

## Characterization of the MexC-MexD-OprJ Multidrug Efflux System in $\Delta$ *mexA-mexB-oprM* Mutants of *Pseudomonas aeruginosa*

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**Expression of the multidrug efflux system MexC-MexD-OprJ in *nfxB* mutants of *Pseudomonas aeruginosa* contributes to resistance to fluoroquinolones and the “fourth-generation” cepheims (cefpime and ceftazidime), but not to most  $\beta$ -lactams, including the ordinary cepheims (ceftazidime and cefoperazone). *nfxB* mutants also express a second multidrug efflux system, MexA-MexB-OprM, due to incomplete transcriptional repression of this operon by the *mexR* gene product. To characterize the contribution of the MexC-MexD-OprJ system to drug resistance in *P. aeruginosa*, a site-specific deletion method was employed to remove the *mexA-mexB-oprM* region from the chromosome of wild-type and *nfxB* strains of *P. aeruginosa*. Characterization of mutants lacking the *mexA-mexB-oprM* region clearly indicated that the MexC-MexD-OprJ efflux system is involved in resistance to the ordinary cepheims as well as fluoroquinolones and the fourth-generation cepheims but not to carbapenem and aztreonam. Rabbit polyclonal antisera and murine monoclonal antibody against the components of the MexA-MexB-OprM system were prepared and used to demonstrate the reduced production of this efflux system in the *nfxB* mutants. Consistent with this, transcription of the *mexA-mexB-oprM* operon decreased in an *nfxB* mutant. This reduction appears to explain the hypersusceptibility of the *nfxB* mutant to  $\beta$ -lactams, including ordinary cepheims.**

The clinically important opportunistic pathogen *Pseudomonas aeruginosa* exhibits intrinsic multiple-antibiotic resistance, which has been assumed to result from the low permeability of its outer membrane (21, 35). The OprM-overproducing *nalB*-type mutants show increased resistance to quinolones,  $\beta$ -lactams, tetracycline, and chloramphenicol (14) as well as trimethoprim and sulfamethoxazole (9). Recent identification of the *mexA-mexB-oprM* operon on the *P. aeruginosa* chromosome (24, 25), whose products show significant homology to other bacterial efflux systems such as those encoded by *acrAB* (16) and *mtrCDE* (23), suggests that antibiotic resistance is caused by this system via drug efflux. Indeed, overexpression of this operon by a mutation in the regulator gene *mexR*, which exists upstream of *mexA-mexB-oprM* operon and transcribed in the opposite direction, affords *nalB*-type multidrug resistance to *P. aeruginosa* (27). Moreover, disruption of each gene of this operon, which is expressed in wild-type cells, increases susceptibility of both wild-type and *nalB*-type strains to the same level, suggesting that the intrinsic resistance of this bacterium results, in part, from the function of this efflux system (4, 12, 13, 24).

Two homologs of the *mexA-mexB-oprM* operon, *mexC-mexD-oprJ* and *mexE-mexF-oprN*, have recently been identified and are overexpressed in *nfxB* and *nfxC* mutant cells, respectively (10, 26). Transcription of the *mexC-mexD-oprJ* operon is strictly repressed by a negative regulator encoded by the *nfxB* gene (22, 26, 29), and overexpression of this operon, as a result of the *nfxB* mutation, confers on cells resistance to fluoroquinolones, the “fourth-generation” cepheims, tetracycline, and chloramphenicol and hypersusceptibility to most other  $\beta$ -lactams (19). The *nfxC* mutants expressing the *mexE-mexF-*

*oprN* operon are resistant to fluoroquinolones and imipenem (2, 18).

Expression of the *mexC-mexD-oprJ* and *mexE-mexF-oprN* operons is not detectable in the wild-type *P. aeruginosa* (10, 26), while the *mexA-mexB-oprM* operon is expressed constitutively (4). Therefore, resistance profiles characterized in the *nfxB* and *nfxC* mutants might be attributed to a combination of the MexA-MexB-OprM system and either the MexC-MexD-OprJ or MexE-MexF-OprN system.

One of us has recently devised a general mutagenesis system to delete a large and defined chromosomal fragment by using the site-specific resolution system encoded by a class II transposon (34). In this system, two copies of the site-specific resolution (*res*) site are inserted at two defined chromosomal positions so that the *res* sites are in the same orientation. Provision of the site-specific TnpR recombinase leads to very efficient excision of the DNA fragment flanked by the two *res* sites. An improved version of this system was developed in the present study and used to isolate mutants specifically lacking the *mexR-mexA-mexB-oprM* region from the wild-type and *nfxB* mutant strains of *P. aeruginosa*. The study of such deletion mutants elucidated the role of the MexC-MexD-OprJ efflux system in resistance to various antibiotics.

### MATERIALS AND METHODS

**Bacterial strains and media.** The bacterial strains used in this study are listed in Table 1. Bacterial cells were grown in L broth (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, and 0.5% [wt/vol] NaCl) or L agar (L broth plus 1.5% [wt/vol] agar) at 37°C. BM2 minimal medium (3) was used for selection of *P. aeruginosa* since *Escherichia coli* cannot utilize citrate. The following antibiotics were added to media at the indicated concentrations: ampicillin, 100  $\mu$ g/ml for *E. coli*; carbenicillin, 200  $\mu$ g/ml for *P. aeruginosa*; streptomycin, 30  $\mu$ g/ml for *E. coli* and 100  $\mu$ g/ml for *P. aeruginosa*; tetracycline, 10  $\mu$ g/ml for *E. coli* and 100  $\mu$ g/ml for *P. aeruginosa*; and chloramphenicol, 30  $\mu$ g/ml for *E. coli* and 200  $\mu$ g/ml for *P. aeruginosa*. L agar was supplemented with 5% (wt/vol) sucrose as required.

**Recombinant DNA techniques.** Transformation of *E. coli*, Southern hybridization, isolation of chromosomal DNA and plasmids, and restriction endonuclease digestions were carried out according to standard protocols (28). For PCR

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description <sup>a</sup>	Source or reference
<i>P. aeruginosa</i> strains		
PAO1	Prototroph	
OCR1	MexA-MexB-OprM-overproducing, <i>nalB</i> -type multidrug-resistant derivative of PAO1	17
KG2212	<i>mexR::res-Ω</i> of PAO1	This study
KG2239	<i>ΔmexR-mexA-mexB-oprM</i> of PAO1	This study
KG3052	Intermediately MexC-MexD-OprJ-overproducing, type-A <i>nfxB</i> multidrug-resistant derivative of PAO1 (formerly COR2)	19
KG2217	<i>mexR::res-Ω</i> of KG3052	This study
KG2240	<i>ΔmexR-mexA-mexB-oprM</i> of KG3052	This study
KG3056	Highly MexC-MexD-OprJ-overproducing, type-B <i>nfxB</i> multidrug-resistant derivative of PAO1 (formerly COR6)	19
KG2236	<i>mexR::res-Ω</i> of KG3056	This study
KG2259	<i>ΔmexR-mexA-mexB-oprM</i> of KG3056	This study
Plasmids		
pMT5059	pBend2 derivative carrying the multiple-cloning site and <i>NotI</i> site; Ap <sup>r</sup>	33
pKMM089	pMT5059 derivative carrying a 2.6-kb <i>XhoI-HindIII</i> fragment from pPV20; Ap <sup>r</sup>	This study
pKMM091	pKMM089 derivative carrying <i>NotI</i> -flanked <i>res-tnpR</i> block from pMT5085; Ap <sup>r</sup> Tc <sup>r</sup>	This study
pKMM102	pMT5059 derivative carrying <i>mexR</i> , <i>mexA</i> , <i>mexB</i> , and a part of <i>oprM</i> on a 24-kb <i>PvuII-XhoI</i> fragment; Ap <sup>r</sup>	This study
pKMM127	pKMM102 derivative carrying <i>SacI</i> -flanked <i>res-Ω</i> cassette from pMT5096 and <i>NotI</i> -flanked <i>Mob</i> cassette from pMT5071; Ap <sup>r</sup> Cm <sup>r</sup>	This study
pKMM151	pMT5059 <i>ori</i> -carrying plasmid rescued from KG2213 chromosome; Ap <sup>r</sup>	This study
pKMM157	pKMM089 derivative carrying <i>SacI</i> -flanked <i>res-Ω</i> cassette from pMT5095 and <i>NotI</i> -flanked <i>res-tnpR</i> block from pMT5085; Ap <sup>r</sup> Sm <sup>r</sup> Tc <sup>r</sup>	This study
pMP190	Broad-host-range, low-copy-number <i>lacZ</i> fusion vector; Cm <sup>r</sup> Sm <sup>r</sup>	31
pKMM301	pMP190 derivative carrying the <i>mexR-mexA</i> intergenic region on a 276-bp PCR product amplified with <i>mexR7-mexA3</i> , with the <i>mexA</i> promoter oriented towards the promoterless <i>lacZ</i> gene; Cm <sup>r</sup> Sm <sup>r</sup>	This study

<sup>a</sup> Abbreviations: Ap<sup>r</sup>, ampicillin resistant; Cb<sup>r</sup>, carbenicillin resistant; Cm<sup>r</sup>, chloramphenicol resistant; Km<sup>r</sup>, kanamycin resistant; Sm<sup>r</sup>, streptomycin resistant; and Tc<sup>r</sup>, tetracycline resistant.

amplification of chromosomal DNA sequences (6), a bacterial colony was directly suspended in reaction mixture (6) lacking the primers and *Taq* polymerase. The mixture was boiled for 10 min and used as the template for amplification. The primer pairs used were as follows: *mexR1* (5'-ATGAACCTACCCCGTGAATCCC-3') and *mexR2* (5'-TTAAATATCCTCAAGCGGTTGC-3') for *mexR*, 1611 and 1612 for *mexA*, 1613 and 1614 for *mexB*, and 1615 and 1680 for *oprM*, all of which have been described previously (6). The sizes of the amplified DNA fragments obtained by using these primer pairs were about 0.7, 1.5, 3.2, and 1.4 kb, respectively.

**Cloning of chromosomal regions flanking the *mexR-mexA-mexB-oprM* operon.** To clone the chromosomal region located downstream of *mexR*, the chromosomal DNA of PAO1 was digested completely with *XhoI* and then partially with *PvuII*. The restriction fragments with sizes around 20 kb were collected and ligated with pMT5059 (33) that had been treated with *XhoI* and *PvuII*. The ligation mixture was used for transformation of *E. coli* DH5α (1) to select Ap<sup>r</sup> clones on L agar plates. The *mexR* gene was successfully amplified by PCR (see above) from one of the 48 Ap<sup>r</sup> clones, and the plasmid obtained from such a clone was termed pKMM102. This plasmid carried an ~24-kb chromosomal fragment extending from a *PvuII* site downstream of *mexR* to the *XhoI* site in *oprM*. The chromosomal region located downstream of *oprM* was cloned as follows. Since the KG2213 (see below) chromosome carried a part of the pMT5059-derived *oriV* and *bla* gene, the chromosomal DNA of KG2213 was digested with *SacI*, self-ligated, and used to transform DH5α to obtain Ap<sup>r</sup> clones. One of the plasmids thus recovered, termed pKMM151, contained an ~10-kb chromosomal fragment extending from the *XhoI* site in *oprM* to a *SacI* site located downstream of *oprM*.

**Construction of recombinant plasmids.** The *SacI*-digested *res-Ω* cassette from pMT5096 (34) was inserted into the *SacI* site in the *mexR* gene of pKMM102, followed by insertion of the pMT5071-derived, *NotI*-flanked *Mob* cassette (34) into the *NotI* site to generate pKMM127 (see Fig. 1). The 8.4-kb *SacI-HindIII* fragment encompassing a *mexA-mexB-oprM* region on pPV20 (25) was inserted into pAK1900 (25), and the resulting plasmid contains a 2.6-kb *XhoI-HindIII* fragment that covers the 3' part of *oprM* and its downstream region. A *NotI* site in this fragment was disrupted by blunt ending with T4 DNA polymerase, and such a modified fragment was inserted into pMT5059 to construct pKMM089. pKMM096 was constructed by insertion of the pMT5095-derived, *XhoI*-flanked *res-Ω* cassette into the *XhoI* site of pKMM089. The *NotI*-flanked *res-tnpR* block from pMT5085 (34) was inserted into the pMT5059-derived *NotI* site of pKMM089 and pKMM096 to construct pKMM091 and pKMM157, respectively.

The chromosomal region between the start codon of *mexR* and *mexA* was

amplified by using primers *mexR7* (5'-ATTGTTGGCCGAGTAAACC-3') and *mexA3* (5'-TAGCGTTGCTCATGAGCG-3') and inserted into the blunt-ended *SacI* site of pMP190 (31) to yield pKMM301, such that the promoterless *lacZ* gene was transcribed from the *mexA* promoter.

**Mobilization of recombinant plasmids to *P. aeruginosa* chromosome.** An appropriate plasmid residing in an *E. coli* strain, S17-1 (30), was conjugationally mobilized to *P. aeruginosa* cells. After mating on L agar at 37°C for 4 h, the cell mixture was suspended in 0.4 ml of physiological saline. Aliquots (0.1 ml) of the 10- or 100-fold-diluted suspensions were plated on BM2 minimal agar plates (3) supplemented with appropriate antibiotics and incubated at 30°C for 2 days. The transconjugants thus obtained were purified once on the same selective plates, and examined for resistance to streptomycin, tetracycline, chloramphenicol, and/or sucrose on L agar plates. Clones indicating appropriate resistance to these selective markers were used in subsequent experiments.

**Deletion of chromosomal *mexR-mexA-mexB-oprM*.** Plasmid pKMM127 (Fig. 1 and Table 1) carries a *res-Ω* cassette in the *mexR* gene and a *Mob* cassette, and this plasmid was mobilized from S17-1 to PAO1 (a wild-type strain), KG3052 (a type-A *nfxB* strain), and KG3056 (a type-B *nfxB* strain) to select Sm<sup>r</sup> transconjugants. Among the transconjugants, those showing resistance to sucrose (e.g., KG2212, KG2217, and KG2236 from PAO1, KG3052, and KG3056, respectively) were presumed to have been formed by allelic exchange of the wild-type *mexR* gene with the mutant allele. This was confirmed by the fact that the *mexR* region could not be amplified by PCR from KG2212, KG2217, or KG2236 chromosome, consistent with an increase in the size of the *mexR* region in these strains as a result of insertion of the *res-Ω* cassette (data not shown). The argument for the expected allelic exchange was further supported by Southern hybridization experiments (data not shown).

To obtain the chromosomal deletion by using the original TnpR-mediated deletion system (34), pKMM091 (Table 1) carrying a pMT5085-derived *res-tnpR* block was transferred from *E. coli* S17-1 to KG2212 (PAO1 *mexR::res-Ω*) to select Tc<sup>r</sup> transconjugants on BM2 agar plates. These plasmid integrants were unstable, and their cultivation on L agar plates generated segregants which commonly lost the Sm<sup>r</sup> and Tc<sup>r</sup> markers but not the *sacB* gene (e.g., KG2213).

To eliminate, in the process of deletion mutagenesis, the plasmid-derived sequence including the Cb<sup>r</sup> marker (*bla*), we developed a new improved method that involved mobilization of pKMM157 (Fig. 1 and Table 1) from S17-1 to the three *mexR::res-Ω* mutants of *P. aeruginosa* (KG2212, KG2217, and KG2236) and selection of Tc<sup>r</sup> transconjugants on BM2 agar plates. Phenotypic characterization and Southern analysis indicated that such transconjugants were formed by integration of pKMM157 into the chromosomal *oprM* gene (Fig. 1) (data not shown).

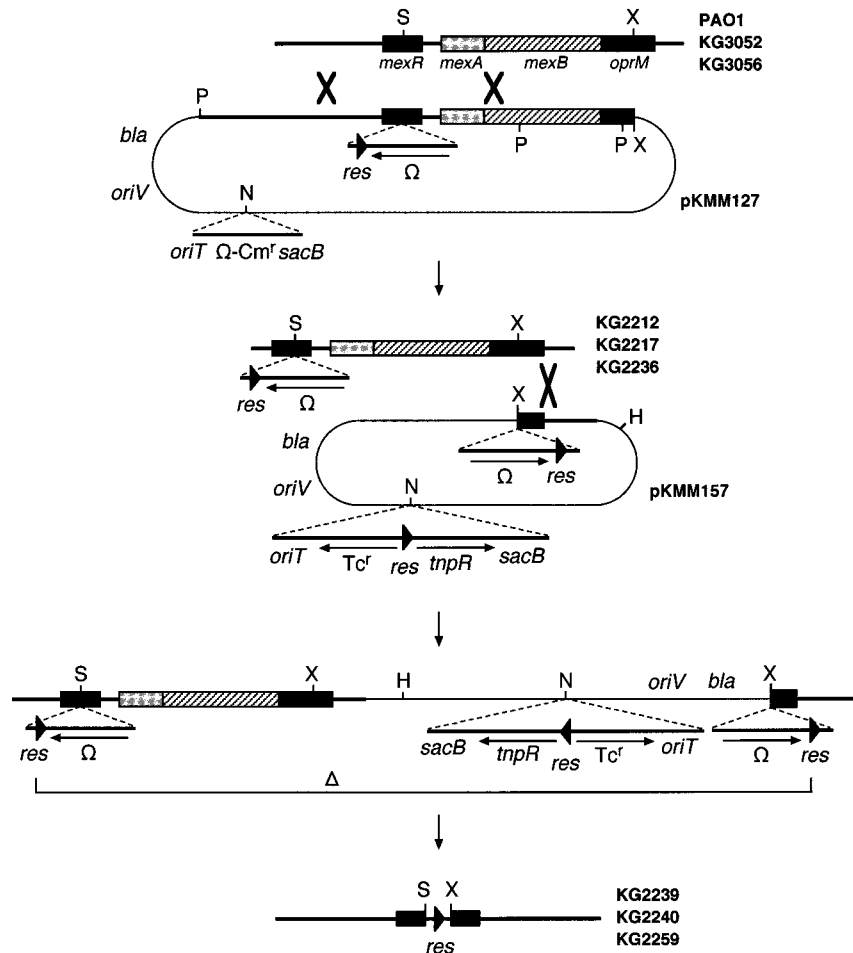


FIG. 1. Schematic models of the TnpR-mediated site-specific deletion based on an improvement of the deletion system developed by Tsuda (34). Specifically, the plasmid carrying an additional *res-Ω* cassette and a *res-tnpR* block (pKMM157) was integrated into the *oprM* gene of the chromosome containing another *res-Ω* cassette in the *mexR* gene. Deletion between the two copies of the *res* site led to removal of the intervening chromosomal region and the integrated plasmid sequence, leaving one copy of the *res* site. Abbreviations for restriction endonuclease sites: P, *Pvu*II; S, *Sac*I; X, *Xho*I; H, *Hind*III; and N, *Not*I.

These transconjugants gave rise to Tc<sup>s</sup> segregants at high frequencies after single-colony isolation on L agar plates. Each of three segregants obtained from the three crosses were resistant to sucrose and sensitive to streptomycin and carbenicillin. Such segregants derived from KG2212, KG2217, and KG2236 were designated KG2239, KG2240, and KG2259, respectively (Fig. 1).

**Design of oligopeptides and development of polyclonal antisera specific to MexA and MexB.** To avoid potential problems with the purification of MexA and MexB, oligopeptides based on the deduced amino acid sequences of these proteins (24, 25) were synthesized and used to immunize rabbits. Hydrophathy analysis was performed with the Analysis Plot program (the Kyte and Doolittle algorithm [11]) included in the GeneWorks software package (Intelligenetics), and the amino acid sequences predicted to belong to the hydrophilic region of each protein were used for design of the oligopeptides. Multiple antigen peptides (32) composed of these oligopeptides linked to poly-lysine carrier were manually synthesized on a Multiple Peptide Synthesizer (Shimadzu Model PSSM-8) by solid-phase peptide synthesis on TAK08-WTGS resin (Shimadzu). New Zealand White rabbits (female; 10 weeks old) were immunized with 20 μg of the prepared antigen at weeks 1, 3, and 6. The first injection was in Freund's complete adjuvant, the second and third ones were in Freund's incomplete adjuvant, and in all cases the antigen was injected subcutaneously. Titers were determined by an enzyme-linked immunosorbent assay using total membranes of the *P. aeruginosa* OCR1 cells, prepared by sonication and subsequent centrifugation (see below), as the antigen.

**Development of murine monoclonal antibody specific to OprM.** BALB/c mice were immunized with 20 μg of purified OprM (4) on days 1, 7, 14, and 17 and an anti-OprM antibody-producing clone was prepared as described previously (5). A monoclonal antibody secreted by this clone was termed TM001.

**Isolation of total membranes, SDS-PAGE, and immunoblot assay.** Cells grown in L broth were harvested by centrifugation at 5,000 × g for 10 min at 4°C. The cells were resuspended in 10 mM Tris-HCl, pH 8.0, and were broken by soni-

cation. After the removal of unbroken cells by centrifugation, total membranes (cell envelopes) were pelleted from the resulting supernatant by centrifugation at 20,000 × g for 30 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (4), with 10% (wt/vol) acrylamide in the running gel. Proteins fractionated by SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane (0.45-μm pore size; Bio-Rad) as described previously (5). Binding of the primary antibodies was detected as described previously (4), with alkaline phosphatase-conjugated goat antibodies to rabbit immunoglobulin G (Cappel) or alkaline phosphatase-conjugated goat antibodies to mouse immunoglobulin G (Cappel) as the secondary antibodies and an Ap Conjugate Substrate Kit (Bio-Rad) for color development.

**β-Galactosidase assays.** Bacteria harboring the plasmid pKMM301 were cultured overnight at 37°C in A medium (28) supplemented with 0.4% (wt/vol) glucose, thiamine (1 μg/ml), 1 mM MgSO<sub>4</sub>, and chloramphenicol (12.5 μg/ml for KG2239 or 400 μg/ml for KG2259) and subsequently diluted 50-fold into fresh medium consisting of the same solutes. Following growth to mid-log phase (*A*<sub>600</sub>, 0.3 to 0.6), cultures were assayed for β-galactosidase activity as described previously (20).

## RESULTS AND DISCUSSION

**Construction of Δ*mexR-mexA-mexB-oprM* strains.** To characterize the precise nature of the contribution of MexC-MexD-OprJ to antibiotic resistance, we attempted to delete the *mexA-mexB-oprM* operon and the *mexR* gene from the chromosomes of two types of *nfxB* mutants that have different expression levels of *mexC-mexD-oprJ* (19). An improved method developed in this study facilitated our subsequent isolation of the

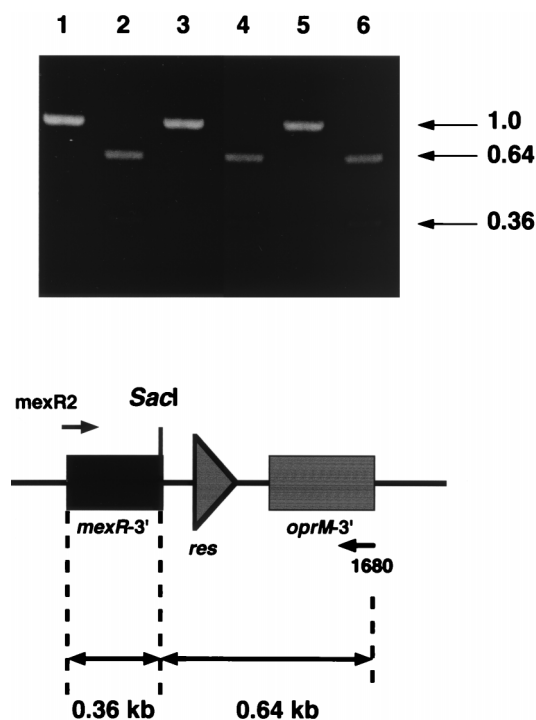


FIG. 2. Amplification of chromosomal fragments containing deletion endpoints. Approximately 1-kb fragments containing the remaining *res* site were amplified by PCR using primer sets that annealed at the 3' ends of *mexR* and *oprM* (primers mexR2 and 1680, respectively). These fragments were digested with *SacI* and electrophoresed on a 1% (wt/vol) agarose gel. Lanes: 1 and 2, KG2239; 3 and 4, KG2240; 5 and 6, KG2259. Lanes 1, 3, and 5 show the ca. 1-kb amplified fragments, and lanes 2, 4, and 6 show the *SacI*-digested PCR products. Boxes labeled *mexR*-3' and *oprM*-3' refer to the 3' ends of each gene. The sizes of the fragments are indicated in kilobases at the right of the figure.

deletion mutants (KG2239, KG2240, and KG2259 from PAO1, KG3052 [type-A *nfxB*], and KG3056 [type-B *nfxB*], respectively) that carried only a single copy of the *res* site (Fig. 1). The PCR amplification of the chromosomal DNAs of the three segregants by using mexR2 (antisense primer for *mexR*) and 1680 (antisense primer for *oprM*) generated 1.0-kb fragments, which generated 0.36- and 0.64-kb fragments by *SacI* digestion (Fig. 2). In contrast, no PCR products were detected when the primer pairs for *mexR*, *mexA*, *mexB*, and *oprM* were used (data not shown). Southern hybridization experiments of *SacI*-di-

gested chromosomal DNAs of the three segregants further demonstrated the presence of the *res* site and the absence of the *tnpR-sacB-oriV-bla* region (data not shown). All of these results confirm that the segregants did in fact contain the chromosomal structures as depicted in Fig. 1. This improved system will be of use in many bacterial species to construct chromosomal deletion mutants that contain only the *res* site, and such mutant strains should prove suitable for biotechnological use in environmental bacteria.

**Development of antibodies specific to MexA, MexB, and OprM.** To detect the production of MexA-MexB-OprM in the mutants isolated in this study, we prepared a murine monoclonal antibody specific to OprM and rabbit antisera specific to MexA and MexB. For this purpose, purified OprM (4) and synthetic oligopeptides were used as antigens for immunization. An immunoblot assay using an OprM-specific monoclonal antibody, TM001, as the primary antibody showed slight production of 49-kDa OprM in PAO1 and its overproduction in the *nalB*-type mutant OCR1 derived from PAO1 (data not shown). Similar results were obtained with antisera from rabbits immunized with oligopeptide 92106 (YQIDPATYEA DYQSA) for MexA and oligopeptide 423437 (EGLSPREAA RKSMGQ) for MexB (data not shown).

**Susceptibility testing and substrate specificity of the two efflux systems.** Table 2 shows the susceptibilities of the constructed mutants and their parent strains. Expression levels of various components of multidrug efflux systems were confirmed by immunoblot assays using rabbit polyclonal antisera for MexA (92106 [see above]) for MexB (423437 [see above]) for MexC (MEXC7 [7]) and for MexD (MEXD7 [7]), and murine monoclonal antibodies for OprM (TM001 [see above]) and for OprJ (HJ001 [8]). The *nfxB* mutants KG3052 (type A) and KG3056 (type B) showed resistance to fluoroquinolones, the fourth-generation cepheims, tetracycline, and chloramphenicol and hypersusceptibility to ordinary cepheims, carbenicillin, and aztreonam, concomitant with production of MexC-MexD-OprJ (Fig. 3, lanes 4 and 7). Elimination of *mexR* by insertion of *res-Ω* resulted in overproduction of MexA-MexB-OprM in the *nfxB* strains and PAO1 (Fig. 3, lanes 2, 5, and 8), as reported by Poole et al. (27), and increased resistance to all antimicrobials tested (see KG2212 [Table 2]). In contrast, the susceptibility to all antimicrobials tested increased in the *mexR-mexA-mexB-oprM*-deficient PAO1 strain, KG2239.

Susceptibility tests also produced information on the substrate specificity of the MexA-MexB-OprM and MexC-MexD-OprJ efflux systems. Comparison of the levels of resistance to

TABLE 2. Susceptibilities of constructed mutants to various antibiotics

Strain	Relevant genotype <sup>a</sup>	MIC (μg/ml) of <sup>b</sup> :											
		SPFX	CPFX	NFLX	LVFX	CZOP	CPR	CAZ	CPZ	CBPC	AZT	TC	CP
PAO1	Prototroph	0.39	0.10	0.39	0.39	0.39	1.56	1.56	6.25	12.5	1.56	6.25	12.5
OCR1	<i>nalB</i>	1.56	0.78	3.13	3.13	1.56	6.25	6.25	25	100	12.5	25	100
KG2212	PAO1 <i>mexR::res-Ω</i>	1.56	0.39	1.56	1.56	0.78	3.13	3.13	12.5	50	6.25	12.5	50
KG2239	PAO1ΔRABM	0.05	0.012	0.05	0.05	0.10	0.39	0.20	0.39	0.39	0.10	0.20	0.78
KG3052	Type-A <i>nfxB</i>	6.25	1.56	6.25	3.13	3.13	6.25	0.78	3.13	6.25	0.78	12.5	25
KG2217	KG3052 <i>mexR::res-Ω</i>	6.25	1.56	6.25	3.13	3.13	6.25	3.13	12.5	50	6.25	25	50
KG2240	KG3052ΔRABM	6.25	1.56	6.25	3.13	3.13	6.25	0.39	0.78	0.39	0.10	3.13	12.5
KG3056	Type-B <i>nfxB</i>	12.5	3.13	12.5	6.25	6.25	12.5	0.78	3.13	1.56	0.39	25	50
KG2236	KG3056 <i>mexR::res-Ω</i>	12.5	3.13	12.5	6.25	6.25	12.5	3.13	25	50	6.25	25	50
KG2259	KG3056ΔRABM	12.5	3.13	12.5	6.25	6.25	12.5	0.78	1.56	0.39	0.10	25	50

<sup>a</sup> ΔRABM, deletion of *mexRAB-oprM* operon.

<sup>b</sup> Abbreviations: SPFX, sparfloxacin; CPFX, ciprofloxacin; NFLX, norfloxacin; LVFX, levofloxacin; CZOP, ceftazidime; CPR, cefpirome; CAZ, ceftazidime; CPZ, cefoperazone; CBPC, carbenicillin; AZT, aztreonam; TC, tetracycline; and CP, chloramphenicol.

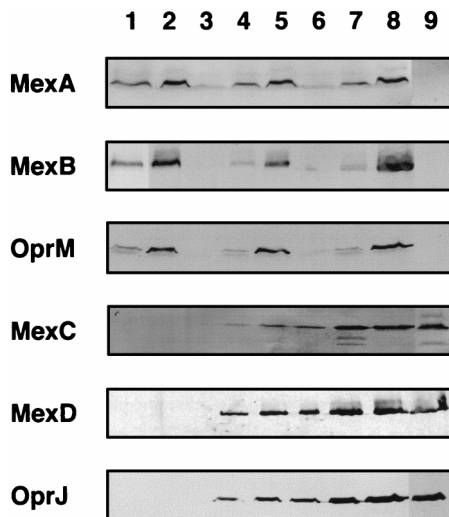


FIG. 3. Detection of MexA-MexB-OprM and MexC-MexD-OprJ component proteins with antisera directed against synthetic oligopeptides containing part of the amino acid sequences of MexA, MexB, MexC, or MexD or monoclonal antibodies specific to OprM or OprJ. Each lane contains 30  $\mu$ g of cell envelope protein, as determined by the method of Lowry et al. (15). Lanes: 1, PAO1; 2, KG2212 (PAO1 *mexR::res- $\Omega$* ); 3, KG2239 (PAO1  $\Delta$ *mexR-mexA-mexB-oprM*); 4, KG3052 (PAO1 type-A *nfxB*); 5, KG2217 (KG3052 *mexR::res- $\Omega$* ); 6, KG2240 (KG3052  $\Delta$ *mexR-mexA-mexB-oprM*); 7, KG3056 (PAO1 type-B *nfxB*); 8, KG2236 (KG3056 *mexR::res- $\Omega$* ); and 9, KG2259 (KG3056  $\Delta$ *mexR-mexA-mexB-oprM*).

tetracycline and chloramphenicol between KG2239 and KG2240 (Table 2) revealed that the MexC-MexD-OprJ system could contribute to the extrusion of these agents. However, the resistance levels to tetracycline and chloramphenicol, but not those to the fluoroquinolones or the fourth-generation cepheims, differed among the three type-A *nfxB* strains, KG3052, KG2217, and KG2240 (Table 2), that were distinguished from one another by the amounts of each component of the MexA-MexB-OprM system (Fig. 3). These results suggested that the MexC-MexD-OprJ system had, in comparison with the MexA-MexB-OprM system, a higher specificity to cause the extrusion of the fluoroquinolones and the fourth-generation cepheims and a lower specificity to cause the extrusion of tetracycline and chloramphenicol. The three strains KG2239, KG2240, and KG2259 commonly lacked the *mexA-mexB-oprM* operon, and the last two of these strains, which express the MexC, MexD, and OprJ components showed higher resistance to ordinary cepheims but not to carbenicillin and aztreonam (Table 2). This suggests the involvement of the MexC-MexD-OprJ system in the extrusion of ordinary cepheims.

**Expression of MexA-MexB-OprM in *nfxB* strains.** Immunoblot assays unexpectedly demonstrated that the amount of MexA-MexB-OprM produced in both *nfxB* mutants KG3052 and KG3056 was less than that produced in PAO1 (Fig. 3, lanes 1, 4, and 7). To confirm this, pKMM301 carrying the promoterless *lacZ* gene downstream of the *mexA* promoter was constructed and introduced into KG2239 (PAO1  $\Delta$ *mexR-mexA-mexB-oprM*) and KG2259 (KG3056  $\Delta$ *mexR-mexA-mexB-oprM*). The latter strain showed lower  $\beta$ -galactosidase activity (474  $\pm$  59 Miller units) than the former strain (1,013  $\pm$  59 Miller units). This indicated that the decreased production of MexA-MexB-OprM in the *nfxB* mutant KG3056 is at least partly due to the reduced transcription of the *mexA-mexB-oprM* operon. Decreased production of MexA-MexB-OprM

explains the hypersusceptibility of the *nfxB* mutants to  $\beta$ -lactams, because in the absence of MexA-MexB-OprM, *nfxB* mutation did not confer hypersusceptibility to the  $\beta$ -lactams tested (KG2239, KG2240, and KG2259 [Table 2]).

In the present study, the characterization of the MexC-MexD-OprJ efflux system in *nfxB* strains that lacked the *mexA-mexB-oprM* region revealed that (i) the MexC-MexD-OprJ efflux system works to cause the extrusion of not only fluoroquinolones, fourth-generation cepheims, tetracycline, and chloramphenicol but also ordinary cepheims (KG2240 and KG2259 [Table 2]); (ii) this system apparently does not function in the efflux of carbenicillin and aztreonam (KG2240 and KG2259 [Table 2]); and (iii) hypersusceptibility to  $\beta$ -lactams, including ordinary cepheims, in *nfxB* mutants is due to decreased expression of MexA-MexB-OprM rather than being a direct function of MexC-MexD-OprJ. Previous studies (8, 18, 19, 26) suggested that the ordinary cepheims are saved from efflux by the MexC-MexD-OprJ efflux system, because those studies used *nfxB* mutants that still expressed MexA-MexB-OprM. By construction of MexA-MexB-OprM-lacking *nfxB* mutants, we have demonstrated that the MexC-MexD-OprJ system also functions for extrusion of the ordinary cepheims. In addition, *nfxC* mutants exhibit hypersusceptibility to  $\beta$ -lactams, including ordinary cepheims (2, 10). The *nfxC* mutation causes expression of a third efflux system, MexE-MexF-OprN, in MexA-MexB-OprM-producing (i.e., wild-type) strains. Construction of MexA-MexB-OprM-deficient mutants may be required to characterize the precise nature of the contribution of MexE-MexF-OprN to antibiotic resistance.

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