ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2003, p. 27–33 Vol. 47, No. 1
0066-4804/03/$08.00+0 DOI: 10.1128/AAC.47.1.27–33.2003

Contributions of MexAB-OprM and an EmrE Homolog to Intrinsic Resistance of *Pseudomonas aeruginosa* to Aminoglycosides and Dyes

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Received 9 July 2002/Returned for modification 9 September 2002/Accepted 24 September 2002

Of the six putative small multidrug resistance (SMR) family proteins of *Pseudomonas aeruginosa*, a protein encoded by the PA4990 gene ($emrE_{Pae}$) shows the highest identity to the well-characterized EmrE efflux transporter of *Escherichia coli*. Reverse transcription-PCR confirmed the expression of $emrE_{Pae}$ in the wild-type strain of *P. aeruginosa*. Using isogenic $emrE_{Pae}$, *mexAB-oprM*, and/or *mexB* deletion mutants, the contributions of the EmrE protein and the MexAB-OprM efflux system to drug resistance in *P. aeruginosa* were assessed by a drug susceptibility test carried out in a low-ionic-strength medium, Difco nutrient broth. We found that $EmrE_{Pae}$ contributed to intrinsic resistance not only to ethidium bromide and acriflavine but also to aminoglycosides. In this low-ionic-strength medium, MexAB-OprM was also shown to contribute to aminoglycoside resistance, presumably via active efflux. Aminoglycoside resistance caused by these two pumps could not be demonstrated in high-ionic-strength media, such as Luria broth or Mueller-Hinton broth. The EmrE-dependent efflux of ethidium bromide was confirmed by a continuous fluorescence assay.

Pseudomoas aeruginosa is a major opportunistic human pathogen that displays high-level multiple intrinsic resistance to a variety of structurally unrelated antimicrobial agents (10, 11, 25, 30). It is now known that this multidrug resistance phenotype of *P. aeruginosa* results from the presence of broad-specificity drug efflux systems and low-permeability outer membrane (20, 25). To date, antimicrobial efflux systems in bacteria have been grouped into five (super)families (28), although the efflux of most of the clinically relevant agents seems to occur in *P. aeruginosa* through drug-proton antiporters of the resistance-nodulation-division (RND) family (25).

The MexAB-OprM system, which is the major, constitutively expressed, multidrug efflux pump and the first discovered member of RND family exporter in *P. aeruginosa*, is known to pump out mostly lipophilic and amphiphilic drugs (11). In contrast, another RND pump complex, MexXY, together with OprM, was found to pump out aminoglycosides, a group of highly hydrophilic compounds, and contribute to the intrinsic resistance of *P. aeruginosa* to these agents (1). Interestingly, MexXY were originally identified as exporters of fluoroquinolones, erythromycin, and ethidium bromide (16), typical amphiphilic substrates of MexAB-OprM (11). This suggests that there is no fundamental difference between the RND pumps for amphiphilic compounds and those for hydrophilic compounds and that the efflux of the latter class of compounds by MexAB-OprM should be examined.

In addition to the RND efflux family, the small multidrug resistance (SMR) family is also a drug resistance determinant (6, 24). A well-characterized member of this family is the EmrE protein of *Escherichia coli* ($EmrE_{Eco}$) (29), which contributes to resistance of the organism to multiple toxic compounds, particularly lipophilic cations such as ethidium bromide and methyl viologen (24). The SMR transporters function as drug-proton antiport systems and display four transmembrane segments within ca. 110 amino acid residues (6, 29). The *P. aeruginosa* genome sequences reveal the presence of several putative SMR transporters, that is, homologues of $EmrE_{Eco}$ (30; http://www.pseudomonas.com). Of these homologues, the PA4990 gene encodes a protein with the highest identity to $EmrE_{Eco}$ (we tentatively designate this *P. aeruginosa* SMR protein $EmrE_{Pae}$ here). Interestingly, a plasmid (integron)-borne *emrE* homolog, *qac*G, was identified on a 36-kb plasmid that was isolated from a clinical strain of *P. aeruginosa* (7). The chromosomally encoded $EmrE_{Pae}$ protein was recently expressed in *E. coli* and purified and reconstituted into proteoliposomes, where the protein was shown to pump out toxic compounds such as methyl viologen (21). Still, the role of $EmrE_{Pae}$ in the intrinsic resistance to antimicrobial agents, especially aminoglycosides (since these are also cationic compounds), remains unknown. We evaluate here the contributions of MexAB-OprM and $EmrE_{Pae}$ in intrinsic drug resistance in *P. aeruginosa*, with emphasis on aminoglycosides. We show that the both MexAB-OprM and $EmrE_{Pae}$ play important roles in the intrinsic resistance of *P. aeruginosa* to aminoglycosides and cationic dyes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in the present study are listed in Table 1. Bacteria were grown at 37°C in Luria-Bertani (LB) broth and agar. In some cases, Difco nutrient broth (NB) and Difco Mueller-Hinton broth (MHB) were also used. Divalent cation contents of NB was reported as 0.29 mM Mg^{2+} and 0.03 mM Ca^{2+} (15). The particular batch of MHB we used contained 0.20 mM Mg^{2+} and 0.10 mM Ca^{2+} according to the manufacturer. LB medium is estimated (31) to contain 0.34 mM Mg^{2+} and 0.06 mM Ca^{2+} if we assume that Difco tryptone contains levels of these ions similar to Difco peptone. The conductivities of NB, MHB, and LB

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TABLE 1. *P. aeruginosa* strains and plasmids used in this study

P. aeruginosa strain or plasmid	Description	Source or reference
P. aeruginosa		
PAO1	Prototroph	10
HN1112	Spontaneous streptomycin-resistant (*rpsL*) derivative of PAO1	This study
HN1113	HN1112 Δ*emrE*	This study
K1119	PAO1 Δ*mexAB-oprM*	9
HN1114	Spontaneous streptomycin-resistant (*rpsL*) derivative of K1119	This study
HN1115	HN1113 Δ*mexAB-oprM*	This study
K1589	Spontaneous streptomycin-resistant (*rpsL*) derivative of PAO1 made Δ*mexR* Δ*mexB*	K. Poole
HN1116	K1589 Δ*emrE*	This study
Plasmids		
pEX18Tc	Broad-host-range gene replacement vector; *sacB,* tetracycline resistant	4
pXZL1307	pEX18Tc::Δ*emrE*	This study
pELCT04	pK18mob *sacB*::ΩHg^r and Δ*mexAB-oprM,* kanamycin and $HgCl_2$ resistant	9
pBluescript II SK(+)	Cloning vector, ampicillin resistant	Stratagene
pXZL1582	pBluescript II SK(+)::$emrE_{Pae}$	This study

medium, which reflects the ionic strength, were equivalent to 13, 102, and 112 mM NaCl, respectively, when measured with the conductivity meter (Radiometer, Copenhagen, Denmark). In the ethidium bromide accumulation experiments, M63 minimal medium [13.6 g of KH_2PO_4, 2 g of $(NH_4)_2SO_4$, and 0.5 mg of $FeSO_4$ per liter; pH 7.0] supplemented with glucose (0.2%) and $MgSO_4$ (10 mM) was used.

Plasmids were maintained in *E. coli* with appropriate selection [pEX18Tc, 10 μg of tetracycline per ml; pECLT04, 50 μg of kanamycin per ml or 15 μg of $HgCl_2$ per ml; and pBluescript II SK(+), 100 μg of ampicillin per ml]. Spontaneous streptomycin-resistant (*rpsL*) derivatives of *P. aeruginosa* strains PAO1 and K1119 (a Δ*mexAB-oprM* derivative of PAO1) were selected by plating cells on LB agar containing streptomycin (1,000 μg/ml for PAO1 and 500 μg/ml for K1119).

Antimicrobial susceptibility assay. Susceptibility testing was carried out by twofold serial dilution in NB with an inoculum of 5×10^5 cells/ml. Growth was scored after an overnight incubation at 37°C.

Cloning of the $emrE_{Pae}$ gene. The genomic DNA of *P. aeruginosa* was extracted as described previously (12). The intact *P. aeruginosa* PA4990 gene ($emrE_{Pae}$) was amplified by PCR from the genomic DNA of strain PAO1 by using the primers paemre7xz (5′-ATTAGGATCCAGCCGAGGTTACGCATCA-3′; complementary to 767 to 786 bp upstream of the start codon; the *Bam*HI site is underlined) and paemre6xz ((5′-ACGTAAGCTTCGCCTTGACCATCACC ATGT-3′; complementary to the sequence 746 to 765 bp downstream from the *emrE* stop codon; the *Hin*dIII site is underlined). The PCR mixture contained 50 ng of *P. aeruginosa* chromosomal DNA, 40 pmol of each primer, 0.2 mM concentrations of each deoxynucleoside triphosphate, 2 mM $MgSO_4$, 10% (vol/vol) dimethyl sufoxide, and 2 U of *Vent* DNA polymerase in 1× Thermo reaction buffer (New England Biolabs, Beverly, Mass.). The reaction was heated for 3 min at 94°C; then processed for 30 cycles of 40 s at 94°C, 40 s at 56°C, and 1 min at 72°C; and finally finished with 8 min at 72°C. The PCR product was purified by using QiaQuick PCR purification kit (Qiagen, Inc.), digested with *Bam*HI and *Hin*dIII, and then cloned into pBluescript SK(+) digested by the same enzymes. The recombinant plasmid pXLZ1582 was transformed into an *E. coli* triple efflux deletion strain (Δ*acrAB* Δ*acrEF* Δ*emrE*) constructed from a Δ*acrAB* Δ*acrEF* strain AG100AX (17) by the use of vector pKO3 (13) carrying a 246-bp in-frame deletion in the $emrE_{Eco}$ gene.

Construction of Δ*emrE* mutants. To delete $emrE_{Pae}$ (PA4990) in *P. aeruginosa*, a gene replacement suicide vector pEX18Tc (4) carrying an $emrE_{Pae}$ deletion (pXZL1307) was constructed as follows. Two PCRs were performed to amplify sequences upstream (1.3 kb) and downstream (0.85 kb) from the $emrE_{Pae}$ gene by using the *P. aeruginosa* PAO1 genomic DNA (see above) as a template. The upstream fragment was amplified with the primers paemre3xz (5′-ACTGGAG CTCGGACCTGCTCGCCGAACAAT-3′; complementary to the sequence from 1,268 to 1,288 bp upstream from the *emrE* start codon; the *Sac*I site is underlined) and paemre4xz (5′-ACGATCTAGAGGCGAGGTAGAGATAGT TGG-3′; complementary to the sequence 5 to 24 bp downstream from the *emrE* start codon; the *Xba*I site is underlined), whereas the downstream fragment was amplified with primers, paemre5xz (5′-ACGATCTAGAAGCCTGGTGGCGA TGTTCGT-3′; complementary to the sequence 81 to 100 bp upstream from the *emrE* stop codon; the *Xba*I site is underlined) and paemre6xz (see above). PCR mixtures were formulated and processed as described above. The PCR products were purified, digested with appropriate restriction enzymes, and cloned into the *Sac*I-*Hin*dIII-digested pEX18Tc via a three-piece ligation. The resultant plasmid, pXZL1307, was introduced into *E. coli* S17-1 by transformation and mobilized into *P. aeruginosa* strains HN1112 and K1589 via conjugation (12). Transconjugants carrying pXZL1307 in the chromosome were selected on LB agar containing tetracycline (25 μg/ml for HN1112 and 10 μg/ml for K1589) and streptomycin (1,000 μg/ml; for counterselection). They were then streaked onto LB agar containing 10% (wt/vol) sucrose, and sucrose-resistant colonies arising after overnight incubation at 37°C were screened for the presence of the *emrE* deletion by using PCR.

Construction of Δ*mexAB-oprM* mutants. To obtain a Δ*emrE* Δ*mexAB-oprM* double-knockout mutants, gene replacement vector pELCT04 was used to construct *mexAB-oprM* deletion mutants of strain HN1113 as described previously (9). The mutants carrying a *mexAB-oprM* deletion were identified by their drug hypersusceptibility phenotype.

RT-PCR. Total bacterial RNA was isolated from LB medium-grown, log-phase or overnight stationary-phase cultures (1 to 2 ml) of *P. aeruginosa* PAO1 by using the Promega SV total RNA isolation system (Promega, Madison, Wis.). After treatment of the RNA samples with RNase-free DNase (Promega; 2 U of enzyme/μg RNA for 60 min at 37°C), the DNase was inactivated at 65°C for 20 min. A 0.1-μg sample of DNase-treated RNA was used as a template for reverse transcription-PCR (RT-PCR) with the Promega access RT-PCR system according to the protocol supplied by the manufacturer. A pair of primers specific for and internal to *emrE* (paemre8xz, 5′-CCGCCATGACCAACTATCTC-3′ [forward]; and paemre9xz, 5′-GCTGGCCGTAGACGAACATC-3′ [reverse]) was used to amplify mRNA as a measure of $emrE_{Pae}$ expression. As a control, the mRNA of the constitutively expressed *rpsL* gene was amplified and quantitated by RT-PCR with the primers rpsl1xz (5′-GCAACTATCAACCAGGCTG-3′ [forward]) and rpsl2xz (5′-GCTGTGCTCTTGCAGGTTGTG-3′ [reverse]). A total of 50 pmol of each primer was used per reaction (final volume, 50 μl), which involved a 45-min incubation at 48°C, followed by 2 min at 94°C; 40 cycles of 40 s at 94°C, 40 s at 56°C, and 30 s at 68°C; and a final step at 8 min at 68°C. RT-PCR products were analyzed by agarose (1.7% [wt/vol]) gel electrophoresis for the expected RT-PCR products (*emrE*, 249 bp; *rpsL*, 220 bp). To control for DNA contamination of RNA samples, non-RT reactions (i.e., PCRs) were carried out. In no instance was a product obtained in the absence of a RT reaction.

Ethidium accumulation assay. Bacterial cells were cultivated overnight at 37°C in M63-glucose medium or LB broth and subsequently diluted 50-fold into M63 medium or LB broth, respectively. After growth to late exponential phase (i.e., an optical density at 600 nm [OD_{600}] of ca. 0.8 to 1.0 for M63 cultures or of ca. 1.2 to 1.5 for LB cultures), cells were harvested at 5,000 × *g* for 10 min at room temperature, washed once with 50 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl and 0.1% (vol/vol) glycerol (23), and resuspended in the same buffer at a cell OD_{600} of 0.5 to 1. The accumulation of ethidium bromide by *P. aeruginosa* cells was monitored with a Shimadzu RF-5301PC spectrofluorometer (Shimadzu Scientific Instruments, Inc., Columbia, Md.) at room temperature. The final concentration of ethidium bromide used was 5 μM,

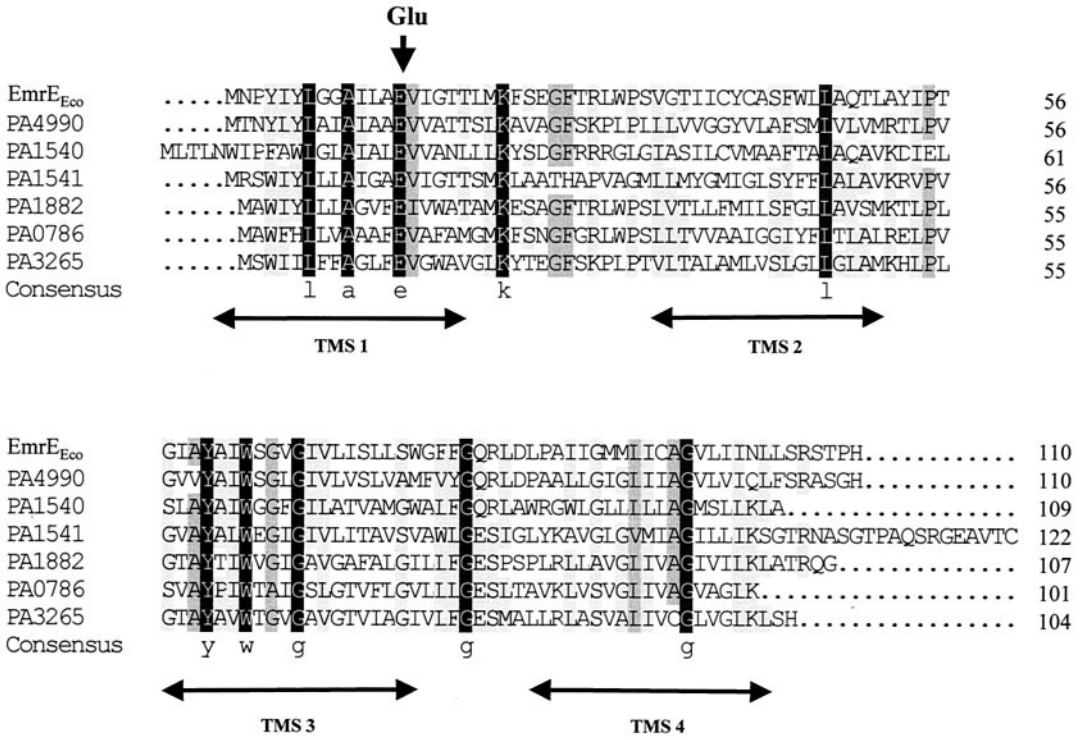

FIG. 1. Multiple alignment of EmrE of *E. coli* and its SMR homologues in *P. aeruginosa*. Residues conserved in all proteins are highlighted in black, whereas those conserved in 75 and 50% of the indicated proteins are highlighted in dark and light gray, respectively. The *P. aeruginosa* SMR proteins are given by their gene names as indicated. All of the proteins are predicated as strong hydrophobic proteins with four transmembrane segments (TMS 1 to 4). The four transmembrane segments of the PA4990 protein (EmrE$_{Pae}$) are highlighted, whereas other proteins possess similar transmembrane-spanning regions. A conserved, charged glutamyl residue in TMS 1 is indicated by an arrow. Numbers at the right show the position of the last residue in each line within the protein sequences.

and bacterial cells were used at an OD_{600} of 0.1 or 0.2. The excitation and emission wavelengths were 520 and 590 nm, respectively, with slit widths at 5 nm for excitation and 10 nm for emission. In some cases, cells were pretreated with a proton conductor, CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), at 100 to 400 μM before the addition of ethidium bromide.

RESULTS

SMR proteins in *P. aeruginosa*. A BLAST search of the *P. aeruginosa* genome with the well-characterized EmrE$_{Eco}$ protein (b0543; GenBank accession number AE000160) revealed the presence of six genes coding for homologous proteins in *P. aeruginosa* (Fig. 1). This result is certainly not surprising since, for example, *E. coli* and *Bacillus subtilis* have at least five and seven SMR proteins, respectively (5, 6). Compared with EmrE$_{Eco}$, the six putative *P. aeruginosa* proteins share amino acid identities of 24 to 46% (Fig. 1 and data not shown), and the program TMpred (http://www.ch.embnet.org) predicted all as integral membrane proteins, each with four transmembrane segments. All of these putative SMR proteins contain, in their first transmembrane segments, a conserved glutamic acid residue that functions as a part of the ligand-binding domain in EmrE$_{Eco}$ and thus mediates the coupling of proton influx to toxicant extrusion (19, 29). To date, some of the chromosomally encoded *E. coli* SMR proteins, including EmrE and SugE, were shown to provide drug resistance in intact cells (3, 22), although the latter (initially known as a suppressor of *groEL* mutations) had to be overexpressed on a plasmid to reveal its export function (3). In the present study, we examined the function of EmrE$_{Pae}$ encoded by the PA4990 gene, which showed the highest identity to EmrE$_{Eco}$.

Role of EmrE$_{Pae}$ in the intrinsic resistance in *P. aeruginosa*. Initial examination of the upstream sequence of the *emrE*$_{Pae}$ gene by neural network prediction method (http://www.fruitfly.org/seq_tools/promoter.html) identified a strong candidate promoter sequence between 10 and 50 bp upstream of the *emrE* start codon with a possible transcript starting from 10 bp upstream from the PA4990 gene, suggesting that the *emrE* gene is likely expressed in *P. aeruginosa*. Indeed, RT-PCR with *emrE*$_{Pae}$-specific primers demonstrated *emrE*$_{Pae}$ expression in uninduced wild-type *P. aeruginosa* (Fig. 2). With the constitutively expressed *rpsL* gene as control, it was found that *emrE*$_{Pae}$ expression was increased in the stationary phase of growth (Fig. 2).

The cloned *emrE*$_{Pae}$ gene was indeed effective in producing ethidium bromide resistance in *E. coli*. Thus, when pXZL1582 containing this gene (see Materials and Methods) was introduced into a Δ*acrAB* Δ*acrEF* Δ*emrE* strain of *E. coli*, the ethidium bromide MIC increased eightfold in comparison with the same strain containing the vector only (0.25 versus 2 μg/ml).

To assess the role of the EmrE$_{Pae}$ protein in antimicrobial resistance, we constructed a Δ*emrE* mutant carrying 189-bp in-frame deletion by using a gene replacement vector as described in Materials and Methods (Fig. 3). As shown in the first

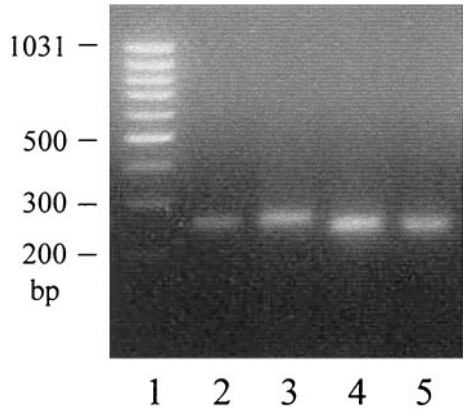

FIG. 2. $emrE_{Pae}$ expression in *P. aeruginosa* PAO1 (HN1112) as measured by RT-PCR of RNA isolated from exponential-phase (lane 2) and stationary-phase (lane 3) cultures. RT-PCR with primers specific for the *rpsL* gene was included as controls (lanes 4 [exponential phase] and 5 [stationary phase]). DNA size markers (100-bp ladder) is shown on lane 1.

two rows of Table 2, the strain containing the $emrE_{Pae}$ deletion, HN1113, displayed hypersusceptibility to ethidium bromide and acriflavine (fourfold decrease in the MIC compared to those of the parental strain HN1112), two basic dyes known to be the substrates of $EmrE_{Eco}$ (24). Interestingly, when tested in NB, the $\Delta emrE_{Pae}$ mutant strain was also hypersusceptible to several aminoglycosides (gentamicin, kanamycin, and neomycin) that are also cationic antimicrobials (two- to fourfold decrease in the MICs; Table 2). Because the differences were small, we repeated the MIC determination at least three times, with consistent results. It should also be noted that no difference in MIC was observed when high-ionic-strength media, MHB and LB medium, were used (Table 2, values in parentheses). We are not aware of any previous report indicating that the aminoglycoside susceptibility was affected by the loss of EmrE homologs. There was no alteration, however, in resistance of the *emrE* mutant to methyl viologen (Table 2) and many other antimicrobial agents listed in footnote *a* of Table 2.

Roles of $EmrE_{Pae}$ and MexAB-OprM in intrinsic drug resistance. The MexAB-OprM multidrug efflux system plays a dominant role in the intrinsic multidrug resistance of *P. aeruginosa* as a constitutively expressed pump with a very wide substrate specificity (11, 25). As described in the introduction above, we cannot completely disregard the possibility that MexAB-OprM may also contribute to the resistance to cationic agents, including hydrophilic aminoglycosides. To compare the role of $EmrE_{Pae}$ with that of MexAB-OprM, we constructed a double mutant, HN1115 (Δ*mexAB-oprM* Δ*emrE*). The deletion of *mexAB-oprM* in this strain abolishes at least the function of the two multidrug efflux systems, MexAB-OprM and MexXY-OprM (1, 12), and possibly also others (see below) since OprM may function with other uncharacterized efflux systems (25). Because of this complication, we constructed another double mutant strain, HN1116 (Δ*mexB* Δ*emrE*), carrying an in-frame *mexB* deletion. This mutation does not affect OprM expression (R. Srikumar and K. Poole, unpublished data), and strains K1589 and HN1116 are expected to indicate the contribution of MexAB-OprM system (alone) and $EmrE_{Pae}$ to intrinsic resistance.

As expected, mutant K1589 (Δ*mexB*) became highly susceptible to a variety of antibiotics such as to fusidic acid (8-fold decrease in MIC), phosphonomycin (2-fold decrease in MIC), trimethoprim (2-fold decrease in MIC), β-lactams (8-, 16-, >8-, 8-, 4-, and 2-fold decreases in MIC for azlocillin, carbenicillin, cloxacillin, cefsulodin, cefuroxime, and ceftriaxone, respectively), the fluoroquinolones norfloxacin and ciprofloxacin (both 2-fold decreases in MIC), novobiocin (4-fold decrease in MIC), chloramphenicol (2-fold decrease in MIC), tetracycline (2-fold decrease in MIC), sodium dodecyl sulfate (4-fold decrease in MIC), and triclosan (>2-fold decrease in MIC) (not shown), and the dyes ethidium bromide and acriflavine (Table 2). It was also somewhat surprising that the MICs of all aminoglycosides were decreased (two- to fourfold) in this strain compared to the parent strain HN1112 when the MIC was determined in NB (Table 2). Aminoglycoside MICs were unchanged in MHB and LB medium, an observation consistent with our previous data (11). The additional deletion of $EmrE_{Pae}$ in HN1116 further decreased MICs of dyes and several aminoglycosides in comparison with K1589, mostly in NB (Table 2).

HN1114, deleted not only for MexAB but also for OprM, showed in general MICs identical to those found in K1589. However, there were notable exceptions in which HN1114 was more susceptible than K1589: examples in which the differences were >2-fold included norfloxacin (0.025 versus 0.2 μg/ml), ciprofloxacin (0.013 versus 0.05 μg/ml), enoxacin (0.032 versus 0.25 μg/ml), chloramphenicol (0.5 versus 2 μg/ml), tetracycline (0.125 versus 1 μg/ml), and trimethoprim (1 versus 16 μg/ml). It is likely that other efflux system(s) that also utilize OprM as the outer membrane channel are being expressed constitutively and are extruding these agents. In any case, the additional deletion of $EmrE_{Pae}$ in HN1115 decreased the MICs of ethidium bromide, acriflavine, and some aminoglycosides further from the values found in HN1114 (Table 2).

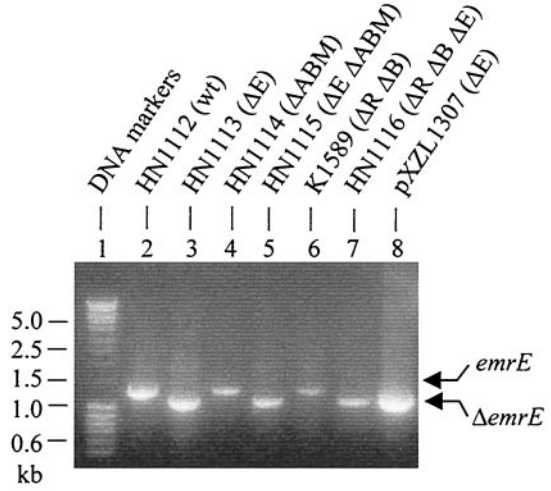

FIG. 3. Confirmation of $emrE_{Pae}$ deletion by PCR amplification of the *emrE* gene of *P. aeruginosa* strains with genomic DNA as templates and primer pair paemre1xz and paemre6xz. Strain designations are indicated above the lanes, and DNA size markers are shown at the left (lane 1).

TABLE 2. MICs of various agents for *P. aeruginosa* producing different combinations of $EmrE_{Pae}$ and MexAB-OprM[a]

Strain (genotype)	MIC (μg/ml)[b]									
	EtBr	AF	MV	AMK	GEN	KAN	NEO	TOB	NAL	PMB
HN1112 (wild type)	256	32	8	0.06 (2, 8)	0.128 (0.5, 4)	4 (64, 128)	0.25 (16, 64)	0.064 (0.5, 2)	32	0.25
HN1113 (Δ*emrE*$_{Pae}$)	64	8	8	0.06 (2, 4)	0.064 (0.5, 4)	2 (64, 128)	0.064 (16, 64)	0.064 (0.5, 2)	16	0.25
HN1114 (Δ*mexAB-oprM*)	64	8	8	0.03 (2, 2)	0.016 (0.25, 0.5)	2 (64, 128)	0.128 (8, 16)	0.032 (0.5, 0.5)	4	0.125
HN1115 (Δ*mexAB-oprM* Δ*emrE*$_{Pae}$)	32	4	8	0.03 (1, 2)	0.016 (0.25, 0.5)	1 (32, 128)	0.032 (8, 8)	0.016 (0.25, 0.5)	4	0.125
K1589 (Δ*mexR* Δ*mexB*)	32	16	8	0.03 (1, 2)	0.032 (0.25, 2)	2 (64, 128)	0.128 (16, 32)	0.032 (0.5, 1)	8	0.25
HN1116 (Δ*mexR* Δ*mexB* Δ*emrE*$_{Pae}$)	16	4	8	0.03 (1, 2)	0.016 (0.25, 2)	1 (64, 128)	0.064 (8, 16)	0.016 (0.25, 1)	4	0.125

[a] MIC values were determined in NB, except the values in parentheses. In addition to the agents listed here, the MICs of the following agents were determined, and some results for the following agents are mentioned in the text: spectinomycin, azlocillin, carbenicillin, cloxacillin, cefsulodin, cefuroxime, ceftriaxone, norfloxacin, ciprofloxacin, enoxacin, novobiocin, chloramphenicol, erythromycin, fusidic acid, tetracycline, trimethoprim, phosphonomycin, rifampin, vancomycin, sodium dodecyl sulfate, and triclosan. Abbreviations: EtBr, ethidium bromide; AF, acriflavine; MV, methyl viologen; AMK, amikacin; GEN, gentamicin; KAN, kanamycin; NEO, neomycin; TOB, tobramycin; NAL, nalidixic acid; PMB, polymyxin B.

[b] Values in parentheses are MIC values obtained in MHB and LB medium as follows: MHB MIC, LB medium MIC.

Ethidium bromide accumulation in intact cells. Susceptibility data suggested that both the $EmrE_{Pae}$ and MexAB-OprM efflux systems contributed to resistance to cationic dyes and aminoglycosides. To confirm that the increased resistance caused by the presence of $EmrE_{Pae}$ was due to active efflux, we examined the accumulation, i.e., the spontaneous influx minus the active efflux, of ethidium bromide by intact cells. The cells were grown in M63-glucose medium, washed, and incubated with ethidium bromide, and the fluorescence caused by the entry of ethidium into cells and its binding to DNA was monitored (Fig. 4). Since the strains were isogenic, spontaneous entry of ethidium can be expected to occur at the same rate, and the net entry rates observed therefore reflect the magnitude (in the negative way) of the active efflux rates. The accumulation was the slowest, and therefore the efflux was most efficient in the wild-type HN1112 strain, whereas the efflux was least efficient in the Δ*emrE*/Δ*mexAB-oprM* double mutant HN1115. HN1113 with the single Δ*emrE* mutation and HN1114 with the single Δ*mexAB-oprM* mutation showed intermediate efflux levels, and the efflux catalyzed by the MexAB-OprM appeared to be slightly more important than the one catalyzed by $EmrE_{Pae}$. Entry was accelerated to nearly the same level in all strains in the presence of proton conductor CCCP (Fig. 4). Finally, the emission spectra of cells incubated in the presence of ethidium bromide confirmed that fluorescence came from ethidium-DNA complex (result not shown).

DISCUSSION

In the present study, we showed that the *P. aeruginosa* homolog of *E. coli* EmrE, $EmrE_{Pae}$, is constitutively expressed and plays a major role in the intrinsic resistance of this organism to cationic dyes ethidium bromide and acriflavine. The contribution of EmrE here was comparable in magnitude to that of the MexAB-OprM system (Table 2). These dyes are well-known substrates of *E. coli* EmrE (19, 24). (Recently, these dyes were shown to induce the production of the normally repressed MexCD-OprJ system, which extrude these dyes efficiently [18]. This system was thus presumably functioning in all of our strains.) In contrast, the deletion of Emr_{Pae} had no effect on the susceptibility to methyl viologen (Table 2), another substrate of *E. coli* EmrE (21, 24). This is consistent with the report of Ninio et al. (21) that methyl viologen was not a good substrate for the purified *P. aeruginosa* $EmrE_{Pae}$.

We also examined the aminoglycoside susceptibility of our mutants. Because aminoglycoside action is antagonized by high ionic strength and by high divalent cation concentrations (15, 27), we used NB with a low ionic strength for MIC determinations. The use of this medium is known not only to lower the aminoglycoside MICs (15) but also to make the contribution of efflux pumps often more visible, as shown earlier for *E. coli acrD* system (27). In this medium, the deletion of *emrE*$_{Pae}$ gene

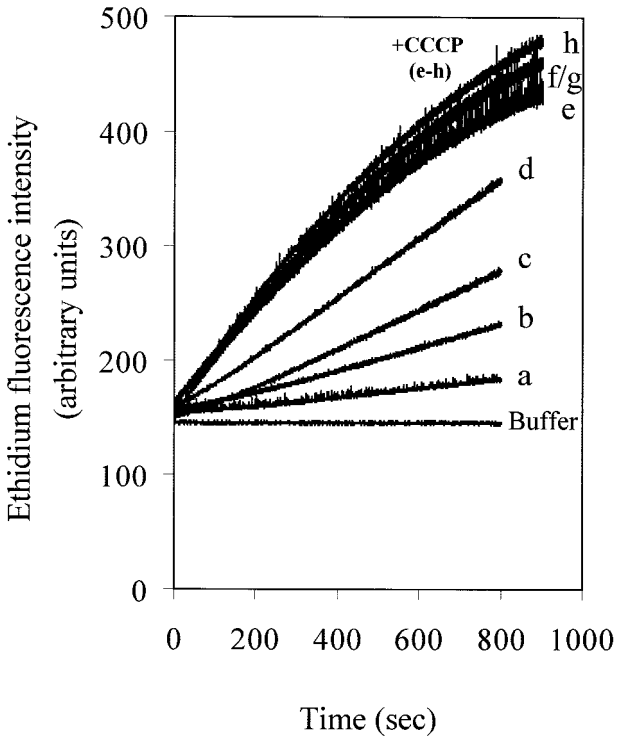

FIG. 4. Ethidium bromide accumulation by intact cells of *P. aeruginosa* monitored in time course by a spectrofluorometer. The isogenic strains compared are HN1112 (wild type [a and e]), HN1113 (Δ*emrE* [b and f]), HN1114 (Δ*mexAB-oprM* [c and g]), and HN1115 (Δ*emrE* Δ*mexAB-oprM* [d and h]). Cells were grown in M63 medium, harvested, washed, and resuspended in a phosphate buffer as described in Materials and Methods. Accumulation was followed in cells not treated by CCCP (panels a to d) or cells pretreated by CCCP (250 μM) (panels e to h). A no-cell control is included (Buffer). Ethidium bromide was used at a final concentration of 5 μM.

was shown to decrease the MICs of the aminoglycosides, except that of amikacin, by two- to fourfold (Table 2). No changes in MICs were observed if LB medium or MHB was used (Table 2). Although neither Emr_{Ec} nor Emr_{Pae} has been known to play a role in aminoglycoside efflux, a recent report (5) showed that an overexpression of a pair of SMR-type pump genes from *B. subtilis* created a multidrug resistance phenotype of unusually broad specificity, including resistance to streptomycin (as well as chloramphenicol and tetracycline).

Interestingly, it became clear that in NB the MexAB-OprM system, which was not known to pump out such hydrophilic agents as aminoglycosides (10, 11, 26), contributed to aminoglycoside resistance to about the same, or even a higher, degree as the $EmrE_{Pae}$ transporter (compare K1589 with HN1112 in Table 2). Other RND family pumps, MexY of *P. aeruginosa* (1, 16) and AcrD of *E. coli* (21, 27), apparently transport both hydrophilic aminoglycosides and amphiphilic or lipophilic agents such as fluoroquinolones and organic cations. Thus, the wider specificity of MexAB-OprM system is probably not too surprising.

There are interesting differences in the respective contribution of MexAB-OprM and Emr_{Pae} to aminoglycoside resistance. Thus, the presence of the former seems to contribute strongly to the resistance to amikacin, tobramycin, and gentamicin, as judged by the two- to fourfold increases in MICs caused by the presence of MexAB-OprM in the $\Delta emrE_{Pae}$ background (compare HN1113 versus HN1116 in Table 2). In contrast, Emr_{Pae} may be important for resistance to especially neomycin, because the additional presence of Emr_{Pae} in K1589 increased neomycin MIC in comparison with HN1116, and the deletion of Emr_{Pae} from the wild type decreased the MIC fourfold (compare HN1113 versus HN1112, Table 2), whereas the presence of the functional MexAB-OprM in HN1113 (lacking Emr_{Pae} alone) did not increase neomycin resistance in comparison with HN1116 (lacking both MexB and Emr_{Pae}) (Table 2). These data are consistent with the notion that MexAB-OprM prefers a slightly more lipophilic members of the aminoglycoside family, whereas Emr_{Pae} prefers strongly hydrophilic members.

MexAB-OprM system also appeared to affect the resistance to amikacin, gentamicin, and tobramycin slightly more strongly in LB medium than in other media. The reason for this difference is unclear.

The MexAB-OprM system is a tripartite pump that extrudes the drug molecules directly into the medium. In contrast, $EmrE_{Pae}$ is a simple pump that transports the drug across the cytoplasmic membrane into the periplasm. Lee et al. (8) showed that the presence of these two types of pumps, if they both act on the same substrate, has a "multiplicative effect" on resistance. Unfortunately the effect of deletion of Emr_{Pae} and MexAB-OprM on the MIC of dyes and aminoglycosides was usually too small to test this idea.

SMR proteins are widely distributed in bacteria and mediate drug resistance via active efflux. Our data indicate that $EmrE_{Pae}$ confers intrinsic drug resistance not only to ethidium bromide but also to aminoglycosides in *P. aeruginosa.* We further showed that MexAB-OprM makes a significant contribution to the intrinsic resistance of this organism to aminoglycosides. However, the effect of both of these efflux pumps became apparent only in a low-ionic-strength medium, NB. The requirement for low ionic strength for the detection of aminoglycoside efflux activity leads to a legitimate question as to the clinical relevance of in aminoglycoside resistance. Human body fluids are high in ionic strength and in the content of both Mg^{2+} and Ca^{2+} (2), and it is difficult to imagine that the efflux by these pumps plays a major role in the intrinsic resistance of this organism under such conditions. However, if these pumps are overproduced, perhaps they may be able to increase resistance levels even under such conditions.

The reason why the aminoglycoside efflux activity becomes visible only in low-ionic-strength media is currently unclear. This may be because the efflux activities of MexAB-OprM and Emr_{Pae} are overshadowed by that of the already known aminoglycoside efflux pump, MexXY-OprM, which is apparently activated by the presence of millimolar concentrations of Mg^{2+} and Ca^{2+} (14). Although the effect of monovalent cations and ionic strength was not studied by Mao et al. (14), it is reasonable to expect that high ionic strength (such as 0.5% NaCl present in LB medium) would mimic the high concentrations of divalent cations (see reference 15). Thus, possibly in low-ionic-strength media such as NB, the MexXY-mediated efflux is poorly functional, and therefore the weaker efflux activity of MexAB-OprM and $EmrE_{Pae}$ becomes detectable. This hypothesis, however, appears to be inconsistent with the observation that even in LB medium or MHB the additional deletion of OprM (which would be necessary for MexXY-OprM activity) did not change the aminoglycoside MICs of *mexB* strain (compare K1589 and HN1114 in Table 2). Possibly, in our strains MexXY was not expressed at detectable levels, as seen earlier (32). In any case, low ionic strength is expected to increase electrostatic repulsion between highly negatively charged lipopolysaccharide molecules and thereby increase the nonspecific permeability of the outer membrane. The increased influx of drugs, including aminoglycosides, across the outer membrane should obscure, rather than reveal, differences caused by the active efflux. Thus, the observed results cannot be explained currently in a straightforward manner. In any case, the interaction of aminoglycoside efflux system(s) with ionic strength and divalent cations is an important topic that requires further study.

ACKNOWLEDGMENTS

We thank R. Srikumar for constructing strain K1589.

This study was supported by a grant from the U.S. Public Health Service (AI-09644) (for work at Berkeley) and by a grant from the Canadian Cystic Fibrosis Foundation (for work at Kingston).

REFERENCES

1. **Aires, J. R., T. Köhler, H. Nikaido, and P. Plésiat.** 1999. Involvement of an active efflux system in the natural resistance of *Pseudomonas aeruginosa* to aminoglycosides. Antimicrob. Agents Chemother. **43:**2624–2628.
2. **Anonymous.** 1986. Case records of Massachusetts General Hospital: weekly clinicopathological exercises. Normal reference values. N. Engl. J. Med. **314:**39–46.
3. **Chung, Y. J., and M. H. Saier, Jr.** 2002. Overexpression of the *Escherichia coli sugE* gene confers resistance to a narrow range of quaternary ammonium compuonds. J. Bacteriol. **184:**2543–2545.
4. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-*FRT* recombination system for site-specific excision of chromosomally located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77–86.
5. **Jack, D. L., M. L. Storms, J. H. Tchieu, I. T. Paulsen, and M. H. Saier, Jr.** 2000. A broad-specificity multidrug efflux pump requiring a pair of homologous SMR-type proteins. J. Bacteriol. **182:**2311–2313.
6. **Jack, D. L., N. M. Yang, and M. H. Saier, Jr.** 2001. The drug/metabolite transporter superfamily. Eur. J. Biochem. **268:**3620–3639.

7. **Laraki, N., M. Galleni, I. Thamm, M. L. Riccio, G. Amicosante, J.-M. Frere, and G. M. Rossolini.** 1999. Structure of In31, a *bla*IMP-containing *Pseudomonas aeruginosa* integron phyletically related to In5, which carries an unusual array of gene cassettes. Antimicrob. Agents Chemother. **43:**890–901.
8. **Lee, A., W. Mao, M. S. Warren, A. Mistry, K. Hoshino, R. Okumura, H. Ishida, and O. Lomovskaya.** 2000. Interplay between efflux pumps may provide either additive or multiplicative effects on drug resistance. J. Bacteriol. **182:**3142–3150.
9. **Li, X.-Z., N. Barré, and K. Poole.** 2000. Influence of the MexA-MexB-OprM multidrug efflux pumps on the expression of the MexC-MexD-OprJ and MexE-MexF-OprJ systems of *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. **46:**885–893.
10. **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.
11. **Li, X.-Z., H. Nikaido, and K. Poole.** 1995. Role of *mexA-mexB-oprM* in antibiotic efflux in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**1948–1953.
12. **Li, X.-Z., and K. Poole.** 2001. Mutational analysis of the OprM outer membrane component of the MexA-MexB-OprM multidrug efflux system of *Pseudomonas aeruginosa.* J. Bacteriol. **183:**12–27.
13. **Link, A. J., D. Phillips, and G. M. Church.** 1997. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. J. Bacteriol. **179:**6228–6237.
14. **Mao, W., M. S. Warren, A. Lee, A. Mistry, and O. Lomovskaya.** 2001. MexXY-OprM efflux pump is required for antagonism of aminoglycosides by divalent cations in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **45:**2001–2007.
15. **Medeiros, A. A., T. F. O'Brien, W. E. C. Wacker, and N. F. Yulug.** 1971. Effect of salt concentration on the apparent in vitro susceptibility of *Pseudomonas* and other gram-negative bacilli to gentamicin. J. Infect. Dis. **124:**S59-S64.
16. **Mine, T., Y. Morita, A. Kataoka, T. Mizushima, and T. Tsuchiya.** 1999. Expression in *Escherichia coli* of a new multidrug efflux pump MexXY from *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **43:**415–417.
17. **Miyamae, S., O. Ueda, F. Yoshimura, J. Hwang, Y. Tanaka, and H. Nikaido.** 2001. A MATE family multidrug efflux transporter pumps out fluoroquinolones in *Bacteroides fragilis*. Antimicrob. Agents Chemother. **45:**3341–3346.
18. **Morita, Y., Y. Komori, T. Mima, T. Kuroda, T. Mizushima, and T. Tsuchiya.** 2001. Construction of a series of mutants lacking all of the four major *mex* operons for multidrug efflux pumps or possessing each one of the operons from *Pseudomonas aeruginosa* PAO1: MexCD-OprJ is an inducible pump. FEMS Microbiol. Lett. **202:**139–143.
19. **Muth, T. R., and S. Schuldiner.** 2000. A membrane-embedded glutamate is required for ligand binding to the multidrug transporter EmrE. EMBO J. **19:**234–240.
20. **Nikaido, H.** 2001. Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. Semin. Cell Dev. Biol. **12:**215–223.
21. **Ninio, S., D. Rotem, and S. Schuldiner.** 2001. Functional analysis of novel multidrug transporters from human pathogens. J. Biol. Chem. **276:**48250–48256.
22. **Nishino, K., and A. Yamaguchi.** 2001. Analysis of a complete library of putative drug transporter genes in *Escherichia coli*. J. Bacteriol. **183:**5803–5812.
23. **Ocaktan, A., H. Yoneyama, and T. Nakae.** 1997. Use of fluorescence probes to monitor function of the subunit proteins of the MexA-MexB-OprM drug extrusion machinery in *Pseudomonas aeruginosa*. J. Biol. Chem. **272:**21964–21969.
24. **Paulsen, I. T., R. A. Skurray, R. Tam, M. H. Saier, Jr., R. J. Turner, J. H. Weiner, E. B. Goldberg, and L. L. Grinius.** 1996. The SMR family: a novel family of multidrug efflux proteins involved with the efflux of lipophilic drugs. Mol. Microbiol. **19:**1167–1175.
25. **Poole, K.** 2001. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. J. Mol. Microbiol. Biotechnol. **3:**255–264.
26. **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.
27. **Rosenberg, E. Y., D. Ma, and H. Nikaido.** 2000. AcrD of *Escherichia coli* is an aminoglycoside efflux pump. J. Bacteriol. **182:**1754–1756.
28. **Saier, M. H., Jr., and I. Paulsen.** 2001. Phylogeny of multidrug transporters. Semin. Cell Dev. Biol. **12:**205–213.
29. **Schuldiner, S., D. Granot, S. Steiner, S. Ninio, D. Rotem, M. Soskin, and H. Yerushalmi.** 2001. Precious things come in little packages. J. Mol. Microbiol. Biotechnol. **3:**155–162.
30. **Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, K., D. Spencer, G. K. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. W. Hancock, S. Lory, and M. V. Olson.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature **406:**959–964.
31. **Wee, S., and B. J. Wilkinson.** 1988. Insights into the cell envelope of *Paracoccus denitrificans*, a member of the α-subdivision of purple bacteria, through studies of its lysozyme susceptibility. Can. J. Microbiol. **34:**952–959.
32. **Westbrock-Wadman, S., D. R. Sherman, M. J. Hickey, S. N. Coulter, Y. Q. Zhu, P. Warrener, L. Y. Nguyen, R. M. Shawar, K. R. Folger, and C. K. Stover.** 1999. Characterization of a *Pseudomonas aeruginosa* efflux pump contributing to aminoglycoside impermeability. Antimicrob. Agents Chemother. **43:**2975–2983.