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Assignment of the Substrate-Selective Subunits of the MexEF-OprN Multidrug Efflux Pump of *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa **expresses a low level of the MexAB-OprM efflux pump and shows natural resistance to many structurally and functionally diverse antibiotics. The mutation that has been referred to previously as *nfxC* expresses an additional efflux pump, MexEF-OprN, exhibiting resistance to fluoroquinolones, imipenem, and chloramphenicol and hypersusceptibility to β-lactam antibiotics. To address the antibiotic specificity of the MexEF-OprN efflux pump, we introduced a plasmid carrying the *mexEF-oprN* operon into *P. aeruginosa* lacking the *mexAB-oprM* operon. The transformants exhibited resistance to fluoroquinolones, trimethoprim, and chloramphenicol but, unlike most *nfxC*-type mutants, did not show β-lactam hypersusceptibility. The transformants exhibited additional resistance to tetracycline. In the next experiment, we analyzed the MexEF-OprN pump subunit(s) responsible for substrate selectivity by expressing MexE, MexF, OprN, and MexEF in strains lacking MexA, MexB, OprM, and MexAB, respectively. The MexEF-OprM/ΔMexAB transformants exhibited MexEF-OprN-type pump function that rendered the strains resistant to fluoroquinolones and chloramphenicol but did not change susceptibility to β-lactam antibiotics compared with the host strain. The MexAB-OprN/ΔOprM, MexAF-OprM/ΔMexB, and MexEB-OprM/ΔMexA mutants exhibited antibiotic susceptibility indistinguishable from that in the mutant lacking both types of efflux pumps. The results imply that the MexEF-OprM pump selects substrates by a MexEF functional unit. Interestingly, OprN did not link functionally with the MexAB complex, despite the fact that OprM interacted functionally with MexEF.**

Infections by *Pseudomonas aeruginosa* in patients with low immune activity are a major problem in hospitals, in part because this organism exhibits natural (inherent) as well as acquired resistance to a broad spectrum of antibiotics. The naturally occurring antibiotic resistance of this organism is attributable mainly to the interplay of tight outer membrane permeability and low-level expression of the MexAB-OprM efflux pump (13, 14, 18). All *nalB* mutants previously reported overexpress the MexAB-OprM pump and become highly resistant to a wide variety of antimicrobial agents, including most β-lactam antibiotics, fluoroquinolones, tetracycline, chloramphenicol, and others (13, 18, 21, 28). In contrast, mutations in the *nfxB* (7, 15) and *nfxC* (3) loci located near the *ilvB* (0 min) and *catA* (46 min) genes, respectively, of the *P. aeruginosa* chromosome render the bacterium hypersusceptible to β-lactam antibiotics and resistant to fluoroquinolones, chloramphenicol, and other antibiotics. The resistance is mainly attributable to the expression of the MexAB-OprM, MexCD-OprJ, and MexEF-OprN efflux pumps, respectively (8, 13, 17–19). Among these efflux pump systems, only the MexAB-OprM pump is expressed in most, if not all, strains of *P. aeruginosa* so far tested, including laboratory and clinical strains (8, 17), and thus most, if not all, *nfxB* and *nfxC* mutations occur in strains producing at least low levels of MexAB-OprM pump. Therefore, the antibiotic resistance profile of the *nfxB* and *nfxC* mutants might be a consequence of the expression of the MexCD-OprJ and MexEF-OprN systems, respectively, plus a low level of MexAB-OprM pump expression.

MexB and its homologues span the cytoplasmic membrane 12 times (6, 16) and have been assumed to function as the substrate-exporting subunit across the cytoplasmic membrane. MexA and its homologues are membrane fusion proteins associated with the cytoplasmic membrane via the fatty acid residue, and the peptide moiety extends almost to the periplasmic space (20, 30). OprM and its homologues are outer membrane proteins that are assumed to form a channel to facilitate the exit of substrates through the outer membrane (10).

Since the subunit proteins of the three efflux pumps are similar to each other, it has been assumed that a subunit of one pump system could be substituted for the homologous subunit of another pump system. Experiments exchanging the subunits of MexAB-OprM and MexCD-OprJ have been carried out and revealed that replacement of OprM with OprJ or vice versa partially complemented the pump function (5, 26, 27); however, replacing the inner membrane subunits totally abolished the pump function. These experiments still left the following important questions unanswered. (i) What antibiotics are the substrates of the MexEF-OprN pump? (ii) Does expression of the MexEF-OprN pump suppress OprD production and confer carbapenem resistance? (iii) Can *nfxC*-type β-lactam hypersusceptibility be surmounted by overexpression of the MexEF-OprN pump without *nfxC* mutation?

In this study, we addressed these issues by expressing the MexEF-OprN pump in a strain lacking the MexAB-OprM pump. Moreover, we assigned the subunit protein(s) of the MexEF-OprN pump responsible for substrate recognition based on the results of the subunit swapping experiment.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* XL10 Gold was the host in the DNA manipulation.

Recombinant DNA techniques. We manipulated recombinant DNA by the standard procedures described previously (22). Chromosomal DNA from the *P. aeruginosa* cells was isolated by the procedure described by Ausubel et al. (1). For Southern blotting, DNA fragments were blotted onto Hybond-N+ (Amersham Life Science, Arlington Heights, Ill.) by the capillary method and visualized with a digoxigenin-labeled probe (Boehringer Mannheim, Laval, Quebec, Canada) prepared according to the manufacturer's instructions. Nucleotide sequencing of

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid(s)	Relevant properties	Source (reference)
P. aeruginosa		
PAO4009	*leu-9018 nir-9006*; $FP5^+$	H. Matsumoto (7)
KH4014a	*nfxC* mutant of PAO4009	H. Fukuda (3)
PAO4290	*leu-10 argF10 aph-9004*; FP^-	H. Matsumoto (28)
TNP070	Δ*mexA*; derivative of PAO4290	H. Yoneyama (28)
TNP071	Δ*mexB*; derivative of PAO4290	H. Yoneyama (28)
TNP072	Δ*oprM*; derivative of PAO4290	H. Yoneyama (28)
TNP073	Δ*mexA* Δ*mexB*; derivative of PAO4290	H. Yoneyama (28)
TNP077	Δ*mexA* Δ*mexB* Δ*oprM*; derivative of PAO4290	This study
TNP0701	TNP070 derivative harboring pMEXA1	H. Yoneyama (27)
TNP0705	TNP070 derivative harboring pMEXE1	This study
TNP0703	TNP070 derivative harboring pMMB67HE	H. Yoneyama (27)
TNP0711	TNP071 derivative harboring pMEXB1	H. Yoneyama (27)
TNP0715	TNP071 derivative harboring pMEXF1	This study
TNP0713	TNP071 derivative harboring pMMB67EH	H. Yoneyama (27)
TNP0721	TNP072 derivative harboring pOPRM1	H. Yoneyama (27)
TNP0725	TNP072 derivative harboring pOPRN1	This study
TNP0723	TNP072 derivative harboring pMMB67EH	H. Yoneyama (27)
TNP0731	TNP073 derivative harboring pMEXAB1	This study
TNP0735	TNP073 derivative harboring pMEXEF1	This study
TNP0733	TNP073 derivative harboring pMMB67EH	H. Yoneyama (27)
TNP0771	TNP077 derivative harboring pMEXAB-OPRM1	This study
TNP0775	TNP077 derivative harboring pMEXEF-OPRN1	This study
TNP0773	TNP077 derivative harboring pMMB67EH	This study
TNP0776	TNP077 derivative harboring pVLT33	This study
TNP0777	TNP077 derivative harboring pMEXEF-OPRN-KM1	This study
E. coli		
XL10 Gold	*E. coli* strain for transformation	Stratagene
S17-1	Mobilizer strain	R. Simon (25)
Plasmids		
pUC19	Cloning vector; Amp^r	Toyobo
pNOT19	pUC19 derivative with *Not*I site; Amp^r	H. P. Schweizer (24)
pKF18K	pUC18 derivative cloning vector; Amp^r	Toyobo
pBluescript II SK(+)	Cloning vector; Amp^r	Stratagene
pMOB3	pHSS21 derivative carrying MOB cassette; Cm^r Km^r	H. P. Schweizer (24)
pQE30, pQE31	Expression vectors carrying a six-His affinity tag; Amp^r	Qiagen, Inc.
pMMB67EH/HE	Broad-host-range vector; Amp^r IncQ	Fürste (4)
pVLT33	Broad-host-range vector; Km^r IncQ	V. de Lorenzo (2)
pMEXA1	pMMB67EH derivative carrying *mexA* gene	H. Yoneyama (27)
pMEXB1	pMMB67EH derivative carrying *mexB* gene	H. Yoneyama (27)
pOPRM1	pMMB67EH derivative carrying *oprM* gene	H. Yoneyama (27)
pMEXE1	pMMB67EH derivative carrying *mexE* gene	This study
pMEXF1	pMMB67EH derivative carrying *mexF* gene	This study
pOPRN1	pMMB67EH derivative carrying *oprN* gene	This study
pMEXAB1	pMMB67EH derivative carrying *mexA* and *mexB* genes	This study
pMEXEF1	pMMB67EH derivative carrying *mexE* and *mexF* genes	This study
pMEXAB-OPRM1	pMMB67EH derivative carrying *mexA*, *mexB*, and *oprM* genes	This study
pMEXEF-OPRN1	pMMB67EH derivative carrying *mexE*, *mexF*, and *oprN* genes	This study
pMEXEF-OPRN-KM1	pVLT33 derivative carrying *mexE*, *mexF*, and *oprN* genes	This study
pΔMexA-B-OprM	pNOT19 derivative carrying defective *mexA*, *mexB*, and *oprM*; Amp^r Km^r Cm^r	This study

the recombinant DNA was conducted by the dideoxy chain-termination method (23). PCR amplification of chromosomal DNA was carried out using the LA *Taq* kit (Takara Shuzo, Osaka, Japan) according to the manufacturer's instructions. The primers used were as follows: mexE1 (5′-GCGGTACCGACTGGCGGAG TCAAGCA-3′) and mexF2 (5′-CGAAGCTTGCGCGTGAATCAT-3′) for *mexEF* and oprN1 (5′-GTGGTACCCTGCCAGAGGTGCATGCAT-3′) and oprN2 (5′-AACAAGCTTCAGGCGCTGGGTTGCCAG-3′) for *oprN*. The sizes of the amplified DNA fragments obtained using these primers were about 4.5 and 1.4 kbp, respectively.

Preparation of rabbit antisera against MexE, MexF, and OprN. Using the PCR technique, we amplified 1.1-, 0.8-, and 1.3-kbp fragments encoding deduced amino acid sequences from positions 42 to 413, 47 to 317, and 27 to 472 of MexE, MexF, and OprN, respectively. The following primer pairs were used: mexE3 (5′-GCGGTACCGCCGAAGTCATCGAACAAC-3′) and mexE2 (5′-GCAAG CTTCGGTTCTTCCTATCGCCGC-3′) for MexE, mexF3 (5′-GCGGTACCGG TCCGCGCCAACTTCCC-3′) and mexF4 (5′-CGAAGCTTTCAGCTCGGCC ATCTTCTC-3′) for MexF, and oprN3 (5′-AAGGATCCACGGTGGGTCCGG ACTAC-3′) and oprN2 (see above) for OprN. The amplified fragments were subcloned into the pQN30 or pQN31 (Qiagen, Inc., Chatsworth, Calif.) expression vector carrying a sequence coding for the polyhistidine affinity tag, to which the N terminus of the desired DNA fragment was fused. By nucleotide sequencing, we confirmed that the reading frames of all these genes were correctly maintained. Fully grown *E. coli* cells, which harbored an appropriate plasmid, were diluted 50-fold with fresh medium and incubated in the presence of appropriate concentrations of antibiotics.

At an A_{600} of 0.5, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added and the culture was continued for an additional 5 h under vigorous shaking. The hybrid protein was purified by affinity chromatography using an Ni-nitrilotriacetic acid column (Qiagen, Inc.) according to the manufacturer's manual. The protein was subjected to preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (3-mm thickness) and stained, and then the desired protein band was excised and eluted electrophoretically. Rabbits were immunized, and antisera were obtained.

Deletion of chromosomal *mexAB-oprM*. We subcloned a 6.5-kbp fragment containing the *mexAB-oprM* genes from the previously cloned 26-kbp fragment (13) on pNOT19-ΔRI yielding pMex(sac/Hind)/ΔRI-pNOT19. To disrupt the chromosomal *mexAB-oprM*, pMex(sac/Hind)ΔRI-pNOT19 was treated with *Eco*RI and *Xho*I and then self-ligated after blunt-ending with T4 DNA polymerase. The resulting plasmid, pNOT19-ΔMexABM, and pMOB3 were ligated at the *Not*I site and transferred into the mobilizer strain *E. coli* S17-1. The resulting plasmid pΔMexA-B-OprM was transferred to *P. aeruginosa* PAO4290 by conjugation as reported earlier (as shown in Fig. 1A) (29). The deletion was confirmed by PCR (data not shown). MexA, MexB, and OprM proteins were undetectable in the mutant TNP077, tested by an immunoblot assay using polyclonal antisera (Fig. 2). Determination of MICs of antibiotic agents revealed that the MexAB-OprM deletion mutant TNP077 was hypersusceptible to fluoroquinolones, chloramphenicol, and β-lactams, except for imipenem, as reported previously (Table 2) (28).

Cloning of the *mexEF-oprN* genes. The *mexEF-oprN* genes were obtained by PCR and colony hybridization techniques. A 4.5-kbp DNA fragment encoded by the *mexEF* genes and a 1.4-kbp DNA fragment encoded by the *oprN* gene were amplified with primer sets mexE1 and mexF2 and oprN1 and oprN2, respectively, as mentioned above, by using the LA *Taq* kit. The 4.5- and 1.4-kbp fragments were cloned onto pNOT19 and pKF18, respectively, to yield pUC19-*mexEF* and pKF18-*oprN*. Nucleotide sequencing of these fragments revealed that the 4.5-kbp fragment contained two incorrect nucleotides. To obtain the correct 4.5-kbp DNA fragment, the 3.4-kbp *Sph*I-*Sph*I fragment from the genomic DNA encoded by a part of *mexEF* was cloned on pBluescript II SK(+) as follows. Total DNA prepared from *P. aeruginosa* PAO1 was digested with *Sph*I and separated by gel electrophoresis. Fragments of around 3.4 kbp were collected; this was followed by ligation with *Sph*I-treated pBluescript II SK(+). The ligation mixture was used for transformation of *E. coli* XL10 Gold. One clone that harbored recombinant pBluescript II SK(+) with a *Sph*I-*Sph*I fragment was selected from 684 Amp^r clones using the labeled pUC19-*mexEF* as a probe in the colony hybridization method. Next, we replaced the 3.4-kbp *Sph*I-*Sph*I region on pUC19-*mexEF* with this newly cloned fragment on pBluescript II SK(+) and constructed the pUC19-*mexEF* plasmid with the correct *mexEF*. Finally, to join *mexEF* with *oprN*, the new pUC19-*mexEF* was digested with *Eco*T22I and *Hin*dIII and ligated to a 1.4-kbp *oprN* gene fragment from pKF18-oprN, resulting in pUC19-*mexEFN*. The nucleotide sequence of cloned *mexEF-oprN* was identical to that of the strain PAO1 gene.

Construction of recombinant plasmids. To construct pMEXEF1, a *Kpn*I-*Hin*dIII fragment of about 4.5 kbp from pUC19-*mexEF* was inserted into pMMB67EH. This plasmid DNA was digested with *Kpn*I and *Bam*HI, followed by blunt-ending with T4 DNA polymerase and self-ligating to yield the pMEXF1 (Fig. 1B). The 2.2-kbp *Kpn*I-*Bgl*II fragment from pUC19-*mexEF* was inserted at *Kpn*I and *Bam*HI sites of the pMMB67EH to construct pMEXE1. A *Kpn*I-*Hin*dIII fragment of about 6 kbp from pUC19-*mexEFN* was ligated to pMMB67EH and pVLT33 and then digested with *Kpn*I and *Hin*dIII to construct pMEXEF-ORRN1 and pMEXEF-OPRN-KM1, respectively. A 1.4-kbp *Kpn*I-*Hin*dIII fragment from pKF18-*oprN* was inserted into pMMB67EH to construct pOPRN1. To construct pMEXAB-OPRM1, pMex(sac/Hind)/Δ RI-pNOT19 was partially digested and a *Sac*I-*Hin*dIII fragment of about 6.5 kbp was separated by agarose gel electrophoresis and inserted into pMMB67EH.

Other techniques. Western blot analysis has been described before (28). The MICs of the antibiotics were determined by the agar dilution method using Mueller-Hinton agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.). Protein was quantified by the method of Lowry et al. (9).

RESULTS

Expression of MexEF-OprN in the mutant lacking MexAB-OprM and examination of antibiotic susceptibilities. To express the *mexE*, *mexF*, and *oprN* genes under the control of the *tac* promoter in *P. aeruginosa*, the DNA fragment containing the *mexE*, *mexF*, and *oprN* genes was excised from pUC19-*mexEFN*. This fragment was subcloned into the broad-host-range vector, pMMB67EH, yielding pMEXEF-OPRN1 (Fig. 1B). Introduction of pMEXEF-OPRN1 into TNP077 led to expression of substantial amounts of MexE, MexF, and OprN (Fig. 2, lane 3) and an undetectable level of MexAB-OprM. Similarly, strain TNP0771 carrying pMEXAB-OPRM produced substantial levels of MexAB-OprM proteins, but MexEF-OprN was undetectable (data not shown).

To elucidate which antibiotic might be a valid substrate of the MexEF-OprN pump, the antibiotic susceptibilities of these strains were examined. Strain TNP0773 harboring the pMMB67EH plasmid barely restored resistance to any of the antibiotics except for carbenicillin and cefoperazone. This resistance is most likely due to the selective Amp^r marker. On the other hand, TNP0775 harboring pMEXEF-OPRN restored resistance to norfloxacin, ofloxacin, chloramphenicol, and tetracycline (Table 2), suggesting that these antibiotics are substrates of the MexEF-OprN efflux pump. Interestingly, TNP0775 did not show resistance to gentamicin, kanamycin, erythromycin, novobiocin, ceftazidime, cefpirome, cefozopran, aztreonam, or meropenem, clearly indicating that these antibiotics are not substrates of the MexEF-OprN pump. Importantly, TNP0775 did not show resistance to imipenem. These findings showed that this carbapenem is not a substrate of the MexEF-OprN pump and suggested that carbapenem resistance in the *nfxC* mutant is not due to expression of MexEF-OprN. Consequently, it became clear that low-level expression of OprD is not linked to the expression of MexEF-OprN. Earlier studies showed inconsistent tetracycline susceptibility among the *nfxC* mutants (3, 11). However, the present study clarified this ambiguity and demonstrated that tetracycline is an excellent substrate of the MexEF-OprN pump.

A previously described *nfxC* mutant was hypersusceptible to β-lactam antibiotics (3). In this study, TNP0775 harboring the plasmid containing *mexEF-oprN* showed susceptibility to β-lactam antibiotics comparable to that of the strain harboring the plasmid only. To ascertain whether or not the MexEF-OprN pump is capable of recognizing and exporting the β-lactam antibiotics, we inserted *mexEF-oprN* genes into the pVLT33 plasmid with the Km^r marker instead of the Amp^r marker (2) and the resulting plasmid was introduced into TNP077. The recombinant strain (TNP0776) showed susceptibility to β-lactam antibiotics equal to that of the strain without pMEXEF-OPRN-KM1, besides demonstrating resistance to the fluoroquinolone antibiotics, chloramphenicol, trimethoprim, and tetracycline. Based on these results, we concluded that the MexEF-OprN pump does not export β-lactam antibiotics. In addition, the result showed that β-lactam hypersusceptibility in the *nfxC* mutant was not attributable to expression of the MexEF-OprN pump.

Assignment of the subunit protein(s) that recognizes the MexEF-OprN substrate. Whereas both MexAB-OprM and MexEF-OprN pumps export flluoroquinolone antibiotics, chloramphenicol, trimethoprim, and tetracycline, only the former, not the latter, exports β-lactams, novobiocin, and erythromycin. To determine which subunit of the MexEF-OprN pump recognizes substrates, we constructed an efflux pump consisting of mixed subunit proteins from MexAB-OprM and MexEF-OprN and conducted an assay for antibiotic selectivity. Expression of the desired subunit from two different efflux pump systems was confirmed by immunoblotting assays (Fig. 2). Mutants lacking both pump subunits were hypersusceptible to all antibiotics tested. Strains TNP0701, TNP0711, TNP0721, and TNP0731, which were MexA/ΔMexA, MexB/ΔMexB, OprM/ΔOprM, and MexAB/ΔMexAB recombinants, respectively, produced MexAB-OprM proteins and restored antibiotic resistance.

Next, strains with deletions of only one subunit, TNP070, TNP071, and TNP072, lacking MexA, MexB, and OprM, respectively, were transformed with a plasmid carrying genes encoding MexE, MexF, and OprN, respectively. The antibiotic susceptibility test with these constructs revealed that replacement of MexA with MexE and OprM with OprN resulted in total pump dysfunction. Two important observations were recorded. First, strain TNP0715, which expresses MexAF-OprM, showed a MIC of aztreonam that was two and four times higher, respectively, than those of TNP0713 and TNP071 (Table 3). This result implies that the MexAM complex may recognize β-lactam antibiotics. Second, replacement of OprM with OprN totally abolished the pump function, suggesting that

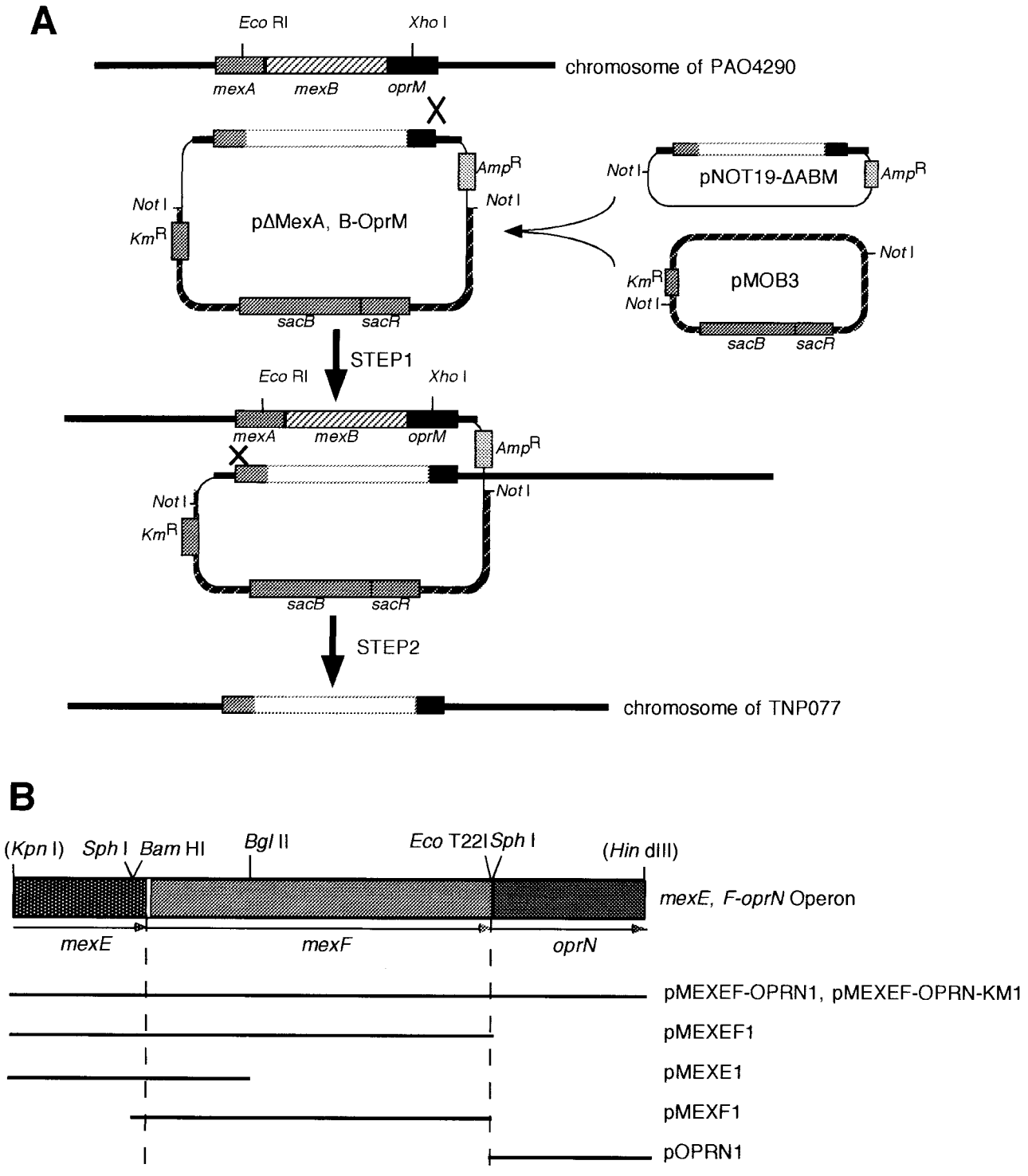

FIG. 1. Schematic representation of the procedure for deleting the chromosomal *mexAB-oprM* genes and subcloning DNA fragments into shuttle vector pMMB67EH. (A) Deletion of chromosomal *mexAB-oprM*. *Not*I-treated pNOT19-ΔABM was ligated to the *Not*I site of pMOB3, and the resulting pΔMexA,B-OprM was inserted into chromosomal *mexAB-oprM* by homologous recombination. The transconjugant was Km^r and sucrose-sensitive and contained an unwanted DNA fragment, which was excised by selecting for sucrose resistance (29). (B) Physical mapping of the restriction fragments subcloned into the shuttle vector. Solid lines represent the restriction fragments cloned into pMMB67EH (pMEXE1, pMEXF1, pOPRN1, pMEXEF1, and pMEXEF-OPRN1) and pVLT33 (pMEXEF-OPRN-KM1). Physical distances of the lines to the *mexEF-oprN* genes are arbitrary.

the MexAB unit does not interact functionally with OprN. Replacement of OprN with OprM fully restored the MexEF-OprN-type pump function, in which the strain was resistant to norfloxacin and chloramphenicol but susceptible to novobiocin and aztreonam. This phenotype is different from that of TNP0755 but is distinguishable from the MexAB-OprM-type pump function. The results imply that the MexEF unit may select the substrate for the MexEF-OprN pump.

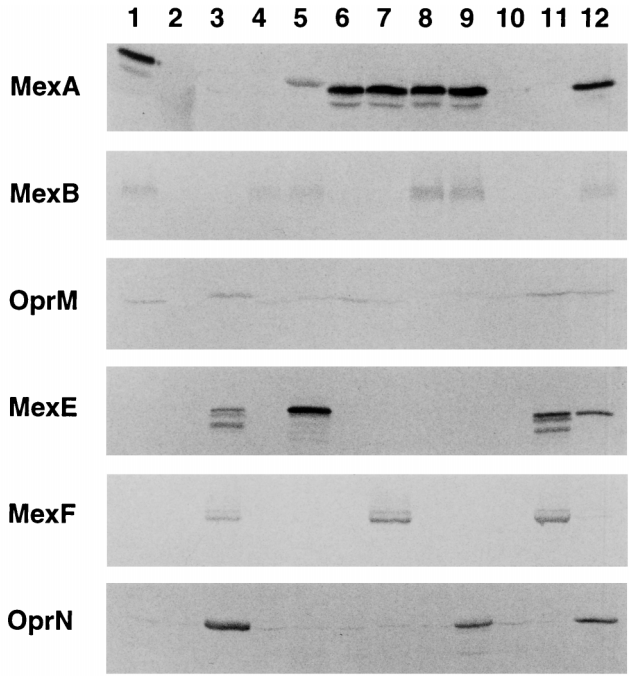

FIG. 2. Immunoblotting visualization of MexAB-OprM and MexEF-OprN proteins expressed in the constructs. Total cell lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the protein bands were visualized by the immunoblotting method. The amount of protein applied per lane was 8 μg for MexA, about 20 μg each for MexE, MexF, and OprN, and about 40 μg each for MexB and OprM. The antibody used to visualize a protein(s) is marked at the far left as the membrane protein. Lanes: 1, PAO4290(pMMB67EH); 2, TNP0773; 3, TNP0775; 4, TNP0703; 5, TNP0705; 6, TNP0713; 7, TNP0715; 8, TNP0723; 9, TNP0725; 10, TNP0733; 11, TNP0735; 12, KH4014a. Unexpected protein bands that appeared, for example, MexA in lane 5, where the MexE protein was stained by the anti-MexA protein, were most likely due to cross-reactivity of the antibody with highly homologous protein(s).

DISCUSSION

This study was conducted to clarify antibiotic selectivity of the MexEF-OprN pump and to assign a subunit protein(s), which filters the substrate antibiotics. The rationale for such a study is as follows. (i) The *nfxC* mutant expresses the MexEF-OprN efflux pump in the presence of a low level of MexAB-OprM. Therefore, the valid substrate for the MexEF-OprN pump was not clear. (ii) The *nfxC* mutant exhibits particularly intriguing properties, such as expressing the MexEF-OprN pump and gaining resistance to fluoroquinolone antibiotics, chloramphenicol, and trimethoprim; lacking the OprD protein, resulting in imipenem resistance; and showing hypersusceptibility to β-lactam antibiotics by an unknown mechanism. Therefore, the question of whether β-lactam hypersusceptibility is attributable to expression of MexEF-OprN or the *nfxC* mutation remains unanswered.

To address the former issue, we expressed the MexEF-OprN pump in the strain lacking MexAB-OprM and, importantly, without an *nfxC* mutation. Under these conditions, the MICs of the antibiotics appeared to be determined by the MexEF-OprN pump without any influence of the MexAB-OprM pump and the *nfxC* mutation. The results were that the transformant exhibited resistance to fluoroquinolones, chloramphenicol, trimethoprim, and tetracycline but was susceptible to β-lactam antibiotics, including carbapenem, at the level of the host strain. One may argue that β-lactamase interferes with the efflux pump function. However, this is unlikely because the level of β-lactamase in the *nfxC* mutant was fully comparable

TABLE 2. Antibiotic susceptibility of the strains constructed to test the substrate selectivity of the MexEF-OprN pump[a]

Strain	Plasmid	Pump component(s) expressed	MIC (μg/ml)																
			NFLX	OFLX	CP	TET	GM	KM	EM	NOV	CAZ	CPZ	CPR	CZOP	CAR	AZT	MPM	IPM	TMP
PAO4290		ABM	0.78	0.78	100	25	3.13	3.13	400	1,600	1.56	6.25	3.13	0.78	25	3.13	0.39	1.56	200
TNP077			0.1	0.05	3.13	0.39	0.39	3.13	25	100	0.39	1.56	1.56	0.39	3.13	0.2	0.2	1.56	12.5
TNP0773	pMMB67EH		0.1	0.05	3.13	0.39	0.39	3.13	12.5	100	0.78	25	3.13	0.39	>800	0.2	0.2	1.56	12.5
TNP0771	pMEXAB-OPRM1	ABM	3.13	3.13	800	50	3.13	3.13	400	1600	3.13	25	6.25	1.56	>800	25	1.56	1.56	>1,600
TNP0775	pMEXEF-OPRN1	EFN	3.13	3.13	1,600	3.13	0.39	3.13	12.5	100	0.39	25	1.56	0.39	>800	0.2	0.2	1.56	>1,600
TNP0776	pVLT33		0.1	0.1	3.13	0.39	0.39	400	12.5	100	0.39	3.13	3.13	0.39	6.25	0.2	0.39	1.56	12.5
TNP0777	pMEXEF-OPRN-KM1	EFN	6.25	3.13	1,600	1.56	0.39	400	12.5	100	0.39	3.13	1.56	0.39	3.13	0.2	0.2	1.56	>1,600
PAO4009		ABM	0.39	0.78	50	25	3.13	100	400	800	0.78	6.25	3.13	0.78	25	3.13	0.78	0.78	200
KH4014a		ABM, EFN	12.5	12.5	1,600	25	1.56	25	200	400	0.78	3.13	0.78	0.39	12.5	1.56	0.78	3.13	>1,600

[a] Mueller-Hinton agar contained 2 mM IPTG. Abbreviations: NFLX, norfloxacin; OFLX, ofloxacin; CP, chloramphenicol; TET, tetracycline; GM, gentamicin; KM, kanamycin; EM, erythromycin; NOV, novobiocin; CAZ, ceftazidime; CPZ, cefoperazone; CPR, cefpirome; CZOP, cefozopran; CAR, carbenicillin; AZT, aztreonam; MPM, meropenem; IPM, imipenem; TMP, trimethoprim. Pump components expressed are shown as varying combinations of the following subunits: A, MexA; B, MexB; E, MexE; F, MexF; M, OprM; and N, OprN.

TABLE 3. Antibiotic susceptibility of the strains constructed to assign the substrate-selective subunit(s)[a]

Strain	Plasmid	Pump component expressed	MIC (μg/ml)			
			NFLX	CP	NOV	AZT
TNP070		-BM	0.39	12.5	100	0.2
TNP0703	pMMB67EH	-BM	0.39	12.5	100	0.39
TNP0701	pMEXA1	ABM	0.39	100	1,600	1.56
TNP0705	pMEXE1	EBM	0.39	12.5	100	0.39
TNP071		A-M	0.39	12.5	100	0.2
TNP0713	pMMB67EH	A-M	0.39	12.5	100	0.39
TNP0711	pMEXB1	ABM	0.78	100	1,600	3.13
TNP0715	pMEXF1	AFM	0.39	12.5	50	0.78
TNP072		AB-	0.1	3.13	100	0.2
TNP0723	pMMB67EH	AB-	0.1	3.13	100	0.39
TNP0721	pOPRM1	ABM	0.78	50	200	3.13
TNP0725	pOPRN1	ABN	0.1	3.13	50	0.2
TNP073		--M	0.39	12.5	100	0.2
TNP0733	pMMB67EH	--M	0.39	12.5	100	0.39
TNP0731	pMEXAB1	ABM	0.78	200	1,600	3.13
TNP0735	pMEXEF1	EFM	3.13	1,600	100	0.2

[a] Mueller-Hinton agar contained 2 mM IPTG. Abbreviations: NFLX, norfloxacin; CP, chloramphenicol; NOV, novobiocin; AZT, aztreonam. Pump components expressed are shown as various combinations of the following subunits: A, MexA; B, MexB; E, MexE; F, MexF; M, OprM; and N, OprN. A hyphen indicates a missing subunit.

with that in the strain having wild-type *nfxC* in the presence of carbenicillin (3).

Tetracycline resistance in *nfxC* mutants has been shown to be strain dependent (3, 8, 11). Our study clearly demonstrated that tetracycline is an excellent substrate of the MexEF-OprN pump. On the other hand, MexEF-OprN pump expression caused neither β-lactam hypersusceptibility nor carbapenem resistance.

To assign the subunit protein responsible for substrate selectivity, we carried out a subunit swapping experiment similar to earlier investigations (5, 26, 27). We found that the MexEF unit acts synergistically with OprM and that the hybrid pump functioned equally to the MexEF-OprN pump. Three independent groups of investigators have carried out experiments to exchange the subunit proteins OprM and OprJ (5, 26, 27). They concluded that OprM fully functions with the MexCD unit and OprJ partially functions with the MexAB unit (5, 26, 27). More recently, interplay between the MexXY pump and OprM in *E. coli* cells was demonstrated (12). Therefore, OprM seems to be a universal outer membrane subunit for most *P. aeruginosa* efflux pumps.

To our surprise, OprN showed undetectable collaboration with the MexAB unit. The reason for the dysfunction of the MexAB-OprN hybrid pump is not known at present. We tested the possibility of a dominant-negative phenotype by expressing OprN in the strain expressing the MexAB-OprM but could not find such interaction (data not shown). We assumed until recently that two efflux pump systems, MexAB-OprM and MexEF-OprN, functioned in the *nfxC* mutant. However, the present study revealed that the *nfxC* mutant expresses three efflux pumps simultaneously: MexAB-OprM, MexEF-OprN, and an additional pump, MexEF-OprM.

Another interesting finding of the subunit swapping experiment was that strain TNP0725 expressing MexAB-OprM showed a slightly higher aztreonam MIC than either TNP070 or TNP0715, both of which lack MexB. This result implies that the hybrid pump consisting of MexAB-OprM might recognize β-lactam antibiotics. Since the MexF subunit in the MexEF-OprN pump is not involved in the export of β-lactam antibiotics, it is possible that MexA in combination with MexF is involved in β-lactam recognition. If so, it is conceivable that MexA in the MexAB-OprM pump plays a role in β-lactam selectivity. Though the difference is rather small, this result was confirmed by repeated experiments.

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