JOURNAL OF BACTERIOLOGY, Dec. 1997, p. 7875–7881 Vol. 179, No. 24
0021-9193/97/$04.00+0

Inner Membrane Efflux Components Are Responsible for β-Lactam Specificity of Multidrug Efflux Pumps in *Pseudomonas aeruginosa*

RAMAKRISHNAN SRIKUMAR, XIAN-ZHI LI, AND KEITH POOLE*

Department of Microbiology and Immunology, Queen's University, Kingston, Ontario K7L 3N6, Canada

Received 23 July 1997/Accepted 14 October 1997

A major feature of the MexAB-OprM multidrug efflux pump which distinguishes it from the MexCD-OprJ and MexEF-OprN multidrug efflux systems in *Pseudomonas aeruginosa* is its ability to export a wide variety of β-lactam antibiotics. Given the periplasmic location of their targets it is feasible that β-lactams exit the cell via the outer membrane OprM without interaction with MexA and MexB, though the latter appear to be necessary for OprM function. To test this, chimeric MexAB-OprJ and MexCD-OprM efflux pumps were reconstituted in Δ*mexCD* Δ*oprM* and Δ*mexAB* Δ*oprJ* strains, respectively, and the influence of the exchange of outer membrane components on substrate (i.e., β-lactam) specificity was assessed. Both chimeric pumps were active in antibiotic efflux, as evidenced by their contributions to resistance to a variety of antimicrobial agents, although there was no change in resistance profiles relative to the native pumps, indicating that OprM is not the determining factor for the β-lactam specificity of MexAB-OprM. Thus, one or both of inner membrane-associated proteins MexA and MexB are responsible for drug recognition, including recognition of β-lactams.

Pseudomonas aeruginosa is an opportunistic human pathogen characterized by an innate resistance to a variety of antimicrobial agents. Previously attributed to a highly impermeable outer membrane (27), this so-called intrinsic multidrug resistance is now known to result from the synergy between broadly specific drug efflux pumps and a low degree of outer membrane permeability (22). One such efflux system, a tripartite pump encoded by the *mexAB-oprM* operon (10, 31, 32), exports a range of antibiotics, including tetracycline, chloramphenicol, quinolones, novobiocin, macrolides, trimethoprim, and β-lactams (10, 15, 20, 32). The β-lactam antibiotics are somewhat unique among these efflux substrates in that their cellular targets are within the periplasm. It is possible, therefore, that these agents are not exported via MexAB-OprM but rather that the efflux pump or the *nalB* mutation associated with MexAB-OprM overexpression (see below) or both somehow influence other β-lactam resistance determinants within *P. aeruginosa*. Still, recent data demonstrating MexAB-OprM-dependent expression of β-lactam resistance in *Escherichia coli* (38) support a role for the efflux system in the export of this class of compounds.

Expressed constitutively in wild-type cells where it contributes to intrinsic drug resistance (5, 20, 32), the *mexAB-oprM* operon is hyperexpressed in *nalB* mutants (33), which are thus resistant to elevated levels of substrate antibiotics (10, 15, 20, 32). Homologous efflux systems encoded by the *mexCD-oprJ* (30) and *mexEF-oprN* (16) operons have also been described. Apparently not expressed during growth under normal laboratory conditions, these systems are expressed in *nfxB* (30) and *nfxC* (16) multidrug-resistant mutants, respectively. *nfxB* strains are resistant to chloramphenicol, tetracycline, quinolones, macrolides, and "fourth-generation" cephalosporins such as cefepime and cefpirome but display hypersusceptibility to most β-lactam antibiotics (11). *nfxC* strains exhibit resistance to chloramphenicol, trimethoprim, quinolones, and carbapenems, including imipenem, although resistance to the latter results from the loss of porin protein OprD in these mutants and not from overexpression of MexEF-OprN (8, 16). These tripartite efflux pumps consist of an inner membrane component (MexB, MexD, or MexF) which functions as a resistance-nodulation-division (RND) family H^+ antiport exporter (26, 34), an outer membrane, presumably a channel-forming component (OprM, OprJ, or OprN) (22, 28), and a so-called membrane fusion protein predicted to link the membrane-associated efflux components (MexA, MexC, or MexE) (22, 28).

Most of the antibiotics which are exported via MexAB-OprM act within the cytoplasm and are thus expected to access the pump at the cytoplasmic face of the inner membrane. β-Lactams, by virtue of the periplasmic nature of their targets, must, however, interact differently with pump components. Possibilities include interaction with inner membrane constituents at the periplasmic face of the cytoplasmic membrane or within the cytoplasmic membrane itself (22) or export via the outer membrane OprM alone. The observation that *mexAB-oprM* but not *oprM* alone promoted β-lactam resistance in *E. coli* (38) suggested that OprM was insufficient for β-lactam efflux. Still, it was possible that OprM was the sole route by which β-lactams exited the cell but that the operation of this protein was dependent upon the activity of and/or its association with the other components of this efflux system.

In an effort to determine if the outer membrane OprM protein was the determining factor for β-lactam recognition and efflux and thus for β-lactam resistance attributed to MexAB-OprM, chimeric pumps were constructed whereby the outer membrane constituents of the MexAB-OprM and MexCD-OprJ pumps were switched and the influence on substrate specificity was assessed.

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. NfxB-type strains were selected from Δ*mexAB-oprM* derivative K1121 by plating on Luria-Bertani (LB) agar (see below) containing 0.2 μg of ciprofloxacin per ml. Hyperexpression of MexCD-OprJ was confirmed in one of these (K1131) by using antisera specific to MexD (Fig. 1C, lane 5) and OprJ (Fig. 1D, lane 5).

* Corresponding author. Phone: 613-545-6677. Fax: 613-545-6796. E-mail: poolek@post.queensu.ca.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[a]	Source or reference
E. coli		
DH5α	*endA hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 deoR* [ϕ80 d*lac*Δ (*lacZ*)*M15*]	1
S17-1	*thi pro hsdR recA* Tra^+	37
MM294	*supE44* λ^- *rfbD1 spoT thi-1 endA1 hsdR17 pro*	25
GM2163	*ara-14 leuB6 thi-1 fhuA31 lacY tsx-78 galK2 galT22 supE44 hisG4 rpsL136 xyl-5 mtl-1 dam-13*::Tn*9 dcm-6 mcrB1 hsdR2 mcrA*	New England Biolabs
KZM120	Δ*acrAB*::*kan*	21
P. aeruginosa		
ML5087	*ilv-220 thr-9001 leu-9001 met-9011 pur-67 aphA*	29
K1114	ML5087 Δ*mexCD-oprJ*	This study
K1126	K1114 *nalB*	This study
K1127	K1126 Δ*oprM*	This study
K1121	ML5087 Δ*mexAB-oprM*	This study
K1131	K1121 *nfxB*	This study
K1132	K1131 Δ*oprJ*	This study
PAO6609	*met-9011 amiE200 rpsL pvd-9*	12
K880	PAO6609 *mexB*::ΩHg	Q. Zhao
Plasmids		
pMMB206	Broad-host-range cloning vector; *lacI* p*lac* MCS Cm^r	24
pRK415	Broad-host-range cloning vector; p*lac* MCS Tc^r	14
pVLT31	Broad-host-range expression vector; *lacI* p*lac* MCS Tc^r	4
pRK2013	Broad-host-range helper vector; Tra^+ Km^r	6
pK18*mobsacB*	Gene replacement vector derived from plasmid pK18; Mob^+ *sacB* Km^r	36
pBR322	ColE1-derived cloning vector; Tc^r Ap^r	35
pAK1900	*E. coli-P. aeruginosa* shuttle cloning vector; Ap^r Cb^r	R. Sharp; this department
pPV20	pAK1900::*mexAB-oprM*; *mexAB-oprM* in the opposite orientation to p*lac*	31
pKMJ002	pAK1900::*mexCD-oprJ*	30
pRSP14	pPV20::Δ*mexAB-oprM*; carries a 4,104-bp deletion from codon 172 of *mexA* to codon 104 of *oprM*	This study
pRSP21	pK18*mobsacB*::Δ*mexAB-oprM*	This study
pRSP02	pBR322::*mexCD-oprJ*	This study
pRSP03	pRSP02::Δ*mexCD-oprJ*; carries a 4,462-bp deletion from codon 218 of *mexC* to codon 263 of *oprJ*	This study
pRSP05	pK18*mobsacB*::Δ*mexCD-oprJ*	This study
pRSP01	pAK1900::*mexAB-oprM*; *mexAB-oprM* in the same orientation as p*lac*	38
pXZL01	pBR322 derivative carrying a deletion of the 0.77-kb *Sal*I-*Ava*I fragment	This study
pXZL02	pXZL1::*oprM*	This study
pXZL03	pXZL2::Δ*oprM*; carries a 517-bp deletion in *oprM*	This study
pXZL04	pK18*mobsacB*::Δ*oprM*	This study
pRSP06	pRK415::*oprJ*	38
pRSP24	pK18*mobsacB*::*oprJ*	This study
pRSP27	pK18*mobsacB*::Δ*oprJ*; carries a 660-bp deletion in *oprJ*	This study
pRSP08	pRK415::*oprM*	38
pKPM-2	pVLT31::*oprM*	39
pT7-7	pBR322 derivative carrying an MCS downstream of a strong phage T7 gene 10 promoter and a ribosome-binding site; Ap^r	S. Tabor
pKPJ-1	pT7-7::*oprJ*	This study
pKPJ-2	pVLT31::*oprJ*	This study
pRSP19	pRK415::*mexAB*	38
pRSP35	pRK415::*mexB*	This study
pRSP25	pRK415::*mexCD*	38
pRSP37	pRK415::*mexD*	This study
pRSP38	pRK415::*mexC*	This study
pRSP39	pMMB206::*mexC*	This study
pQZ05	pMMB206::*oprM*	Q. Zhao

[a] Km^r, kanamycin resistant; Tc^r, tetracycline resistant; Cm^r, chloramphenicol resistant; Ap^r, ampicillin resistant; Cb^r, carbenicillin resistant; MCS, multiple cloning site; p*lac*, *lac* promoter.

Similarly, NalB-type strains were selected from Δ*mexCD-oprJ* derivative K1114 by plating on LB agar containing 0.2 μg of ciprofloxacin and 8 μg of cefoperazone per ml. Hyperexpression of MexAB-OprM was confirmed in one of these (K1126) with antisera specific to MexB (Fig. 1A, lane 4) and OprM (Fig. 1B, lane 4). For the construction of pKPJ-1, the *oprJ* gene was amplified from pKMJ003 with Vent DNA polymerase (New England Biolabs) and primers oprJ-1 (5′-AGCTCATA TGCGCAAACCTGCTTTCG-3′), which carries an embedded *Nde*I site at the ATG start codon of the *oprJ* gene, and oprJ-2

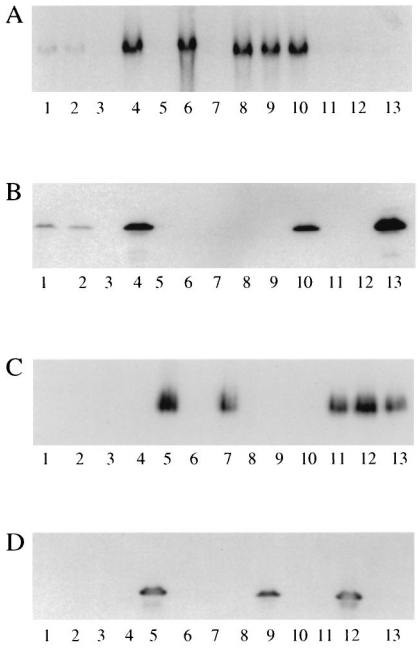

FIG. 1. Western immunoblots of bacterial cell envelopes (10 μg of protein) developed with antibodies raised against MexB (A), OprM (B), MexD (C), and OprJ (D). Lane 1, ML50871; lane 2, K1114 (Δ*mexCD-oprJ*); lane 3, K1121 (Δ*mexAB-oprM*); lane 4, K1126 (Δ*mexCD-oprJ nalB*); lane 5, K1131 (Δ*mexAB-oprM nfxB*); lane 6, K1127 (Δ*mexCD-oprJ nalB* Δ*oprM*); lane 7, K1132 (Δ*mexAB-oprM nfxB* Δ*oprJ*); lane 8, K1127 harboring pRK415 (Δ*mexCD-oprJ nalB* Δ*oprM* plus pRK415); lane 9, K1127 harboring pRSP06 (Δ*mexCD-oprJ nalB* Δ*oprM* plus pRK415::*oprJ*); lane 10, K1127 harboring pRSP08 (Δ*mexCD-oprJ nalB* Δ*oprM* plus pRK415::*oprM*); lane 11, K1132 harboring pVLT31 (Δ*mexAB-oprM nfxB* Δ*oprJ* plus pVLT31); lane 12, K1132 harboring pKPJ-2 (Δ*mexAB-oprM nfxB* Δ*oprJ* plus pVLT31::*oprJ*); lane 13, K1132 harboring pKPM-2 (Δ*mexAB-oprM nfxB* Δ*oprJ* plus pVLT31::*oprM*). In the above lane descriptions, the relevant efflux phenotype or genes expressed are indicated in parenthesis.

(5′-AGCTAAGCTTTCAACTCCTGCCGCCTCGATG-3′), which anneals downstream of *oprJ* and carries an embedded *Hin*dIII site. PCR mixtures (100 μl) contained 20 ng of pKMJ003, a 1 μM concentration of each primer, a 200 μM concentration of each dNTP, 4 mM $MgSO_4$, 5% (vol/vol) dimethyl sulfoxide, and 1 U of Vent polymerase in a 1× reaction buffer. Mixtures were heated at 94°C for 2 min before being subjected to 25 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min; this was followed by 10 min at 72°C. The ca. 1.4-kb *oprJ*-containing PCR product was subsequently purified with the Qiaquick PCR purification kit (Qiagen), digested with *Nde*I and *Hin*dIII, and cloned into *Nde*I-*Hin*dIII-restricted pT7-7 to yield pKPJ-1. Plasmid pKPJ-2 was constructed by cloning a ca. 1.4-kb *Xba*I-*Hin*dIII fragment carrying *oprJ* from pKPJ-1 into pVLT31. Digestion of plasmids with *Xba*I necessitated their being prepared first in a *dam* strain of *E. coli* (e.g., GM2163). The *mexB* gene was cloned into pRK415 on a 4.5-kb *Eco*RI fragment from pRSP19 to yield pRSP35. Similarly, the *mexD* gene was isolated on a 5-kb *Sna*BI-*Bam*HI fragment from pRSP25 and cloned into pRK415, following restriction of the latter vector with *Hin*dIII, end filling with the Klenow fragment, and subsequent restriction with *Bam*HI, to yield pRSP37. Finally, the *mexC* gene was cloned first into pRK415 on a 5-kb *Bam*HI-*Sac*I fragment from pRSP25 (yielding pRSP38) and then into pMMB206 on a 4-kb *Bam*HI-*Pst*I fragment from pRSP38 (yielding pRSP39).

In constructing strain K1121, deletion of the *mexAB-oprM* genes was first carried out on the cloned genes and the deletion was subsequently introduced into the chromosome of *P. aeruginosa* ML5087 by a previously defined gene replacement approach (36). Several *Sac*II sites are present within the *mexAB-oprM* insert present in plasmid pPV20, including one in *mexA*, one in *oprM*, and one downstream of *oprM* in a region not previously sequenced, but none are present in the vector (pAK1900) portion of pPV20. Following digestion of pPV20 with *Sac*II, a 6.9-kb fragment encompassing all of pAK1900 as well as insert sequences to the *Sac*II site at the 5′ end of *mexA* at one end of the fragment and to the *Sac*II site 3′ of *oprM* at the other and a 2-kb fragment encompassing the region from the *Sac*II site in *oprM* to the *Sac*II site 3′ of this gene were recovered and ligated. A recombinant carrying the 2-kb *oprM* *Sac*II fragment in the same orientation as for the original pPV20 vector was identified by restriction mapping and designated pRSP14. This vector carried a 4,104-bp deletion of *mexAB-oprM*, from the *Sac*II site in *mexA* to the *Sac*II site in *oprM* (i.e., from codon 172 of *mexA* to codon 104 of *oprM*). The deletion was subsequently recovered on a ca. 4-kb *Hin*dIII fragment and cloned into *Hin*dIII-restricted pK18*mobsacB* to yield pRSP21. Following transformation of *E. coli* S17-1, pRSP21 was mobilized into *P. aeruginosa* ML5087 via conjugation (31), and transconjugants carrying a copy of pRSP14 in the chromosome were selected on LB agar containing 50 μg of kanamycin per ml. Subsequent streaking of transconjugants onto LB agar containing 10% (wt/vol) sucrose yielded isolated colonies which had lost pK18*mobsacB* sequences (kanamycin sensitive) and which carried either an unaltered wild-type copy of *mexAB-oprM* or the *mexAB-oprM* deletion. Those carrying a deletion of *mexAB-oprM* were identified by PCR. Briefly, chromosomal DNA isolated as described previously (1) was amplified with *Taq* DNA polymerase (Life Technologies, Inc.) and primers ABM-1 (5′-CAGCAGCTCTACCAGATCGAC-3′), which anneals 284 bp downstream of the *mexA* initiation codon, and ABM-2 (5′-GTGTCCTTGGTCAGCTGCAAC-3′), which anneals 844 bp upstream of the *oprM* stop codon. Reaction mixtures (100 μl), including 2.5 U of *Taq* DNA polymerase, a 0.5 μM concentration of each primer, a 0.2 mM concentration of each dNTP, 2 mM $MgCl_2$, 10% (vol/vol) dimethyl sulfoxide, 100 ng of genomic DNA, and 1× PCR buffer (Life Technologies), were heated for 1 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 56°C, and 5 min at 72°C; this was followed by 10 min at 72°C. PCR products were resolved on agarose gels, and strains carrying the appropriately-sized deletion were screened for loss of MexB and OprM in cell envelopes with specific antisera (9, 38) and for supersusceptibility to antibiotics. Procedures for the isolation of cell envelopes, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting have been described previously (38). The susceptibilities of bacterial strains to antimicrobial agents were tested by inoculating 1 ml of LB broth containing serial twofold dilutions of each antimicrobial agent with 10^5 organisms as described previously (20).

The deletion of *mexCD-oprJ* in strain K1114 was carried out by a strategy similar to the one described above. Briefly, the majority of the *mexCD-oprJ* operon was recovered on a 5.8-kb *Ssp*I-*Nhe*I fragment from pKMJ002 and cloned into *Eco*RV-*Nhe*I-restricted pBR322, yielding vector pRSP02. Following the digestion of pRSP02 with *Sac*II and the recovery of a ca. 5.7-kb fragment carrying pBR322 sequences as well as the 5′ region of *mexC* and the 3′ region of *oprJ*, vector recircularization (ligation) yielded vector pRSP03, which carried a 4,462-bp deletion in the *mexCD-oprJ* operon that extended from the 218th codon in *mexC* to the 263rd codon in *oprJ*. This deletion was then recovered on a ca. 1-kb *Bam*HI-*Hin*dIII fragment and cloned into pK18*mobsacB* to yield pRSP05. This vector was

TABLE 2. Antibiotic susceptibilities of *P. aeruginosa* strains expressing a chimeric MexAB-OprJ efflux system[a]

Strain	Relevant genotype[b]	Plasmid[c]	Pump components expressed[d]	MIC[e,f] (μg/ml) of:												
				CAR	CPZ	CEF	CPR	TAX	TAZ	CIP	NOR	CAM	NOV	AZI	ERY	CV
ML5087	Wild type	—	ABM	64	8	2	2	16	2	0.2	0.5	128	128	256	128	ND[g]
K1114	ΔCDJ	—	ABM	64	4	1–2	2	16	2	0.2	0.5	128	128	512	128	ND
K1126	ΔCDJ *nalB*	—	ABM^{++}	512	32	4	4	64	4	0.8	2	512	512	256	512	64
K1127	ΔCDJ *nalB* ΔM	—	AB^{++}	0.5	0.5	0.13	0.06	1	0.5	0.013	0.06	2	8	16	8	<8
K1127	ΔCDJ *nalB* ΔM	pRK415	AB^{++}	0.5	0.25	0.13	0.13	0.25	0.5	0.006	0.03	2	8	32	8	<8
K1127	ΔCDJ *nalB* ΔM	pRSP08	AB^{++}/M^{++}	256	16	2	1	32	2	0.4	2	256	256	128	256	32
K1127	ΔCDJ *nalB* ΔM	pRSP06	AB^{++}/J^{++}	256	16	0.25	0.25	8	0.5	0.2	0.5	256	256	256	256	16

[a] The susceptibilities of the indicated strains carrying the indicated plasmids were assessed as described in the text.
[b] ΔCDJ, Δ*mexCD-oprJ*; ΔM, Δ*oprM*.
[c] pRK415, vector control; pRSP08, pRK415::*oprM*; pRSP06, pRK415::*oprJ*; —, no plasmid.
[d] As assessed with Western immunoblots (Fig. 1). A, MexA; B, MexB; M, OprM; J, OprJ; $^{++}$, hyperexpression. $/M^{++}$ and $/J^{++}$ indicate plasmid-determined hyperexpression.
[e] CAR, carbenicillin; CPZ, cefoperazone; CEF, cefepime; CPR, cefpirome; TAX, cefotaxime; TAZ, ceftazadime; CIP, ciprofloxacin; NOR, norfloxacin; CAM, chloramphenicol; NOV, novobiocin; AZI, azithromycin; ERY, erythromycin; CV, crystal violet.
[f] Carbenicillin, cefoperazone, ceftazidime, ciprofloxacin, norfloxacin, novobiocin, and chloramphenicol were purchased from Sigma Chemical Co., St. Louis, Mo. Crystal violet was purchased from ICN Pharmaceuticals Canada Ltd. Cefepime was provided by Bristol-Meyers Squibb. Azithromycin was a gift from Pfizer. Cefpirome (Roussel Uclaf) and ceftazidime (Glaxo) were also gifts. Erythromycin (Abbott) was purchased from the pharmacy of the Kingston General Hospital.
[g] ND, not determined.

then introduced into *E. coli* S17-1 and used to construct a *mexCD-oprJ* deletion derivative of *P. aeruginosa* ML5087 as described above. The presence of a deletion was confirmed by PCR exactly as described above with primers CDJ-1 (5′-AGC CAGCAGGACTTCGATACC-3′), which anneals 429 bp downstream of the *mexC* start codon, and CDJ-2 (5′-AAGAT CGGCAGCGTCAACGTC-3′), which anneals 409 bp upstream of the *oprJ* stop codon.

To delete *oprM* in derivatives of ML5087, a pK18*mobsac*B vector carrying an *oprM* deletion was constructed and used as described above. Briefly, the *oprM* gene of pRSP01 was cloned on a 3-kb *Sph*I fragment into a pBR322 derivative, pXZL01, which carries a 0.77-kb deletion of the *Sal*I-*Ava*I region of the vector. Digestion of this vector, designated pXZL02, with *Sal*I and *Xho*I released a 517-bp fragment from within the *oprM* coding region. Purification of the plasmid without this fragment and subsequent religation generated vector pXZL03, which carried a 517-bp deletion in *oprM*. This deletion was recovered on a 2.5-kb *Sph*I fragment and cloned into *Sph*I-restricted pK18*mobsacB* to yield pXZL04. The vector was mobilized into *P. aeruginosa* from *E. coli* S17-1 as described above, and *oprM* deletions were confirmed with antiserum specific to OprM.

The approach outlined above was used to construct an *oprJ* deletion strain. Initially, the *oprJ* gene was recovered from pRSP06 on a 2.5-kb *Eco*RI-*Nhe*I fragment and cloned into *Eco*RI-*Xba*I-restricted pK18*mobsacB*. Digestion of this vector, designated pRSP24, with *Eco*RV released a 0.7-kb fragment from within the coding region of *oprJ*. Purification of the vector without this fragment and subsequent religation generated vector pRSP27, which carried the *oprJ* gene with a 0.7-kb internal deletion. Again, mobilization of pRSP27 into a *P. aeruginosa nfxB* strain as described above allowed selection of *oprJ* deletion derivatives which were confirmed with an antiserum specific to OprJ (13).

Introduction of plasmids pRK415, pMMB206, and pVLT31 and their derivatives into *P. aeruginosa* required a triparental mating procedure employing helper vector pRK2013. Briefly, 100 μl of overnight cultures of plasmid-containing *E. coli* DH5α, pRK2013-containing *E. coli* MM294, and ML5087 derivatives of *P. aeruginosa* were pelleted together in a microcentrifuge tube, resuspended in 25 μl of L broth and spotted onto the center of an L agar plate. Following incubation overnight at 37°C, bacteria were resuspended in 1 ml of L broth and appropriate dilutions were plated on L agar containing 25 μg of chloramphenicol (for matings involving pVLT31, pVLT31, and pRK415 derivatives), 1 μg of imipenem (for matings involving pRK415), or 25 μg of tetracycline (for matings involving pMMB206 and its derivatives) per ml to counterselect the *E. coli* and either 16 μg of chloramphenicol (for pMMB206 and its derivatives) or 10 (for pRK415 and its derivatives) to 25 (for pVLT31 and its derivatives) μg of tetracycline per ml to select plasmid-bearing transconjugants. Plasmid DNA was prepared from *P. aeruginosa* recipients by the miniprep procedure to confirm successful plasmid transfer. Plasmid preparation, restriction endonuclease digestions, ligations, end filling with the Klenow fragment of DNA polymerase I, transformations, and agarose gel electrophoresis were performed as described in Sambrook et al. (35) or as outlined in reference 38.

Construction and activity of a chimeric MexAB-OprJ efflux system. In an effort to ascertain whether the outer membrane components, particularly OprM, define the ability of the *P. aeruginosa* multidrug efflux pumps to accommodate β-lactams, a strain hyperexpressing the MexAB, but not the OprM, components of the MexAB-OprM efflux system was constructed and examined for reconstitution of efflux activity, particularly of β-lactams, in the presence of the cloned *oprM* and *oprJ* genes. Initially, the MexCD-OprJ system was removed from ML5087 to offset any possible contribution of this system to resistance and efflux in the final construct. As expected, elimination of MexCD-OprJ (strain K1114) had no effect on resistance (Table 2), consistent with previous reports that the operon is not expressed in wild-type cells (Fig. 1C and D, lane 1) (23). Although MexAB-OprM is expressed in wild-type cells (Fig. 1A and B, lane 1) (9, 33), it was reasoned that the overexpression of efflux components may be necessary to assess the operation of chimeric pumps, which may be less efficient in the efflux process. Thus, a *nalB* derivative of K1114 (designated K1126) which displayed the characteristic resistance profile (Table 2) and hyperexpression of components of the MexAB-OprM system was selected (Fig. 1A and B, lane 4). To replace OprM with a plasmid-encoded OprJ, it was first necessary to delete the *oprM* gene from K1126 (confirmed in Fig. 1B, lane 6), yielding a strain (K1127) which hyperexpressed only MexAB (Fig. 1A, lane 6) and which, as expected, was antibiotic supersusceptible (Table 2). K1127 was also hy-

TABLE 3. Antibiotic susceptibilities of *P. aeruginosa* strains expressing a chimeric MexCD-OprM efflux system

Strain	Relevant genotype[a]	Plasmid[b]	Pump components expressed[c]	MIC (μg/ml)[d] of:												
				CAR	CPZ	CEF	CPR	TAX	TAZ	CIP	NOR	CAM	NOV	AZI	ERY	CV
ML5087	Wild type	—	ABM	64	8	2	2	16	2	0.2	0.5	128	128	256	128	ND[e]
K1121	ΔABM	—	—	1	1	0.13	0.13	1	0.5	0.1	0.25	32	16	32	64	ND
K1131	ΔABM *nfxB*	—	CDJ^{++}	0.5	2	8	16	16	1	1.6	1	128	64	512	>1,024	64
K1132	ΔABM *nfxB* ΔJ	—	CD^{++}	0.5	0.5	0.25	0.25	0.5	0.5	0.05	0.25	16	8	64	64	<8
K1132	ΔABM *nfxB* ΔJ	pVLT31	CD^{++}	0.5	0.5	0.13	0.13	0.25	0.5	0.05	0.25	16	8	64	64	<8
K1132	ΔABM *nfxB* ΔJ	pKPJ-2	CD^{++}/J^{++}	0.5	1	4	8	4	1	1.6	0.5	64	32	512	1,024	64
K1132	ΔABM *nfxB* ΔJ	pKPM-2	CD^{++}/M^{++}	0.5	0.5	1	2	0.5	0.25	0.4	1	32	8	256	512	32

[a] ΔABM, Δ*mexAB-oprM*; ΔJ, Δ*oprJ*.
[b] pVLT31, vector control; pKPJ-2, pVLT31::*oprJ*; pKPM-2, pVLT31::*oprM*; —, no plasmid.
[c] As assessed with Western immunoblots (Fig. 1). C, MexC; D, MexD; —, none of the MexCD-OprJ or MexAB-OprM components expressed. Other symbols and abbreviations are as defined in footnote *d* to Table 2.
[d] Abbreviations are as defined in footnote *e* to Table 2.
[e] ND, not determined.

persensitive to crystal violet, demonstrating that MexAB-OprM accommodates dyes as well as antibiotics. Restoration of antibiotic (and dye) resistance in K1127 could then be used to assess the functionality of a MexAB-OprJ efflux pump. Introduction of the pRK415 vector alone failed to enhance OprM production (Fig. 1B, lane 8) or the antibiotic and dye resistance of this strain (Table 2), while reintroduction of *oprM* on vector pRSP08 (Fig. 1B, lane 10) or pKPM-2 (data not shown) restored OprM production (Fig. 1) and resistance to all agents (Table 2). Although the levels of OprM present in K1127 harboring pRSP08 were comparable to that seen in *nalB* strain K1126 (Fig. 1B; compare lanes 10 and 4), resistance levels were typically twofold lower in K1127 harboring pRSP08 (Table 2). Introduction of *oprJ* vector pRSP06 into K1127 facilitated OprJ expression in this strain (Fig. 1D, lane 9) and increased resistance to most agents, with some exceptions, to levels achieved with OprM (Table 2). Most importantly, the MexAB-OprJ pump accommodated β-lactams, such as carbenicillin and cefoperazone, which are not substrates for MexCD-OprJ but which are substrates for MexAB-OprM (Table 2). Thus, replacement of OprM with OprJ did not alter the substrate specificity of the MexAB pump, and OprM is not the determining factor for the β-lactam specificity of this pump.

Construction and activity of a chimeric MexCD-OprM efflux system. To provide independent confirmation that OprM does not determine the β-lactam specificity of the efflux pumps associated with it and to determine if OprM was able to function with MexCD in mediating antibiotic resistance, a strain hyperexpressing the MexCD components was constructed from a strain ultimately lacking MexA, MexB, OprM, and OprJ and the ability of the plasmid-borne *oprM* gene to reconstitute resistance was assessed. Initially, the *mexAB-oprM* genes were deleted (strain designated K1121) (Fig. 1A and B, lane 3) since they provide substantial antibiotic resistance in wild-type cells (Table 3, compare ML5087 and K1121), and MexCD-OprJ-overexpressing *nfxB* strain K1131 (Fig. 1C and D, lane 5) was selected. As expected, K1121 was antibiotic supersusceptible due to the loss of MexAB-OprM and K1131 displayed a resistance profile typical of NfxB-type multidrug-resistant *P. aeruginosa* (Table 3) (30). To exchange OprJ for OprM, the *oprJ* gene was deleted from K1131 (yielding K1132) (Fig. 1D, lane 7), rendering K1132 antibiotic (and crystal violet) supersusceptible (Table 3) and providing a screen for the activity of OprM with MexCD (i.e., restored resistance). The introduction of pRK415 (data not shown) or pVLT31 failed to alter the antibiotic or dye supersusceptibility of K1132, although reintroduction of *oprJ* on pRSP06 (data not shown) or pKPJ-2 restored OprJ production (Fig. 1D, lane 12) and antibiotic and dye resistance (Table 3). Initial attempts at introducing *oprM* on pRK415-derived plasmid pRSP08 failed, despite repeated attempts. Apparently, the high-level OprM expression afforded by this vector was not accommodated by *P. aeruginosa* strains not also hyperexpressing MexAB. Indeed, this vector was readily introduced into *nalB* Δ*oprM* strains of *P. aeruginosa* (data not shown). Thus, *oprM* was introduced into K1132 on lower-copy-number vector pKPM-2. The resultant strain expressed OprM (Fig. 1B, lane 13) and was antibiotic and dye resistant (Table 3). Again, resistance afforded by OprM (i.e., MexCD-OprM) was specific to those agents to which resistance was afforded by MexCD-OprJ (Table 3), indicating that the outer membrane protein, though necessary, is not a determining factor for the substrate specificity of the *P. aeruginosa* multidrug efflux pumps. In all instances, OprM was less effective at restoring resistance, indicating that the MexCD-OprM pump was less efficient than the native MexCD-OprJ pump. This could be because OprJ more readily associates with MexCD to form a functional efflux system than does OprM or because a MexCD-OprJ pump is simply more efficient than a MexCD-OprM pump. Again, neither the MexCD-OprJ (in strain K1132 harboring pKPJ-2) nor the MexCD-OprM (in strain K1132 harboring pKPM-2) reconstituted pumps provided resistance equivalent to that observed for the original *nfxB* mutant, K1131, despite the fact that levels of OprJ, for example, produced by K1131 were comparable to those produced by K1132 harboring pKPJ-2 (Fig. 1D; compare lanes 12 and 5).

Conclusions and comments. These data indicate that drugs with cytoplasmic as well as periplasmic targets must interact with the inner membrane-associated (e.g., MexAB) components of the multidrug efflux pumps and that these components are thus responsible for defining the specificities of these pumps for all substrates, β-lactams included. Moreover, they indicate that these components are able to recognize and acquire substrates present on either side of the inner membrane. This could be accomplished by having distinct binding sites for the various substrates on, for example, integral membrane pump component MexB, with most of the binding sites occurring on the cytoplasmic face of the protein and those for β-lactams occurring on the periplasmic face. Alternatively, as proposed elsewhere, substrate antibiotics may first partially partition into the inner membrane bilayer, from whence they are "vacuumed" by the inner membrane efflux components (e.g., MexB) (22). As such, a common, membrane-spanning domain or domains might be expected to function in the rec-

ognition of all substrates. MexB, the major integral inner membrane efflux component of the MexAB-OprM pump, undoubtedly defines the substrate specificity for the majority of substrates, most of which act within the cytoplasm, since it is the only pump component with access to the cytoplasm. As such, it is the leading candidate for determining β-lactam specificity as well. Still, we cannot rule out the possibility that β-lactams represent a unique substrate and that their recognition is a function of inner-membrane-associated periplasmic linker component MexA, which certainly would have access to these agents.

The observation that OprJ functions in association with MexCD as well as MexAB and that OprM similarly functions with either MexAB or MexCD indicates that there is a substantial amount of flexibility as regards "recognition" of the outer membrane efflux component by the other components of these pumps. Given current models of the organization of pump components in the cell envelope, this suggests that linker proteins MexA and MexC are capable of interacting, in a functional way, with either OprM or OprJ. Such flexibility in the outer membrane component was previously demonstrated for *E. coli*, where the *mexCD* genes alone promoted resistance to antimicrobial agents in a Δ*acrAB* strain; the resistance was dependent, however, on TolC, the outer membrane counterpart of the AcrAB efflux system (7).

To determine if MexB was the efflux component responsible for the β-lactam specificity of the MexAB-OprM efflux system, attempts were made to construct chimeric pumps in which the MexB and MexD components were exchanged. Cloning of the *mexD* (pRSP37) and *oprM* (pQZ05) genes in *mexB*::ΩHg *P. aeruginosa* strain K880 ($MexA^+$ $MexB^-$ $OprM^-$) in an effort to reconstitute a MexAD-OprM efflux system failed to provide resistance to any antibiotic (data not shown). Similarly, introduction of the *mexC* and *mexB* genes into *E. coli* KZM120 on separate but compatible vectors (pRSP39 and pRSP35), in an effort to reconstitute a MexCB-TolC efflux system, failed to provide any drug resistance, even though introduction of the *mexC* and *mexD* genes into KZM120 on two plasmids (pRSP39 and pRSP37) did provide resistance to those agents for which resistance was previously provided by *mexCD* vector pRSP25 (data not shown). It is unclear, however, whether failure to reconstitute efflux activity upon exchange of the integral inner membrane MexB and MexD components results from failure to assemble an active efflux system or simply from lack of stable expression of efflux components. In any case, the fact that MexB and MexD were readily produced and active when coexpressed with MexA and MexC, respectively, regardless of the outer membrane component, suggests that the activity and/or stability of MexB and MexD may depend upon association with cognate linker proteins MexA and MexC. Thus, it appears that the flexibility permitted as regards the outer membrane components of these tripartite efflux systems and their interaction with the linker proteins may not apply to the integral inner membrane components and their interaction with the linker proteins.

Construction and characterization of chimeric efflux/export systems has been studied previously (2) with components of the *Erwinia chrysanthemi* metalloprotease exporter (encoded by the *prtDEF* genes [17]) and the *Serratia marcescens* HasA heme acquisition protein exporter (encoded by the *hasDEF* genes [3, 19]). The PrtDEF and HasDEF efflux components are highly homologous and are composed of an integral cytoplasmic membrane component (PrtD, HasD), a membrane fusion or linker protein (PrtE, HasE), and an outer membrane protein (PrtF, HasF). HasF is a homolog of the *E. coli* TolC protein (3), and, indeed, TolC (as well as PrtF) was able to functionally reconstitute an active export system with the HasDE components (18). The ability of outer membrane protein PrtF to work in conjunction with PrtDE or HasDE is reminiscent of what was found in the present study and further demonstrates the flexibility of these tripartite efflux/export systems in terms of the outer membrane constituents. Still, TolC was unable to work with PrtDE, indicating that this protein, at least, exhibits some specificity in terms of its probable interaction with linker protein HasE (2). It is not yet clear, however, whether the HasF protein can only interact with HasDE. This "specificity" of TolC is also reflected in a recent study where the protein was shown to function and, presumably, interact with the MexCD efflux components but not the MexAB components (38). Interestingly, integral cytoplasmic membrane proteins PrtD and HasD worked equally well with either PrtEF or HasE-TolC in reconstituting export (2), in contrast to what was shown by data presented here, which demonstrates the specific requirement of integral membrane proteins MexB and MexD for their native linkers, MexA and MexC, respectively, for the operation of the corresponding efflux systems.

The authors thank N. Gotoh for providing anti-OprM, anti-MexD, and anti-OprJ antisera and Q. Zhao for strains and plasmids.

This work was supported by an operating grant from the Canadian Cystic Fibrosis Foundation, whose continuing support is gratefully acknowledged. R.S. is the recipient of a postdoctoral fellowship from the Natural Sciences and Engineering Research Council (NSERC). K.P. is an NSERC University Research Fellow. X.-Z.L. holds a studentship from the Canadian Cystic Fibrosis Foundation.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1992. Short protocols in molecular biology, 2nd ed. John Wiley & Sons, Inc., New York, N.Y.
2. **Binet, R., and C. Wandersman.** 1995. Protein secretion by hybrid bacterial ABC-transporters: specific functions of the membrane ATPase and the membrane fusion protein. EMBO J. **14:**2298–2306.
3. **Binet, R., and C. Wandersman.** 1996. Cloning of the *Serratia marcescens hasF* gene encoding the Has ABC exporter outer membrane component: a TolC analogue. Mol. Microbiol. **22:**265–273.
4. **de Lorenzo, V., L. Eltis, B. Kessler, and K. Timmis.** 1993. Analysis of *Pseudomonas* gene products using *lacI*^{q}/*Ptrp-lac* plasmids and transposons that confer conditional phenotypes. Gene **123:**17–24.
5. **Evans, K., and K. Poole.** Unpublished data.
6. **Figurski, D. H., and E. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
7. **Fralick, J. A.** 1996. Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of *Escherichia coli*. J. Bacteriol. **178:**5803–5805.
8. **Fukuda, H., M. Hosaka, K. Hirai, and S. Iyobe.** 1990. New norfloxacin resistance gene in *Pseudomonas aeruginosa* PAO. Antimicrob. Agents Chemother. **34:**1757–1761.
9. **Gotoh, N., N. Itoh, H. Tsujimoto, J.-I. 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.
10. **Gotoh, N., H. Tsujimoto, K. Poole, J.-I. Yamagishi, and T. Nishino.** 1995. The outer membrane protein OprM of *Pseudomonas aeruginosa* is encoded by *oprK* of the *mexA-mexB-oprK* multidrug resistance operon. Antimicrob. Agents Chemother. **39:**2567–2569.
11. **Hirai, K., S. Suzue, T. Irikura, S. Iyobe, and S. Mitsuhashi.** 1987. Mutations producing resistance to norfloxacin in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **31:**582–586.
12. **Hohnadel, D., D. Haas, and J.-M. Meyer.** 1986. Mapping of mutations affecting pyoverdin production in *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **36:**195–199.
13. **Hosaka, M., N. Gotoh, and T. Nishino.** 1995. Purification of a 54-kilodalton protein (OprJ) produced in NfxB mutants of *Pseudomonas aeruginosa* and production of a monoclonal antibody specific to OprJ. Antimicrob. Agents Chemother. **39:**1731–1735.
14. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene **70:**191–197.
15. **Koehler, T., M. Kok, M. Michea-Hamzehpour, P. Plesiat, N. Gotoh, T. Nishino, L. Kocjanici Curty, and J.-C. Pechere.** 1996. Multidrug efflux in intrinsic resistance to trimethoprim and sulfamethoxazole in *Pseudomonas*

aeruginosa. Antimicrob. Agents Chemother. **40:**2288–2290.
16. **Koehler, T., M. Michea-Hamzehpour, U. Henze, N. Gotoh, L. K. Curty, and J.-C. Pechere.** 1997. Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Mol. Microbiol. **23:**345–354.
17. **Letoffe, S., P. Delepelaire, and C. Wandersman.** 1990. Protease secretion by *Erwinia chrysanthemi*: the specific secretion functions are analogous to those of *Escherichia coli* a-haemolysin. EMBO J. **9:**1375–1382.
18. **Letoffe, S., J. M. Ghigo, and C. Wandersman.** 1993. Identification of two components of the *Serratia marcescens* metalloprotease transporter: protease SM secretion in *Escherichia coli* is TolC dependent. J. Bacteriol. **175:**7321–7328.
19. **Letoffe, S., J. M. Ghigo, and C. Wandersman.** 1994. Secretion of the *Serratia marcescens* HasA protein by an ABC transporter. J. Bacteriol. **176:**5372–5377.
20. **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.
21. **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 system of *Escherichia coli*. Mol. Microbiol. **16:**45–55.
22. **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.
23. **Masuda, N., N. Gotoh, S. Ohya, and T. Nishino.** 1996. Quantitative correlation between susceptibility and OprJ production in NfxB mutants of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **40:**909–913.
24. **Morales, V. M., A. Backman, and M. Bagdasarian.** 1991. A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. Gene **97:**39–47.
25. **Neidhardt, F. C., J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.).** 1987. *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
26. **Nies, D.** 1995. The cobalt, zinc, and cadmium efflux system CzcABC from *Alcaligenes eutrophus* functions as a cation-proton antiporter in *Escherichia coli*. J. Bacteriol. **177:**2707–2712.
27. **Nikaido, H.** 1989. Outer membrane barrier as a mechanism of antimicrobial resistance. Antimicrob. Agents Chemother. **33:**1831–1836.
28. **Nikaido, H.** 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science **264:**382–388.
29. **Okii, M., S. Iyobe, and S. Mitsuhashi.** 1983. Mapping of the gene specifying aminoglycoside 3′-phosphotransferase II on the *Pseudomonas aeruginosa* chromosome. J. Bacteriol. **155:**643–649.
30. **Poole, K., N. Gotoh, H. Tsujimoto, Q. Zhao, A. Wada, T. Yamasaki, S. Neshat, J.-I. Yamagishi, X.-Z. Li, and T. Nishino.** 1996. Overexpression of the *mexC-mex-oprJ* efflux operon in *nfxB* multidrug resistant strains of *Pseudomonas aeruginosa*. Mol. Microbiol. **21:**713–724.
31. **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 pyoverdin. Mol. Microbiol. **10:**529–544.
32. **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.
33. **Poole, K., K. Tetro, Q. Zhao, S. Neshat, D. Heinrichs, and N. Bianco.** 1996. Expression of the multidrug resistance operon *mexA-mexB-oprM* in *Pseudomonas aeruginosa*: *mexR* encodes a regulator of operon expression. Antimicrob. Agents Chemother. **40:**2021–2028.
34. **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.
35. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
36. **Schaefer, A., A. Tauch, W. Jaeger, J. Kalinowski, G. Thierbach, and A. Puehler.** 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145:**69–73.
37. **Simon, R., U. Priefer, and A. Puehler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Biotechnology **1:**784–791.
38. **Srikumar, R., T. Kon, N. Gotoh, and K. Poole.** Expression of *Pseudomonas aeruginosa* multidrug efflux pumps MexA-MexB-OprM and MexC-MexD-OprJ in *Escherichia coli*. Submitted for publication.
39. **Wong, K., K. Poole, N. Gotoh, and R. E. W. Hancock.** 1997. Influence of OprM on multiple antibiotic resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **41:**2009–2012.