Expression of *Pseudomonas aeruginosa* Multidrug Efflux Pumps MexA-MexB-OprM and MexC-MexD-OprJ in a Multidrug-Sensitive *Escherichia coli* Strain

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The *mexCD-oprJ* and *mexAB-oprM* operons encode components of two distinct multidrug efflux pumps in *Pseudomonas aeruginosa*. To assess the contribution of individual components to antibiotic resistance and substrate specificity, these operons and their component genes were cloned and expressed in *Escherichia coli*. Western immunoblotting confirmed expression of the *P. aeruginosa* efflux pump components in *E. coli* strains expressing and deficient in the endogenous multidrug efflux system (AcrAB), although only the Δ*acrAB* strain, KZM120, demonstrated increased resistance to antibiotics in the presence of the *P. aeruginosa* efflux genes. *E. coli* KZM120 expressing MexAB-OprM showed increased resistance to quinolones, chloramphenicol, erythromycin, azithromycin, sodium dodecyl sulfate (SDS), crystal violet, novobiocin, and, significantly, several β-lactams, which is reminiscent of the operation of this pump in *P. aeruginosa*. This confirmed previous suggestions that MexAB-OprM provides a direct contribution to β-lactam resistance via the efflux of this group of antibiotics. An increase in antibiotic resistance, however, was not observed when MexAB or OprM alone was expressed in KZM120. Thus, despite the fact that β-lactams act within the periplasm, OprM alone is insufficient to provide resistance to these agents. *E. coli* KZM120 expressing MexCD-OprJ also showed increased resistance to quinolones, chloramphenicol, macrolides, SDS, and crystal violet, though not to most β-lactams or novobiocin, again somewhat reminiscent of the antibiotic resistance profile of MexCD-OprJ-expressing strains of *P. aeruginosa*. Surprisingly, *E. coli* KZM120 expressing MexCD alone also showed an increase in resistance to these agents, while an OprJ-expressing KZM120 failed to demonstrate any increase in antibiotic resistance. MexCD-mediated resistance, however, was absent in a *tolC* mutant of KZM120, indicating that MexCD functions in KZM120 in conjunction with TolC, the previously identified outer membrane component of the AcrAB-TolC efflux system. These data confirm that a tripartite efflux pump is necessary for the efflux of all substrate antibiotics and that the *P. aeruginosa* multidrug efflux pumps are functional and retain their substrate specificity in *E. coli*.

Pseudomonas aeruginosa is an opportunistic human pathogen characterized by an intrinsic resistance to a variety of antimicrobial agents. Previously attributed to a highly impermeable outer membrane (24), this property is now recognized to result from the synergy between broadly specific drug efflux pumps and low outer membrane permeability (22). One such efflux system, encoded by the *mexAB-oprM* operon (8, 32, 33), acts on a range of antibiotics, including tetracycline, chloramphenicol, quinolones, novobiocin, macrolides, trimethoprim, and, apparently, β-lactams and β-lactamase inhibitors (8, 16–18, 33). The β-lactam–β-lactamase inhibitor group of antibiotics are somewhat unique in that their cellular targets are within the periplasm. In contrast to the remaining antibiotic substrates, which act within the cytoplasm and are thus expected to access the pump at the cytoplasmic face of the inner membrane, the β-lactam–β-lactamase inhibitor compounds, if truly eliminated by MexAB-OprM, must interact differently with pump components. Possibilities include interaction with inner membrane constituents at the periplasmic face of the inner membrane or efflux via the outer membrane OprM alone. Still, it is not yet clear that MexAB-OprM and/or the *nalB* mutation associated with MexAB-OprM overexpression (see below)

does not influence β-lactam resistance via an indirect effect on another resistance determinant within *P. aeruginosa*.

Expressed constitutively in wild-type cells, where it contributes to intrinsic drug resistance (4, 17, 33), the *mexAB-oprM* operon is hyperexpressed in *nalB* mutants (34), producing elevated levels of resistance to substrate antibiotics (8, 16, 17, 33). The MexAB-OprM efflux system is highly conserved in serotype, clinical, and environmental strains (2), indicating that it plays an important role in the intrinsic resistance of all examples of this organism. Homologous efflux systems encoded by the *mexCD-oprJ* (31) and *mexEF-oprN* (15) operons have also been described. Apparently not expressed during growth under normal laboratory conditions, these systems are expressed in *nfxB* (31) and *nfxC* (15) multidrug-resistant mutants, respectively. *nfxB* strains are resistant to chloramphenicol, tetracycline, quinolones, macrolides, novobiocin, and "fourth generation" cephalosporins (such as cefepime and cefpirome) but display hypersusceptibility to most β-lactam antibiotics (10). *nfxC* strains elicit resistant to chloramphenicol, trimethoprim, quinolones, and carbapenems, including imipenem, although the last results from the loss of the porin protein OprD in these mutants and not from overexpression of MexEF-OprN (6, 15).

The tripartite efflux pumps consist of an inner membrane component (e.g., MexB, MexD, or MexF) which functions as an RND family H$^+$ antiport exporter (23, 35), an outer membrane, presumably channel-forming component (e.g., OprM,

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
P. aeruginosa		
ML5087	*ilv-220 thr-9001 leu-9001 met-9011 pur-67 aphA*	29
K1111	ML5087 *nfxB*	This study
K1112	ML5087 *nalB*	This study
E. coli		
DH5α	*supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 acrAB+*	Bethesda Research Laboratories
KZM120[b]	Δ*acrAB*::*kan*	21
LBB1201	KZM120[b] *tolC*::Tn*10*	5
BL21(DE3)	F⁻ *ompT* $r_B^- m_B^-$; DE3 is a lambda derivative carrying *lacI* and T7 RNA polymerase genes under p*lac*UV5 control	40
K113	BL21(DE3) carrying the phage T7 lysozyme vector pLysS; Cm^r	39
Plasmids		
pAK1900	*E. coli-P. aeruginosa* shuttle cloning vector carrying p*lac* upstream of MCS; Ap^r Cb^r	R. Sharp, Queen's University
pKMJ002	pAK1900::*nfxB mexCD-oprJ*; *mexCD-oprJ* in orientation opposite to p*lac*	31
pADD214	Mini-D replicon derived from phage D3112; Tc^r	3
pPV1	pADD214 derivative carrying a *P. aeruginosa* chromosomal fragment which includes *mexAB-oprM*	32
pPV20	pAK1900::*mexAB-oprM mexAB-oprM* in orientation opposite to p*lac*	32
pT7-6	pBR322-based expression vector carrying MCS downstream of the strong gene 10 promoter of phage T7; Ap^r	S. Tabor
pRSK01	pT7-6::*mexAB*	This study
pPV6	pAK1900::*mexAB*; *mexAB* in orientation opposite to p*lac*	32
pRSP01	pAK1900::*mexAB-OprM*	This study
pRK415	Low-copy-number, broad-host-range cloning vector carrying MCS downstream of p*lac*; Tc^r	13
pRSP06	pRK415::*oprJ*	This study
pRSP08	pRK415::*oprM*	This study
pRSP15	pRK415::*nfxB mexCD-oprJ*	This study
pRSP17	pRK415::*mexAB-oprM*	This study
pRSP19	pRK415::*mexAB*	This study
pRSP23	pAK1900::*mexCD*; *mexCD* in orientation opposite to p*lac*	This study
pRSP25	pRK415::*mexCD*	This study
pRSP45	pAK1900::*nfxB mexCD-oprJ*	This study
pRSP46	pAK1900::*mexCD*	This study
pSL1180	Superlinker phagemid cloning vector	Pharmacia
pET21-d(+)	pBR322-derived C-terminal His-Tag vector carrying a T7*lac* promoter upstream of a MCS; *lacI* Ap^r	Novagen
pTK15	pET21-d(+)::*mexB*	This study

[a] MCS, multiple cloning site; Ap^r, ampicillin resistant; Cb^r, carbenicillin resistant; Tc^r, tetracycline resistant; T7*lac*, phage T7 promoter followed by a *lac* operator. Unless otherwise indicated, all genes were cloned in the same orientation as p*lac* of pAK1900 or pRK415.
[b] KZM120 is the same strain as HN818.

OprJ, or OprN) (22, 25), and a so-called membrane fusion protein predicted to link the membrane-associated efflux components (e.g., MexA, MexC, or MexE) (22, 25). Recent data suggest that the operation of MexAB-OprM (and by analogy the remaining efflux systems) is at least partially dependent upon the TonB energy-coupling protein implicated in the opening of outer membrane gated channels responsible for iron-siderophore uptake across the *P. aeruginosa* outer membrane (45). Thus, the outer membrane components of these efflux pumps may be gated channels.

Related efflux systems have been described in *Escherichia coli* (*acrAB* [20, 22] and *acrEF* [22], formerly called *envCD* [14]) and *Neisseria gonorrhoeae* (*mtrCDE* [9]). The AcrAB efflux components appear to function in conjunction with the TolC outer membrane protein (5), a pore-forming protein (1) previously implicated in hemolysin (42) and colicin V (44) export across the outer membrane. The *acrAB-tolC*-encoded efflux system is the primary known efflux system contributing to intrinsic resistance in *E. coli*, and *acrAB* deletion strains are markedly susceptible to a variety of antimicrobial agents (21, 30).

In an effort to assess the role of individual efflux system components in resistance and substrate specificity and to demonstrate that antibiotic resistance attributed to the multidrug efflux pumps in *P. aeruginosa* is a true indication of their efflux by these pumps, and in particular that β-lactams are truly substrates for MexAB-OprM, the *mexAB-oprM* and *mexCD-oprJ* operons were expressed in a multidrug-sensitive Δ*acrAB E. coli* strain. We report here that these systems were indeed expressed in *E. coli*, where they contributed the expected resistance to dyes, detergents, and antibiotics, including β-lactams.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are described in Table 1. For the selection of *nfxB* mutants hyperexpressing MexCD-OprJ (e.g., K1111), overnight cultures of *P. aeruginosa* ML5087 were plated on Luria broth-NaCl (LB [see "Media and culture conditions" below]) plates containing 0.4 μg of ciprofloxacin/ml. MexAB-OprM-hyperexpressing *nalB* mutants of this strain (e.g., K1112) were selected on 0.2 μg of ciprofloxacin/ml and 8 μg of cefoperazone/ml. The strains were confirmed as *nfxB* and *nalB* by antimicrobial susceptibility testing and by Western immunoblotting of cell envelopes with antibodies specific to OprJ and OprM, respectively (see Fig. 1).

The *mexCD-oprJ* operon was previously cloned into plasmid pAK1900 on a ca. 10-kb *Bam*HI fragment to yield pKMJ002. Restriction analysis of pKMJ002 revealed that *mexCD-oprJ* was in an orientation opposite to that of the *lac* promoter (p*lac*) of this vector. To facilitate *mexCD-oprJ* expression in *E. coli*, the 10-kb *Bam*HI fragment containing *mexCD-oprJ* was recloned into two other vectors, pRK415 (yielding pRSP15) and pAK1900 (yielding pRSP45), such that the operon was in the same orientation as p*lac*. The *mexCD* genes were also cloned into pRK415 in the same orientation as p*lac* (yielding pRSP25) on an 8.7-kb *Bam*HI fragment derived from pRSP23, a pKMJ002 derivative in which *oprJ* had been eliminated by the deletion of two internal *Eco*RV fragments (0.7 and 0.8 kb). This same *Bam*HI fragment was cloned into pAK1900, again with the *mexCD* genes in the same orientation as p*lac*, to yield pRSP46. Finally, *oprJ* was also cloned into pRK415 in the same orientation as p*lac* on a 2.5-kb *Kpn*I fragment to yield pRSP06. An 8.5-kb *Hind*III fragment containing the *mexAB-oprM* operon of pPV1 was previously cloned into pAK1900, and the resulting plasmid was designated pPV20. Restriction analysis of pPV20 revealed that *mexAB-oprM* was also transcribed in the opposite direction to the *lac* promoter of this vector. To facilitate *mexAB-oprM* expression in *E. coli*, the 8.5-kb *Hind*III fragment of pPV20 was recloned into pAK1900 (to yield pRSP01) and pRK415 (to yield pRSP17) in the same orientation as p*lac*. pPV6, a pAK1900 derivative carrying *mexAB* on a ca. 5-kb *Hind*III fragment, was initially obtained by cloning a *mexAB*-containing 9-kb *Xho*I fragment from pPV1 into the unique *Sal*I site of pT7-6 (to yield pRSK01) followed by its recovery on a ca. 5-kb *Hind*III fragment (one *Hind*III site upstream of *mexA* and a second in the multiple cloning site [MCS], downstream of the *Sal*I-*Xho*I hybrid site). The 5-kb *mexAB*-containing *Hind*III fragment was subsequently cloned from pPV6 into pRK415 in the same orientation as p*lac* to yield pRSP19. pRSP08 is a pRK415 derivative carrying the *oprM* gene on a 4.2-kb *Pst*I fragment of pRSP01 in the same orientation as p*lac*.

Media and culture conditions. Liquid media for routine culturing of *E. coli* and *P. aeruginosa* strains were prepared by dissolving 15.5 g of Miller's Luria broth base (Difco) and 2 g of NaCl per liter of H_2O (LB). For the selection of chromosomal or plasmid antibiotic resistance markers in *E. coli*, ampicillin at 100 μg per ml, kanamycin at 50 μg per ml, and tetracycline at 10 μg per ml were used in the growth media. Solid media were obtained by the addition of Bacto Agar (1.5% [wt/vol]; Difco).

Molecular biological techniques. Restriction endonuclease digestions, ligations, and transformations were performed as described by Sambrook et al. (36). *E. coli* DH5α was used as the host for large-scale isolation of plasmid DNA with a plasmid maxi kit (Qiagen). DNA fragments used in cloning were extracted from agarose gels with Prep-A-Gene (Bio-Rad) according to the manufacturer's instructions. *E. coli* cells were made competent by the $CaCl_2$ method (36) or, when highly competent *E. coli* were required, by the method of Inoue et al. (12).

Isolation of cell envelopes. Twenty milliliters of bacterial culture was grown in LB to an absorbance at 600 nm (A_{600}) of 1.00, harvested by centrifugation, and stored at −20°C. Cells were later thawed on ice, resuspended in 1 ml of phosphate-buffered saline (1.67 mM $NaH_2PO_4 \cdot H_2O$–8.09 mM Na_2HPO_4–150 mM NaCl [pH 7.4]), and sonicated until the cell suspension was clear. Following centrifugation (8,000 × *g*; 10 min) to pellet unlysed cells and debris, the resulting supernatant was centrifuged (300,000 × *g*; 15 min) and the cell envelope pellet was resuspended in 100 μl of H_2O.

Purification of MexB and generation of rabbit polyclonal antiserum. To generate antibodies to MexB, the protein was purified as polyhistidine-tagged MexB (MexB-His) following the cloning and expression of the gene in plasmid pET21-d(+) (Pharmacia). To clone *mexB* into this vector so that the 3′ end of the gene was in frame with the polyhistidine-encoding sequences of pET21-d(+), the following approach was taken. First, the bulk of *mexB* was released from plasmid pPV6 by digestion with *Eco*RI and *Kpn*I, with *Eco*RI cutting upstream of the gene within *mexA* and *Kpn*I cutting 133 bp upstream of the *mexB* terminus. The 133 bp at the 3′ end of *mexB* were then amplified with Vent polymerase (New England Biolabs) and primers TK-1 (5′-GATCGGTACCGGCGTGATCGGCGGCATGGTCACTGCGACCGTCCTGGCGATCTTCTGGGT**G**CC-3′) and TK-2 (5′-AATTCTCGAGTTGCCCCTTTTCGACGGACG-3′). TK-1 eliminates a *Kpn*I site within the 3′ region of *mexB* as a result of an A-G change (highlighted in bold italics) which does not alter the amino acid sequence of MexB but does permit subsequent digestion of the PCR product with *Kpn*I and *Xho*I so that it can be cloned together with the aforementioned *mexB*-containing *Eco*RI-*Kpn*I fragment to regenerate an intact *mexB* gene. PCR mixtures (100 μl) contained 20 ng of pPV6, 1 μM each primer, 200 μM each deoxynucleoside triphosphate, 4 mM $MgSO_4$, 10% (vol/vol) dimethylsulfoxide, and 1 U of Vent polymerase in 1× reaction buffer. Mixtures were heated at 94°C for 2 min followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min a 72°C before finishing with 10 min at 72°C. The PCR product was then purified with the Qiaquick PCR purification kit (Qiagen), digested with *Kpn*I and *Xho*I, and cloned into *Kpn*I-*Xho*I-restricted pSL1180. The insert was sequenced to ensure that the complete 133-bp 3′ region had been cloned and, except for the elimination of the *Kpn*I site, that no alterations in the *mexB* sequences had been introduced during PCR. The *mexB*-containing 3.8-kb *Eco*RI-*Kpn*I fragment was then cloned into *Eco*RI-*Kpn*I-restricted pSL1180 carrying the cloned PCR product, and the restored *mexB* gene was then recovered on a ca. 3.9-kb *Eco*RI-*Xho*I fragment. Following the cloning of this fragment into *Eco*RI-*Xho*I-restricted pET21-d(+), the resultant vector, pTK15, was introduced into *E. coli* BL21(DE3) carrying the pLysS plasmid (strain K113) and expression of *mexB*

was induced with IPTG (isopropyl-β-D-thiogalactopyranoside). Briefly, overnight cultures of pTK15-carrying K113 in LB containing appropriate antibiotics were diluted 1:49 in the same medium (500 ml) and incubated for 4 h, at which time IPTG was added (0.2 mM final concentration). Two hours later, cells were harvested by centrifugation (8,000 × *g*) and cell envelopes were prepared (as above) and solubilized in 2 ml of 20 mM Tris-HCl (pH 8.0)–100 mM NaCl–2% (vol/vol) Triton X-100. The MexB-containing Triton X-100-soluble material (supernatant fraction) was then recovered, following centrifugation (300,000 × *g*), and applied to a 1.5-ml TALON (Clonetech Laboratories, Inc., Palo Alto, Calif.) column equilibrated with 20 mM Tris-HCl (pH 8.0)–100 mM NaCl–0.5% (vol/vol) Triton X-100 (column buffer). The column was washed with 15 ml of column buffer, and bound protein was eluted with column buffer containing 100 mM imidazole at a flow rate of 0.1 ml/min. Three hundred-microliter fractions were collected, and MexB-containing fractions, identified following analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were pooled and dialyzed against 2 liters of 20 mM Tris-HCl (pH 8.0)–0.5% (vol/vol) Triton X-100. Antibodies to purified MexB-His were subsequently raised in rabbits by L. Mutharia, Department of Microbiology, University of Guelph.

SDS-PAGE and immunoblotting. SDS-PAGE was performed as described previously (19) with 10% (wt/vol) acrylamide in the running gel. Proteins resolved in gels were Coomassie blue stained or transferred electrophoretically to nitrocellulose membranes (BA85; Schleicher & Schuell). Electrophoretic transfer of proteins was as described previously (41) except that SDS (0.1% [wt/vol]) was included in the blotting buffer and transfer was carried out for 16 h at 4°C and 25 mA constant current. Blotted membranes were subsequently incubated in phosphate-buffered saline containing 0.1% (vol/vol) Tween 20 (BDH) (PBST) and 10% (wt/vol) skim milk (Difco) for 60 min. Following two 5-min washes with PBST, the membranes were incubated for 60 min with primary antibody in PBST containing 1% (wt/vol) bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) and then washed four times for 10 min each time with PBST. Secondary antibody conjugated to horseradish peroxidase (HRP) in PBST containing 1% bovine serum albumin was then added to the membranes, which were subsequently washed four times for 10 min each time with PBST. Substrates for HRP were from the ECL Western blotting detection kit (Amersham) and were used according to the manufacturer's instructions. The enzymatic activity of HRP was detected as the emission of chemiluminescence after the exposure of blots to XAR 5 film (Kodak). All incubations and washings in the immunoblot procedure were carried out at room temperature with agitation. The primary antibodies anti-OprJ (11) and anti-OprM (7) were previously described mouse monoclonals, and anti-MexB was a rabbit polyclonal antiserum (see above). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Jackson Laboratories and Amersham, respectively.

Antimicrobial susceptibility testing. The susceptibilities of *E. coli* strains to a number of antimicrobial agents were tested by inoculating 1 ml of LB containing serial twofold dilutions of each antimicrobial agent with 10^5 organisms as described previously (17). Bacterial inocula were derived from stocks prepared from overnight cultures grown in LB which had been harvested and resuspended in an equal volume of 10 mM $MgSO_4$.

Antimicrobial agents. Ampicillin, penicillin G, ticarcillin, cefotaxime, cefoperazone, cefsulodin, cephaloridine, ceftriaxone, ciprofloxacin, norfloxacin, novobiocin, chloramphenicol, and tetracycline were purchased from Sigma. SDS and crystal violet were purchased from ICN Biochemicals, Inc., and Difco, respectively. Cefepime was provided by Bristol-Myers Squibb. Azithromycin was a gift from Pfizer. Sparfloxacin (Rhône-Poulenc Rorer) and pefloxacin (Laboratoire Roger Bellon) were gifts from Microcide Pharmaceuticals Inc., Mountain View, Calif. Cefpirome (Roussel Uclaf) and ceftazidime (Glaxo) were also gifts. Erythromycin (Abbott) and imipenem (Merck Sharpe & Dohme) were purchased from the pharmacy of the Kingston General Hospital.

RESULTS AND DISCUSSION

Expression of MexAB-OprM in *E. coli*. To assess the operation of MexAB-OprM in *E. coli*, it was necessary to express the *P. aeruginosa* multidrug efflux system in this heterologous host. Using antibodies specific to MexB and OprM (Fig. 1A and B, lanes 2), therefore, we examined the production of pump components in *E. coli*. Initially, *mexAB-oprM* was cloned into the low-copy-number, broad-host-range vector pRK415 (to yield pRSP17) in the same orientation as the *lac* promoter of this vector in case it was necessary to induce expression of the operon in order to obtain detectable expression of the pump components. Introduction of pRSP17 into *E. coli* DH5α (AcrAB⁺) (data not shown) or KZM120 (AcrAB⁻) provided for substantial expression of MexAB-OprM without IPTG induction, as evidenced by the production of MexB (Fig. 1A, lane 8) and OprM (Fig. 1B, lane 8) in cell envelopes of these strains. Similarly, *E. coli* KZM120 carrying the *mexAB* vector pRSP19
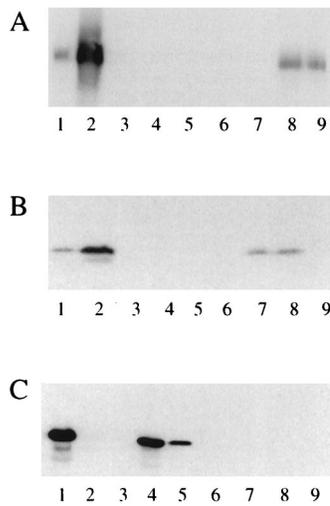
FIG. 1. Western immunoblots of bacterial cell envelopes (10 µg of protein) developed with antibodies raised against MexB (A), OprM (B), and OprJ (C). Lanes 1, K1111 (*nfxB*); lanes 2, K1112 (*nalB*); lanes 3, KZM120 carrying pRK415; lanes 4, KZM120 carrying pRSP06 (*oprJ*); lanes 5, KZM120 carrying pRSP15 (*nfxB mexCD-oprJ*); lanes 6, KZM120 carrying pRSP25 (*mexCD*); lanes 7, KZM120 carrying pRSP08 (*oprM*); lanes 8, KZM120 carrying pRSP17 (*mexAB-oprM*); lanes 9, KZM120 carrying pRSP19 (*mexAB*). (The relevant efflux phenotype or genes expressed are indicated in parentheses.)

TABLE 3. β-lactam susceptibility of *E. coli* strains expressing components of the *P. aeruginosa* multidrug efflux pumps[a]

Plasmid	Efflux genes	MIC (µg/ml)[b,c]				
		AMP	PEN	TIC	CPZ	TAX
pRK415	—[d]	16	32	4	0.128	0.064
pRSP17	*mexAB-oprM*	128	128	32	0.512	0.128
pRSP15	*mexCD-oprJ*	32	32	4	0.256	0.064
pRSP25	*mexCD*	32	32	4	0.256	0.064

[a] The susceptibility of *E. coli* KZM120 (Δ*acrAB*::*kan*) carrying *P. aeruginosa* multidrug efflux genes on the indicated plasmids to various antimicrobial agents was assayed as described in Materials and Methods.
[b] AMP, ampicillin; PEN, penicillin G; TIC, ticarcillin; CPZ, cefoperazone; TAX, cefotaxime.
[c] *E. coli* KZM120 carrying pRSP08 (*oprM*), pRSP19 (*mexAB*) and pRSP06 (*oprJ*) showed the same resistance pattern as that exhibited by KZM120 carrying pRK415. β-lactams for which no change in susceptibility was observed for KZM120 with or without plasmids containing efflux genes included cephaloridine, cefsulodin, cefepime, cefpirome, imipenem, ceftriaxone, and ceftazidime.
[d] —, no plasmid-borne efflux genes.

or the *oprM* vector pRSP08 produced substantial levels of cell envelope-associated MexB (Fig. 1A, lane 9) and OprM (Fig. 1A, lane 7), respectively, without IPTG induction. Thus, *E. coli* carrying *mexAB-oprM* (or *mexCD-oprJ* [see below]) or its components was assessed for antibiotic susceptibility in the absence of IPTG.

Activity of MexAB-OprM in *E. coli*. Despite the expression of MexAB-OprM in *E. coli* DH5α, the presence of pRSP17 failed to enhance the antibiotic resistance of this strain to any of the tested agents (data not shown) (Tables 2 and 3 list the agents tested). In contrast, the *acrAB* deletion strain KZM120 carrying pRSP17 exhibited elevated resistance to a variety of agents, including the more hydrophobic quinolones, novobiocin, macrolides, chloramphenicol, detergents, dyes, and the penicillin subgroup of the β-lactam antibiotics (Tables 2 and 3). Although the influence of pRSP17 on tetracycline resis-

tance could not be assessed (the vector carries a *tet* gene), KZM120 carrying pRSP01, a *bla* vector with the *mexAB-oprM* operon, did show a twofold increase in resistance to tetracycline (data not shown).

This pattern of resistance is less broad than that attributed to MexAB-OprM in *P. aeruginosa*, where resistance to a variety of cephalosporins, including cefotaxime, ceftazidime, cefsulodin, ceftriaxone, cefepime, and cefpirome, and to the quinolones ciprofloxacin and norfloxacin is also afforded by this efflux system (17, 37, 38). Nonetheless, many of the agents to which MexAB-OprM provided resistance in *E. coli* are also accommodated by the *E. coli* acrAB-encoded efflux system, which has been shown to facilitate resistance to chloramphenicol, tetracycline, penicillins (penicillin G and ampicillin), nalidixic acid, novobiocin, erythromycin, SDS, and crystal violet (20, 21, 30). In light of the high degree of homology between MexAB and AcrAB (MexA-AcrA, 57.7% identity; MexB-AcrB, 69.8% identity), it is perhaps not surprising that MexAB-OprM is functional in *E. coli* and that it accommodates a similar range of agents. Still, even for those agents for which resistance is afforded by MexAB-OprM in *E. coli*, resistance levels attributed to this pump are markedly lower in this organism than in *P. aeruginosa* (17, 33, 38). This reduced range of antibiotic

TABLE 2. Antimicrobial susceptibility of *E. coli* expressing components of the *P. aeruginosa* multidrug efflux pumps[a]

Strain	Plasmid	Efflux genes	MIC (µg/ml)[b,c]									
			ERY	AZI	CAM	SDS	CV	CIP	NOR	SPAR	PEF	NOV
KZM120	pRK415	—[d]	16	1	1	32	2	0.008	0.016	0.008	0.032	8
KZM120	pRSP17	*mexAB-oprM*	64	2	2	>256	8	0.008	0.016	0.016	0.064	32
KZM120	pRSP15	*mexCD-oprJ*	>256	8	2	256	>16	0.016	0.064	0.016	0.064	8
KZM120	pRSP25	*mexCD*	>256	8	2	256	>16	0.016	0.064	0.016	0.064	8
KZM120	pAK1900	—; TolC+	4	0.5	ND[e]	128	0.25	ND	0.016	ND	ND	ND
KZM120	pRSP45	*mexCD-oprJ*; TolC+	64	4	ND	>1,024	>16	ND	0.032	ND	ND	ND
KZM120	pRSP46	*mexCD*; TolC+	64	4	ND	>1,024	>16	ND	0.032	ND	ND	ND
LBB1201	pAK1900	—; TolC−	4	0.5	ND	16	0.25	ND	0.016	ND	ND	ND
LBB1201	pRSP45	*mexCD-oprJ*; TolC−	256	16	ND	>1,024	>16	ND	0.128	ND	ND	ND

[a] The susceptibility of *E. coli* KZM120 (Δ*acrAB*::*kan*) and LBB1201 (KZM120 *tolC*::Tn*10*) carrying *P. aeruginosa* multidrug efflux genes on the indicated plasmids to various antimicrobial agents was assayed as described in Materials and Methods.
[b] ERY, erythromycin; AZI, azithromycin; CAM, chloramphenicol; SDS, sodium dodecyl sulfate; CV, crystal violet; CIP, ciprofloxacin; NOR, norfloxacin; SPAR, sparfloxacin; PEF, pefloxacin; NOV, novobiocin.
[c] *E. coli* KZM120 carrying plasmids pRSP08 (*oprM*), pRSP19 (*mexAB*), or pRSP06 (*oprJ*) showed the same pattern of resistance as exhibited by KZM120 carrying the pRK415 vector control. *E. coli* LBB1201 carrying pRSP46 (*mexCD*) showed the same pattern of resistance as LBB1201 carrying the pAK1900 vector control.
[d] —, no plasmid-borne efflux genes.
[e] ND, not determined.

substrates and these lower resistance levels likely reflect the markedly higher outer membrane permeability of *E. coli* compared to that of *P. aeruginosa* (26, 27), which increases drug influx and probably compromises the contribution of MexAB-OprM-mediated drug efflux to resistance. Indeed, it has been proposed that significant intrinsic antibiotic resistance results from a synergy between reduced influx (i.e., low outer membrane permeability) and active efflux and that both determinants are indispensable (22). Certainly, the higher outer membrane permeability of *E. coli* has been used to explain the organism's generally greater susceptibility to antimicrobial agents compared to that of *P. aeruginosa*, despite the presence of multidrug efflux systems in both (22).

The increased outer membrane permeability of *E. coli* reflects an increased permeability to hydrophilic agents, which typically use the porin pathway for uptake across the outer membrane. It is not unexpected, therefore, that resistance afforded by MexAB-OprM (and MexCD-OprJ [see below]) in *E. coli* is generally confined to the more hydrophobic agents, whose uptake will still be somewhat restricted. The contribution of MexAB-OprM to β-lactam resistance in *E. coli*, for example, was generally limited to the penicillins (e.g., ampicillin, penicillin G, and ticarcillin), whose nuclei are more hydrophobic than those of the cephalosporins (43). Moreover, although MexAB-OprM-mediated resistance to the cephalosporins cefotaxime, ceftazidime, ceftriaxone, cefoperazone, cefpirome, and cefepime was minimal or lacking in *E. coli*, resistance to these cephalosporins is readily provided by this efflux system in *P. aeruginosa* (17, 37, 38), indicating that the lack of cephalosporin resistance in MexAB-OprM-expressing *E. coli* is not due to a deficiency of the pump. The observation that MexAB-OprM-expressing *E. coli* is less susceptible (though still not resistant) to cefoperazone is likely explained by the presence of an exceptionally bulky side chain (43), which is expected to impede the drug's passage across the outer membrane, thereby enhancing the contribution of efflux to resistance to this agent.

Arguably, the most significant observation was that β-lactam (penicillin) resistance was afforded by MexAB-OprM in *E. coli* KZM120, since this confirmed that this efflux system can indeed accommodate β-lactams and that it is directly responsible for the β-lactam resistance attributed to it in, e.g., *nalB* strains. Interestingly, however, and despite the periplasmic location of their targets, KZM120 expressing *oprM* alone did not exhibit enhanced resistance to β-lactams (or any other agent) (Tables 2 and 3), indicating that the inner membrane-associated components are essential features of a β-lactam-extruding pump and that OprM alone is unable to facilitate β-lactam efflux and, thus, resistance. Similarly, MexAB alone (pRSP19) also failed to influence antibiotic resistance in the Δ*acrAB* strain (Tables 2 and 3), demonstrating that a tripartite pump is the only efflux-competent entity.

Although not previously reported for *P. aeruginosa*, the observation that MexAB-OprM expression afforded resistance to crystal violet and SDS in *E. coli* indicated that, as with AcrAB, the MexAB-OprM efflux pump can accommodate dyes and detergents. Interestingly, recent comparisons of MexB-OprM$^+$ and MexAB-OprM$^-$ strains of *P. aeruginosa* failed to reveal any differences in susceptibility to either of these agents (data not shown). The MexAB-OprM$^-$ strain was, in fact, very resistant to both SDS and crystal violet (data not shown), presumably due to the presence of additional efflux systems in this strain which are capable of exporting these compounds.

Expression of MexCD-OprJ in *E. coli*. pRSP15 carrying *mexCD-oprJ* was also introduced into *E. coli* DH5α and KZM120, and expression of the efflux system was assessed by immunoblotting with an available anti-OprJ antiserum. As above, *E. coli* DH5α (data not shown) and KZM120 carrying this vector demonstrated OprJ production (Fig. 1C, lane 5) consistent with the expression of the *mexCD-oprJ* operon in this strain. Interestingly, introduction of pKMJ002 (the original *mexCD-oprJ* vector, which carries the efflux genes in an orientation opposite to that of the *lac* promoter on this plasmid) into KZM120 failed to elicit any antibiotic resistance, indicating that, while IPTG induction may not have been necessary for expression of efflux components, expression from the *lac* promoter was probably critical. KZM120 carrying *oprJ* alone on pRSP06 also demonstrated substantial OprJ production (Fig. 1C, lane 4); in fact, levels were markedly higher than those seen for KZM120 carrying pRSP15. This difference in OprJ expression may be due to the presence of the *nfxB* gene, encoding a repressor of *mexCD-oprJ* expression (28, 31), on pRSP15, which might have limited *mexCD-oprJ* (and hence OprJ) expression from this vector. The ability of the *nfxB* gene to repress expression of a *mexC-lacZ* fusion in *E. coli* has been demonstrated (31). In contrast, the *mexAB-oprM*-encoding plasmid pRSP17 lacks the *mexR* repressor gene implicated in regulation of *mexAB-oprM* (34), and accordingly, the levels of OprM detected in KZM120 carrying *oprM* alone (Fig. 1B, lane 7) or *mexAB-oprM* (Fig. 1B, lane 8) were similar.

Activity of MexCD-OprJ in *E. coli*. *E. coli* KZM120 carrying *mexCD-oprJ* on pRSP15 demonstrated elevated resistance to a variety of agents, including macrolides, quinolones, chloramphenicol, SDS, and crystal violet (Table 2). As was seen for MexCD-OprJ in *P. aeruginosa*, this efflux system did not facilitate resistance to novobiocin (Table 2) or most β-lactams (Table 3) in *E. coli*. The decreased susceptibility of MexCD-OprJ-expressing *E. coli* to cefoperazone, however, was consistent with previous observations that *nfxB* strains of *P. aeruginosa* show slightly reduced cefoperazone susceptibility (38). Interestingly, although MexCD-OprJ-expressing strains of *P. aeruginosa* display resistance to the fourth generation cephalosporins cefepime and cefpirome (17, 38), this efflux system failed to alter the susceptibility of *E. coli* KZM120 to these agents (Table 3), again highlighting the point that the *P. aeruginosa* efflux systems are not as effective in providing resistance to substrate antibiotics in *E. coli*. The expression of OprJ alone on pRSP06 (Fig. 1C, lane 4) did not increase the resistance of KZM120 to any tested antimicrobial agent (Table 2), although KZM120 expressing MexCD from pRSP25 exhibited the same profile of resistances as the MexCD-OprJ-expressing KZM120(pRSP15) (Table 2). The dispensability of OprJ suggests that some *E. coli* outer membrane protein substitutes for OprJ in the operation of this efflux system.

Role of TolC in MexCD-mediated antibiotic resistance in *E. coli*. Previous studies have shown that deletion of *oprJ* in a *mexCD-oprJ*-hyperexpressing *nfxB* strain compromised the resistance afforded by the MexCD-OprJ system (38), indicating that the MexCD components are not efflux competent, at least in *P. aeruginosa*, in the absence of an outer membrane pump constituent. The TolC outer membrane protein, implicated as the outer membrane component of the AcrAB efflux system (5), is one possible candidate for association with MexCD in the reconstitution of an efflux system in *E. coli*. Precedents exist for the functioning of such so-called chimeric efflux systems, since functional MexCD-OprM and MexAB-OprJ pumps have been successfully constructed in *P. aeruginosa* (38). To assess the possible involvement of TolC in OprJ-independent, MexCD-mediated multidrug resistance, the *mexCD* genes were introduced into *E. coli* LBB1201, a KZM120 derivative carrying a *tolC*::Tn*10* mutation. Because of the Tn*10*-encoded tetracycline resistance of this strain, how-

ever, it was necessary to introduce these genes on the pAK1900-based *bla* (β-lactam resistance) plasmid pRSP46 rather than on the aforementioned tetracycline resistance vector, pRSP25. The pRSP46 vector failed to increase the resistance of LBB1201 to several agents, including erythromycin, azithromycin, norfloxacin, crystal violet, and SDS, although as expected, it did increase the resistance of the TolC$^+$ strain KZM120 to these agents (Table 2), indicating that the observed MexCD-mediated multidrug resistance in Δ*acrAB* strains of *E. coli* requires TolC. Thus, the latter protein likely functions as the outer membrane efflux system component of a MexCD-TolC pump. Introduction of *mexCD-oprJ* on the pAK1900-based vector pRSP45 also facilitated resistance to erythromycin, azithromycin, norfloxacin, crystal violet, and SDS in both KZM120 and LBB1201 (Table 2), consistent with the native MexCD-OprJ efflux system being operational in *E. coli*, independent of the AcrAB-TolC components, and with strain LBB1201 being capable of reconstituting a functional MexCD-OprJ efflux system. Interestingly, the MexCD-OprJ system appeared to function better in the TolC$^-$ strain LBB1201 than in the TolC$^+$ strain KZM120 (Table 2), suggesting that by virtue of its apparent ability to associate with MexCD, TolC may interfere with the proper association and activity of the MexCD-OprJ components.

These data indicate that the context in which an efflux system operates is important for the expression of resistance and that, limitations in expression of efflux components notwithstanding, MexAB-OprM and MexCD-OprJ in *E. coli* do not facilitate resistance to as many agents or to levels as high as in *P. aeruginosa*. Attempts at reconstituting AcrAB in *P. aeruginosa* (using genes cloned into pRK415 from pUC15C [20]) to see if this system is more active in the less permeable *P. aeruginosa* have so far failed, apparently because of the lethality of these genes on multicopy vectors in this organism (37). The demonstrated ability to reconstitute MexCD-OprJ and MexAB-OprM activity in *E. coli* should nonetheless facilitate intended studies of antibiotic efflux by using everted vesicles, a technology which is better developed for this organism. Such studies of the *P. aeruginosa* pumps should provide novel insights into the molecular and biochemical events that lead to drug efflux.

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