, or in iron-deficient succinate minimal medium supplemented with 1 mM methionine (20), 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 in Luria-Bertani medium or, where indicated, in iron-deficient minimal medium (20). The inoculum was 5×10^4 cells per tube, and the results were read after an overnight incubation at 37°C.

Assay of drug accumulation in intact cells. The entry of radioactive drugs into *P. aeruginosa* cells was measured as described previously (7). Briefly, cells were grown in M63 minimal medium supplemented with glucose (0.2%) and Difco Casamino Acids (0.1%), harvested in the mid-exponential phase, washed, and

TABLE 1. Bacterial strains used

Strain(s)	Description	Reference(s)
K372	PAO *met-9011 amiE200 rpsL pvd-9* (defective in pyochelin production and ferripyochelin receptor)	21
K590	K372 *mexA*::*tet*	21
K613	K372 *oprM*::ΩHg^r	21
PAO4098	PAO *met-9020 pro-9024 blaP9028*	4
PAO4098E	MexAB-OprM-overproducing mutant of PAO4098	4
HN853	PAO4098E *rpsL*	4; this study
HN854, HN856	PAO4098E *oprM*::ΩHg^r *rpsL*	This study

resuspended in a buffer containing 50 mM potassium phosphate (pH 7.0), 1 mM $MgSO_4$, and 0.2% glucose. At intervals after the addition of the radioactive drug, the cells were recovered by centrifugation through silicone-oil, and the drug accumulation was quantitated by liquid scintillation counting (7). At the times indicated in Fig. 2, a proton conductor, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), was added to portions of the suspensions in order to see the effect of membrane deenergization on drug accumulation.

The accumulation of [^{3}H]benzylpenicillin was tested by using cells that had been pretreated for 5 min with 0.1 mM aztreonam, in order to inhibit the low, but significant, uninduced levels of endogenous β-lactamase (8).

RESULTS

Identification of PAO4098E as a MexAB-OprM overproducer. As described in the introduction, it was recently found that the outer membrane protein gene in the *mexAB* operon was *oprM* rather than *oprK*. This means that strain K385, originally thought (21) to be overproducing MexAB and OprK, overproduces another set of putative efflux transporters, rather than MexAB, in addition to OprK. In contrast, a single-step multidrug-resistant mutant of the PAO1 line, PAO4098E, overproduced an outer membrane protein that was indistinguishable from OprM in its mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7). Since the outer membrane proteins overproduced in multidrug-resistant strains are sometimes difficult to distinguish by SDS-PAGE (13), we introduced an *oprM*::ΩHg^r allele on a suicide vector so that it would recombine with the homologous *oprM* gene on the chromosome (see Materials and Methods). When the outer membrane protein patterns of two independently isolated $HgCl_2$- and streptomycin-resistant transconjugants were analyzed by SDS-PAGE, the overproduced 50-kDa protein that was present in the parent strain was completely absent (Fig. 1), showing that PAO4098E was an overproducer of OprM and therefore probably also of the proteins coded for by genes in the same operon, MexA and MexB. The disruption of the *oprM* gene also decreased the MIC of carbenicillin from 1,024 μg/ml (in HN853) to ≤1.6 μg/ml (in HN854 and HN856), as expected from the results of Gotoh et al. (4) and from the data described below.

MIC. The upper part of Table 2 shows the MICs of non-β-lactam antibiotics and therapeutic agents for gene disruption mutants K590 and K613. Since we now know that PAO4098E is an overproducer of MexAB-OprM as described above, the MICs for this strain as well as its immediate parent PAO4098 are also listed for comparison, although many of these data have already been published in a different context (7). In general, the results confirm the earlier observation (21) that disruption of the *mexA* or *oprM* gene makes the mutants hypersusceptible to various agents. The disruption of *mexA* generally had an effect very similar to that of the disruption of *oprM*, suggesting that elimination of any component of this presumed three-protein efflux machinery totally abolishes efflux. However, in the case of norfloxacin, the effect of *oprM* disruption was greater than that of *mexA* disruption. Possible explanations are presented in Discussion.

Comparison of PAO4098E with its parent, PAO4098, also shows that the overexpression of the system makes the organism more resistant to the agents surveyed. The MICs of fluoroquinolones for PAO4098, however, were often significantly different from those for the other wild-type PAO-derived strain, K372. Since *P. aeruginosa* appears to contain at least three different multidrug efflux systems (13), this suggests that the baseline expression levels of these pumps are likely to be different for different strains, presumably as a result of inadvertently introduced, undocumented mutations. Possibly the efflux of fluoroquinolones is especially affected by these other systems. Alternatively, K372 may contain a so-far-undetected mutation in the DNA gyrase genes.

Previous studies did not address the issue of the susceptibility to β-lactam antibiotics of *mexA-mexB-oprM* disruption mutants. As shown in the lower part of Table 2, *mexA* and *oprM* null mutants showed a noticeable increase in susceptibility to a variety of β-lactam antibiotics, including the newer cephalosporins, consistent with a role for the MexA-MexB-OprM pump in β-lactam efflux and resistance. There was no significant change in the MIC of imipenem; this may be due to the

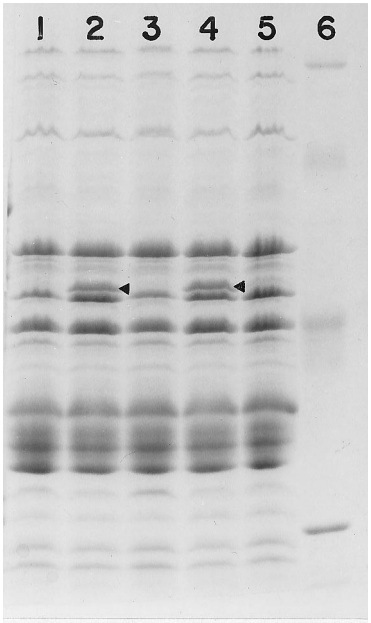

FIG. 1. Outer membrane proteins of HN853 and the two independent $HgCl_2$-resistant transconjugants isolated after the introduction of the *oprM*::ΩHg^r allele, HN854 and HN856. The outer membrane fraction was isolated and analyzed by SDS-PAGE as described previously (7). Lane 1, HN854; lane 2, HN853; lane 3, HN856; lane 4, HN853 (repeated); lane 5, PAO4098; lane 6, molecular mass standards (from the top, 97, 66, 45, and 31 kDa). The arrowheads show the OprM protein band.

TABLE 2. MICs of various antibiotics

Drug	MIC (μg/ml)[a] for strain:				
	K372 (wild type)	K590 (*mexA*::*tetA*)	K613 (*oprM*::ΩHg^r)	PAO4098[b] (wild type)	PAO4098E[b] (MexAB-OprM ↑)
Norfloxacin	>8	1	0.1	0.5	2
Ciprofloxacin	2 (0.2)	0.1 (0.1)	0.1 (0.1)	0.1	0.8
Tetracycline	8–16 (2.5)	ND[c]	0.5 (≤0.2)	8	32
Chloramphenicol	16 (12.5)	4 (3)	8	8	128
Novobiocin	128	16	32	256	512
Penicillin G	>1,024 (512)	512 (256)	512 (256)	>1,024	>1,024
Carbenicillin	32 (16)	≤0.25 (0.12)	≤0.25 (0.12)	32	1,024
Azlocillin	16 (2)	1 (≤0.06)	1 (≤0.06)	8	32
Cefoperazone	4 (2)	0.5 (0.12)	0.5 (0.12)	4	32
Ceftriaxone	64 (4)	8 (0.25)	8 (0.25)	8	64
Carumonam	8 (0.12)	8 (≤0.06)	4 (≤0.06)	8	8–16
Moxalactam	8	2	1	16	128
Cefepime	2 (0.5)	1 (0.25)	0.12 (≤0.06)	1	4
Cefpirome	4 (0.5)	2 (0.25)	0.25 (0.25)	2	8
Imipenem	2	1–2	1–2	1–2	1–2

[a] The values in parentheses are the MICs determined in iron-deficient minimal medium.
[b] Some of the data for this strain are from references 7 and 8.
[c] ND, not determined.

hydrophilic nature of this compound. Interestingly, growth medium-dependent differences in MICs were noted for many antibiotics. The reason for this is not clear, but this result is not unexpected because the expression of AcrAB, a homolog of the MexAB pump in *E. coli*, is known to be influenced strongly by environmental conditions (10). For β-lactams, it may be related also to differences in the induction of the chromosomally coded β-lactamase, as this is a complex process involving the transport of peptidoglycan fragments (6) that are likely to be affected by the nature of the medium. As reported earlier (7), the overproduction of MexAB-OprM makes the strain significantly more resistant to a number of β-lactams.

Accumulation of tetracycline. As shown in Fig. 2A, in the parent strain K372, the steady-state accumulation level of tetracycline, 130 pmol/mg of protein, was much lower than the level of 300 pmol/mg of protein observed previously in a mutant strain with little efflux activity, K799/61 (7). This suggests the presence of energy-dependent efflux, and indeed, deenergization with CCCP increased the accumulation level, presumably by inhibiting the efflux. In the *oprM* null mutant K613, the accumulation level was already around 300 pmol/mg of protein even before the addition of CCCP, and this suggests that little tetracycline is pumped out in the absence of the MexA-MexB-OprM machinery. (The *mexA* mutant K590 could not be tested because it contained a *tet* determinant that codes for tetracycline efflux.) The steady-state accumulation level was lower in the MexAB-OprM overproduction mutant PAO4098E than in its immediate parent, PAO4098 (see Fig. 4 of reference 7).

Efflux of chloramphenicol. Chloramphenicol accumulation in the K372 parent strain attained a steady-state level of about 30 pmol/mg of protein (Fig. 2B), a level which is much lower than the 90 to 100 pmol/mg of protein seen in the efflux-deficient mutant K799/61 (7). This suggests again the significant contribution of an active efflux process, and indeed, the addition of CCCP increased the accumulation to the expected level of 80 pmol/mg of protein (Fig. 2B). The *oprM* mutant K613 and the *mexA* mutant K590, however, showed accumulation levels that were not much different from the level observed in K372 (Fig. 2B). This result may be related to the facts that the total accumulation level is not a very sensitive indicator of the efflux rates (see Discussion) and that the difference in MICs for the null mutants and K372 was rather small (only about fourfold). In any case, the result suggests that chloramphenicol, unlike tetracycline, is still pumped out at significant rates in mutants lacking the MexAB-OprM system. Indeed, we have been able to isolate chloramphenicol-hypersusceptible mutants from K613, which presumably lack the second chloramphenicol efflux pump that is active in K613 or K590 (6a).

In contrast, there was a reproducible difference in the steady-state accumulation level of chloramphenicol between the MexAB-OprM overproduction mutant PAO4098E and its parent, PAO4098 (Fig. 3). In the experiment shown, the chloramphenicol concentration used was much lower (0.15 μM) than usual (5 μM for the example in Fig. 2B), but similar differences were seen at concentrations of 5 and 25 μM (data not shown).

Accumulation of norfloxacin. The *oprM* mutant appeared to accumulate more drug than the K372 parent strain, although there was little difference betwen K372 and the *mexA* mutant (Fig. 2C). This is consistent with the MIC data, as the *oprM* mutant was much more susceptible than the *mexA* mutant (Table 2). Possible interpretations of these results are presented in Discussion. Although not shown in Fig. 2C, the addition of CCCP at 10 min to portions of cell suspensions uniformly resulted in an increase of accumulation, to 1,400 to 1,500 pmol/mg of protein, in all of the strains.

Accumulation of benzylpenicillin. Our previous study suggested that certain β-lactam compounds may also be actively pumped out by *P. aeruginosa* cells (8). We therefore measured the accumulation of [^{3}H]benzylpenicillin by using a previously described procedure (8). The *mexA* and *oprM* null mutants, which were much more susceptible to β-lactams (Table 2), accumulated more of the labeled compound than did K372 (Fig. 2D), indicating an MexA-MexB-OprM-dependent efflux activity in K372. Indeed, the addition of CCCP caused a strong increase in accumulation in K372 (Fig. 2D), supporting the presence of a strong efflux activity in nontreated cells. Interestingly, the accumulation level observed in *mexA* and *oprM* mutants was also increased by the addition of CCCP, suggesting that there is still some residual efflux activity in mutants deficient in the MexA-MexB-OprM pump.

Our previous study showed that a substantial fraction of ^{3}H

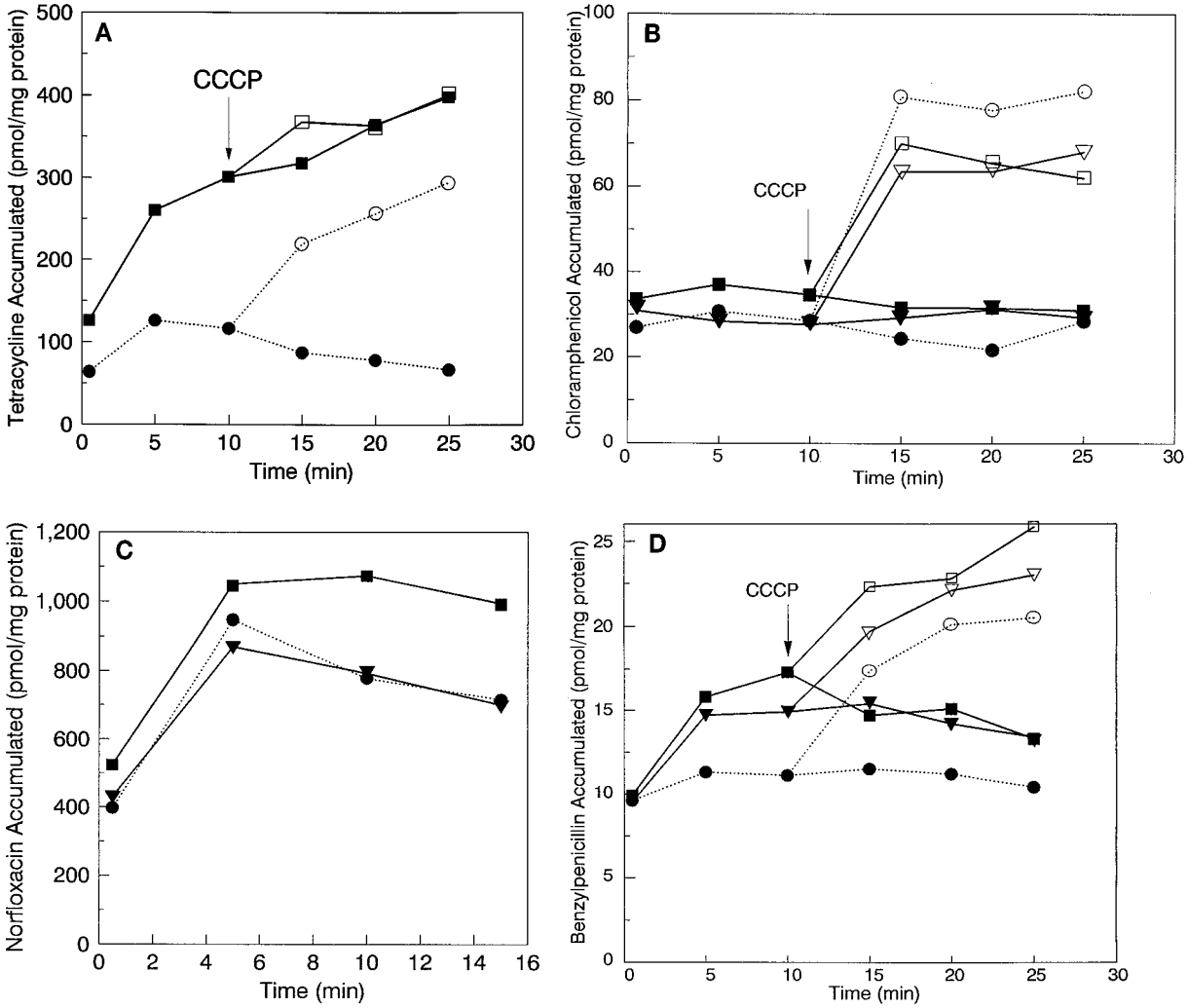

FIG. 2. Accumulation of various drugs in the parental strain K372 (●) and ○), the *mexA* insertion mutant K590 (▼ and ▽), and the *oprM* insertion mutant K613 (■ and □). The cells were grown in M63 medium supplemented with 0.1% Casamino Acids with glucose as the carbon source, harvested, washed, and resuspended in a phosphate buffer containing glucose, as described in Materials and Methods. After 5 min at 37°C, radiolabeled drugs were added, and the cellular accumulation was determined by centrifugation of cells through a silicone-oil mixture. At 10 min, CCCP (0.1 or 0.25 mM) was added to one-half of each reaction mixture. Open symbols show the data for CCCP-treated samples, and closed symbols show the data for samples that did not receive CCCP. The data shown are the averages for three separate experiments, in each of which duplicate samples were taken at each time point. (A) [^{3}H]tetracycline (specific radioactivity, 0.55 Ci/mmol) added at 5 μM. K590 was not tested because it contained the *tetA* determinant. (B) [^{3}H]chloramphenicol, which was previously diluted with the nonradioactive compound to a specific activity of 0.5 Ci/mmol, added at 5 μM. (C) [^{14}C]norfloxacin (specific activity, 14.9 mCi/mmol) added at 20 μM. The addition of CCCP increased the accumulation levels in all strains to about 1,500 pmol/mg of protein, but these results are not shown to avoid overcrowding. (D) [^{3}H]benzylpenicillin, which was previously diluted with the nonradioactive compound to a specific activity of 0.5 Ci/mmol, added at 5 μM.

label inside the cells is in the form of penicilloic acid, the product of hydrolysis of penicillin (8). Most of the label outside the cell is still in the form of benzylpenicillin, however, so there should be a constant influx of benzylpenicillin from the medium. It is thus uncertain whether the assay is measuring the efflux activity for penicilloic acid, benzylpenicillin, or both, but the results nevertheless show a real difference in the drug efflux activity between the null mutants and the wild type.

DISCUSSION

Earlier work has resulted in the cloning and sequencing of the putative operon *mexA-mexB-oprM* from *P. aeruginosa* (20, 21). MexA-MexB appears to belong to a family of gram-negative export machineries (reviewed in reference 11), such as AcrA-AcrB (previously called AcrA-AcrE), AcrE-AcrF (previously called EnvC-EnvD), MtrA-MtrB, CzcB-CzcA, CnrB-CnrA, and NolF-NolGHI, in which the component listed second is a putative 12-transmembrane-segment efflux pump belonging to the RND (resistance-nodulation-division) family (24) and the component listed first belongs to the MFP (membrane-fusion protein) family, which is thought to connect the pump directly to an outer membrane channel (2). The outer membrane protein was called OprK, on the basis of the behavior of the overproduced outer membrane protein in strain K385 (21). However, a recent study (5) of the product of the cloned "*oprK*" gene showed its identity with OprM, which was earlier shown (12) to be overproduced in *nalB* mutants of *P. aeruginosa* (23). The operon is therefore renamed *mexAB-oprM*. The OprM protein presumably serves as the outer membrane channel component in the assembly that functions to pump out solute molecules directly into the medium, circum-

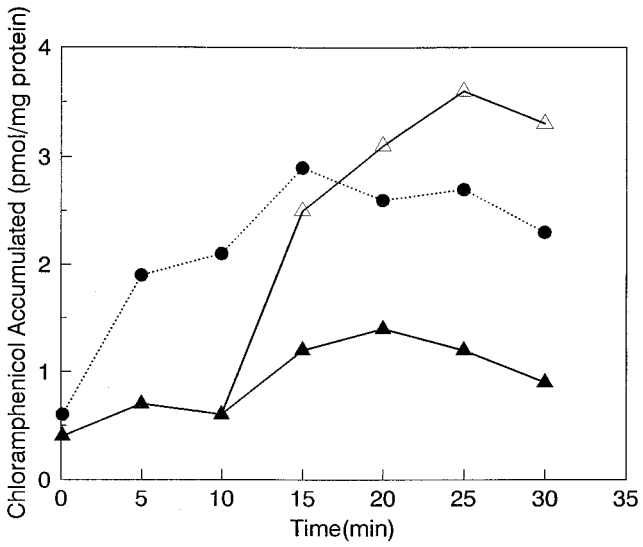

FIG. 3. Accumulation of [^{3}H]chloramphenicol in PAO4098 (●) and an MexAB-OprM overproduction mutant, PAO4098E (▲). The experiments were carried out as outlined in the legend to Fig. 2, except that the concentration of [^{3}H]chloramphenicol was 0.15 μM. At 10 min, CCCP (0.15 mM) was added to one-half of the PAO4098E suspension (△).

venting the outer membrane barrier. This notion is also consistent with the recent report that *oprM* insertion mutants are hypersusceptible to a wide variety of antibiotics (4). For *E. coli*, it was recently shown that chromosomally coded multidrug efflux pumps (including AcrAB) export drugs directly into the medium, whereas plasmid-coded pumps such as TetA pump the substrates mainly into the periplasm (25).

Evidence also suggested strongly that the physiological function of the MexA-MexB-OprM system is to export pyoverdine, a large siderophore molecule, into the medium (20), although it may be more generally involved in the efflux of secondary metabolites (19). At the same time, the involvement of the system in the active efflux of antibiotics was suggested, as null mutations in *mexA* or *oprM* made the mutant *P. aeruginosa* strains hypersusceptible to several antibiotics (20). The results of the present study indeed support the notion that the MexA-MexB-OprM system also functions as a multidrug efflux pump, because steady-state accumulation levels of some drugs are often increased in the null mutants (Fig. 2) and often decreased in the overexpression mutant (Fig. 3; see also references 7 and 8). Moreover, these results agree with previous data which sugested that efflux pumps contribute to β-lactam resistance in *P. aeruginosa*. Indeed, *oprM* and *mexA* null mutants were more susceptible to a variety of β-lactams and accumulated more benzylpenicillin than the parent strain expressing the MexA-MexB-OprM pump.

The drug accumulation data, however, are not always easy to interpret. A complication of one type occurs because we can only measure the sum of accumulations in various compartments of the cell, whereas usually only the accumulation of the free drug in the cytoplasm is the relevant parameter. Thus, even in the well-established case of efflux-based, R plasmid-mediated resistance to tetracycline, a 245-fold increase in MIC was accompanied by only a 7-fold decrease in the cellular accumulation of tetracycline (14). In many situations, therefore, we expect that significant changes in accumulation in the cytoplasm, and therefore in the MIC, would occur without large changes in total cellular accumulation levels, and indeed some of the cases in which we failed to detect much difference in accumulation (for example, for chloramphenicol [Fig. 2B]) could have belonged to this category. Second, *P. aeruginosa* appears to produce more than one multidrug efflux system (7, 13). Similarly, the *E. coli* genome contains genes for at least four homologs of the RND-type pump MexB (11) and in addition contains efflux genes coding for pumps of different types (10, 15, 22). The presence of more than one efflux system with overlapping specificities obviously complicates the interpretation of the accumulation data, especially since each of these systems is likely to be regulated in a distinctive manner. Differences between the *mexA* and *oprM* mutants in the efflux of (Fig. 2C) and susceptibility to (Table 2) norfloxacin, for example, might be explained if OprM is used also by another efflux machinery, which prefers norfloxacin, but not ciprofloxacin, as the substrate. Sharing of one outer membrane channel by multiple efflux systems is known to occur in *E. coli*, in which the TolC channel is used by at least two, and perhaps many more, export systems (3).

In addition to the technical problems mentioned above, there is also potential concern with regard to the expected phenotype of the null mutants. Assuming that MexA-MexB-OprM forms a multisubunit complex that pumps out pyoverdine as well as drugs directly into the medium, one would expect that the steady-state accumulation levels of the drugs will increase in mutants lacking any component of the complex. This effect is caused, in its simplest interpretation, by the absence of the functional efflux apparatus. However, an alternative possibility is that the remaining subunits of the apparatus assemble and produce a highly permeable leakage pathway that increases the influx of drugs into the cytoplasm. We do not believe that this is likely because of the following reasons. (i) We showed recently that deletion of the transporter component of a similar multisubunit efflux machinery, EmrAB (9), does not alter the MICs of commonly used antibiotics in *E. coli* (18a). (ii) *mexA* or *oprK* null mutants are not hypersusceptible to aminoglycosides, for example (20), and chloramphenicol continued to be pumped out effectively in both *mexA* and *oprM* mutants (Fig. 2B). (iii) A one-step mutant, PAO4098E (7), is now known to overproduce MexA, MexB, and OprM (see Results), rather than MexCD-OprM. Thus, the phenotype of this overproducer strain provides a set of data complementary to that from the phenotype of the null mutants examined in this study. As shown earlier (7, 8) and also in the present study (Table 2; Fig. 3), PAO4098E was highly resistant to fluoroquinolones, β-lactams (except imipenem), tetracycline, and chloramphenicol and accumulated lower levels of tetracycline and chloramphenicol than the isogenic parent strain. Taken together, our data indicate that the MexAB-OprM system is intimately involved in the active efflux of various antibiotics and chemotherapeutic agents, thereby contributing significantly to the intrinsic resistance of *P. aeruginosa.*

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REFERENCES

1. **Angus, B. L., A. M. Carey, D. A. Caron, A. M. B. Kropinski, and R. E. W. Hancock.** 1982. Outer membrane permeability in *Pseudomonas aeruginosa*: comparison of a wild type with an antibiotic-supersusceptible mutant. Antimicrob. Agents Chemother. **21:**299–309.
2. **Dinh, T., I. T. Paulsen, and M. H. Saier, Jr.** 1994. A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of gram-negative bacteria. J. Bacteriol. **176:**3825–3831.

3. **Fath, M. J., and R. Kolter.** 1993. ABC transporters: bacterial exporters. Microbiol. Rev. **57:**995–1017.
4. **Gotoh, N., N. Itoh, H. Tsujimoto, J. Yamagishi, Y. Oyamada, and T. Nishino.** 1994. Isolation of OprM-deficient mutants of *Pseudomonas aeruginosa* by transposon insertion mutagenesis: evidence of involvement in multiple antibiotic resistance. FEMS Microbiol. Lett. **122:**267–274.
5. **Gotoh, N., and K. Poole.** 1995. Unpublished observation.
6. **Jacobs, C., C. J. Huang, E. Bartowsky, S. Normark, and J. T. Park.** 1994. Bacterial cell wall recycling provides cytosolic muropeptides as effectors for β-lactamase induction. EMBO J. **13:**4684–4694.

6a. **Johnson, C., and K. Poole.** Unpublished result.

7. **Li, X.-Z., D. M. Livermore, and H. Nikaido.** 1994. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: resistance to tetracycline, chloramphenicol, and norfloxacin. Antimicrob. Agents Chemother. **38:**1732–1741.
8. **Li, X.-Z., D. Ma, D. M. Livermore, and H. Nikaido.** 1994. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: active efflux as a contributing factor to β-lactam resistance. Antimicrob. Agents Chemother. **38:**1742–1752.
9. **Lomovskaya, O., and K. Lewis.** 1992. *emr*, an *Escherichia coli* locus for multidrug resistance. Proc. Natl. Acad. Sci. USA **89:**8938–8942.
10. **Ma, D., D. N. Cook, M. Alberti, N. G. Pon, H. Nikaido, and J. E. Hearst.** 1995. Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. Mol. Microbiol. **16:**45–55.
11. **Ma, D., D. N. Cook, J. E. Hearst, and H. Nikaido.** 1994. Efflux pumps and drug resistance in gram-negative bacteria. Trends Microbiol. **2:**489–493.
12. **Masuda, N., and S. Ohya.** 1992. Cross-resistance to meropenem, cephems, and quinolones in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **36:**1847–1851.
13. **Masuda, N., E. Sakagawa, and S. Ohya.** 1995. Outer membrane proteins responsible for multiple drug resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**645–649.
14. **McMurry, L., R. E. Petrucci, Jr., and S. B. Levy.** 1980. Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **77:**3974–3977.
15. **Morimyo, M., E. Hongo, H. Hama-Inaba, and I. Michida.** 1992. Cloning and characterization of the *mvrC* gene of *Escherichia coli* K-12 which confers resistance against methyl viologen toxicity. Nucleic Acids Res. **20:**3159–3165.
16. **Nikaido, H.** 1989. Outer membrane barrier as a mechanism of antimicrobial resistance. Antimicrob. Agents Chemother. **33:**1831–1836.
17. **Nikaido, H.** 1994. Prevention of drug access to bacterial targets: role of permeability barriers and active efflux. Science **264:**382–388.
18. **Nikaido, H., and S. Normark.** 1987. Sensitivity of *Escherichia coli* to various β-lactams is determined by the interplay of outer membrane permeability and degradation by periplasmic β-lactamases: a quantitative predictive treatment. Mol. Microbiol. **1:**29–36.

18a. **Okusu, H., D. Ma, and H. Nikaido.** Unpublished data.

19. **Poole, K.** 1994. Bacterial multidrug resistance—emphasis on efflux mechanisms and *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. **34:**453–456.
20. **Poole, K., D. E. Heinrichs, and S. Neshat.** 1993. Cloning and sequence analysis of an EnvCD homologue in *Pseudomonas aeruginosa*: regulation by iron and possible involvement in the secretion of the siderophore pyoverdine. Mol. Microbiol. **10:**529–544.
21. **Poole, K., K. Krebes, C. McNally, and S. Neshat.** 1993. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. J. Bacteriol. **175:**7363–7372.
22. **Purewal, A. S.** 1991. Nucleotide sequence of the ethidium efflux gene from *Escherichia* coli. FEMS Microbiol. Lett. **82:**229–232.
23. **Rella, M., and D. Haas.** 1982. Resistance of *Pseudomonas aeruginosa* PAO to nalidixic acid and low levels of β-lactam antibiotics: mapping of chromosomal genes. Antimicrob. Agents Chemother. **22:**242–249.
24. **Saier, M. H., R. Tam, A. Reizer, and J. Reizer.** 1994. Two novel families of bacterial membrane proteins concerned with nodulation, cell division, and transport. Mol. Microbiol. **11:**841–847.
25. **Thanassi, D. G., G. S. B. Suh, and H. Nikaido.** 1995. Role of outer membrane barrier in efflux-mediated tetracycline resistance of *Escherichia coli*. J. Bacteriol. **177:**998–1007.
26. **Yoshimura, F., and H. Nikaido.** 1982. Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes. J. Bacteriol. **152:**636–642.