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The Outer Membrane Protein OprM of *Pseudomonas aeruginosa* Is Encoded by *oprK* of the *mexA-mexB-oprK* Multidrug Resistance Operon

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An outer membrane protein (OprK) overproduced in a multiply antibiotic-resistant strain of *Pseudomonas aeruginosa* was previously identified as the product of the third gene of a multidrug resistance operon, *mexA-mexB-oprK* (K. Poole, K. Krebes, C. McNally, and S. Neshat, J. Bacteriol. 175:7363–7372, 1993). To determine whether this protein was identical to another outer membrane protein (OprM) also overproduced in some multiply resistant strains, attempts were made to map the transposon insertion site of several OprM-deficient mutants to the *mex* operon. Amplification of chromosomal DNA of several Tn*5* insertion OprM-deficient mutants with primers specific to each gene of the *mex* operon revealed that the transposon had inserted into *mexB* in one instance and into *oprK* in two others. Furthermore, introduction of the cloned *mexA-mexB-oprK* operon into these mutants restored expression of multidrug resistance, concomitant with OprM production. These data demonstrated that OprM is encoded by the *mex* operon. OprM and OprK were not, however, immunologically cross-reactive, indicating that they are distinct proteins and that OprK is, in fact, not encoded by the *mex* operon. This operon is thus renamed *mexA-mexB-oprM*.

Pseudomonas aeruginosa, a causative agent of opportunistic infections, exhibits intrinsically high resistance to various antimicrobial agents. Clinical usage of potent antipseudomonal agents such as fluoroquinolones in cases of infection caused by *P. aeruginosa* has been followed by the emergence of clinical isolates resistant to multiple antibiotics, including these agents (7, 10, 11, 17). Mutation of the *nalB* gene on the chromosome of *P. aeruginosa* increased resistance of this bacterium to quinolones, cephems, penams, meropenem, tetracycline, chloramphenicol, and erythromycin, concomitant with overproduction of the outer membrane protein OprM (49 kDa) (3, 11, 15). Gotoh et al. (2) have demonstrated the involvement of OprM in expression of resistance of *P. aeruginosa* to those agents by characterization of transposon insertion OprM-deficient mutants. Poole et al. (13) reported the overproduction of a 50-kDa outer membrane protein (OprK) in multiply antibiotic resistant *P. aeruginosa*. Moreover, the authors identified an operon, *mexA-mexB-oprK*, which apparently includes the OprK gene and whose role in resistance to multiple antibiotics, including ciprofloxacin, tetracycline, and chloramphenicol, has been confirmed (13). The products of the operon show homology to a number of bacterial proteins with export-efflux function (13), and a role in antibiotic efflux has recently been demonstrated (8). In light of the similarities between OprK and OprM, it was of interest to determine whether they were, in fact, the same protein or whether OprM is encoded by an unknown multidrug resistance gene on the *P. aeruginosa* chromosome.

P. aeruginosa PAO strains used in this study are as follows: PAO4141 (*met-9020 proA9024 blaJ9111 blaP9202 aph-9001*) and its OprM-overproducing mutant KG2113 (2) and their respective Tn*5* insertion OprM-deficient mutants KG2109 (2), KG2110 (2), and KG2114 (2); *mexA* mutant K590 (*mexA*::*tet*) (12) and *oprK* mutant K613 (*oprK*::ΩHg) (13) and their parent strain K372 (14); and *mexB* mutant K636 (*mexB*::mini-Tn*10*-Kan) (14) and its parent strain K635 (12). We attempted to identify the location of the transposon on the chromosome of OprM-deficient mutants KG2109, KG2110, and KG2114. Approximately 10^6 cells grown at 37°C overnight on an L broth agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) plate were suspended in 20 μl of sterilized pure water. Following incubation in boiling water for 10 min, 5 μl of the suspension was diluted 10 times with sterilized pure water and used as a template solution in the PCR. The following forward and reverse primer pairs for amplification of the *mexA*, *mexB*, and *oprK* gene segments of *P. aeruginosa* PAO were designated from published DNA sequences of these genes (12, 13): for *mexA*, sense primer 1611, 5′-CAATCGAGCTCGCTCTGG AT-3′, from the *Sst*I site upstream of the initiation codon ATG of the gene, and antisense primer 1612, 5′-TCAGCCCTTGCT GTCGGTTT-3′, including the termination codon TGA of the gene; for *mexB*, sense primer 1613, 5′-TCAAGGGGATTCGT AATGTCG-3′, including the Shine-Dalgarno sequence between *mexA* and *mexB*, and antisense primer 1614, 5′-TCATT GCCCCTTTTCGACGG-3′, including the termination codon TGA of the gene; for *oprK*, sense primer 1615, 5′-TATGAAA CGGTCCTTCCTTTCC-3′, including the initiation codon ATG, and antisense primer 1680, 5′-CTCGCTGGTCAGCTG ATTGA-3′, beginning 64 bp upstream of the TAG termination codon.

PCR was performed on genomic DNA of *P. aeruginosa* strains with a Perkin-Elmer Cetus Thermal Cycler, using *Taq* DNA polymerase (Perkin-Elmer) for 30 cycles in which the conditions were 15 s at 94°C for denaturation, 30 s at 55°C for annealing, and 60 s at 72°C for polymerization. Amplification mixtures were subjected to electrophoresis on a 1.0% agarose gel and stained with ethidium bromide. The specific amplified

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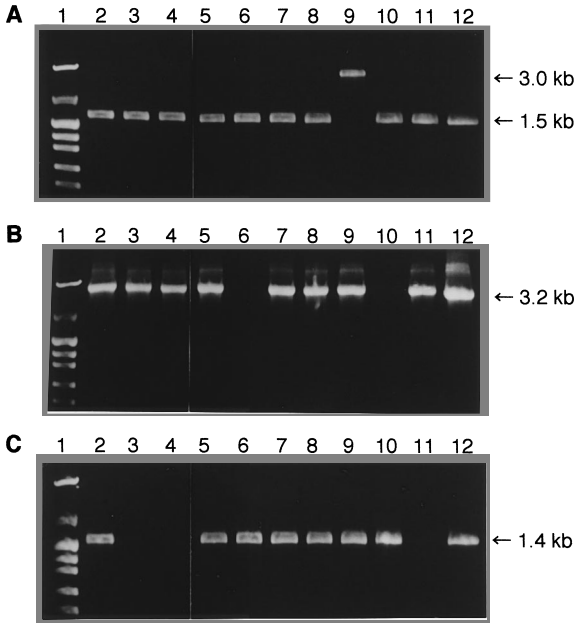

FIG. 1. Detection of the multidrug resistance operon genes *mexA* (A), *mexB* (B), and *oprK* (C) by PCR. PCR experiments were performed by using oligonucleotide DNA synthesized based on DNA sequences in the region including the genes in the *mexA-mexB-oprK* operon (12, 13) as primer pairs and cells incubated in boiling water for 10 min as a template under the conditions described in the text. Lanes: 2, PAO4141 (wild-type producer of OprM); 3, KG2109 (OprM-deficient mutant); 4, KG2110 (OprM-deficient mutant); 5, KG2113 (overproducer of OprM); 6, KG2114 (OprM-deficient mutant); 7, K372 (parent strain of K590 and K613); 8, K635 (parent strain of K636); 9, K590 (*mexA*::*tet*); 10, K636 (*mexB*::mini-Tn*10*-Kan); 11, K613 (*oprK*::ΩHg); 12, pPV20. Lanes 1, size references (PHY marker; Takara), 4,870, 2,016, 1,360, 1,107, 926, 658, and 489 bp.

DNA fragments were visualized by UV illumination (Fig. 1). The authenticity of all PCR products was tested via restriction digestion, and fragments expected from DNA sequences of *mexA*, *mexB*, and *oprK* were observed in all respective PCR products (data not shown). When *mexA* primers were used, ca. 1.5-kb segments were amplified in OprM-deficient mutants KG2109, KG2110, and KG2114 and their respective parent strains PAO4141 (first two mutants) and KG2113 and in K636 (*mexB*::mini-Tn*10*-Kan) and K613 (*oprK*::ΩHg) and their respective parent strains K372 and K636. In K590, however, amplification of a ca. 3.0-kb segment was observed, consistent with the insertion of a *tet* gene into *mexA* on the chromosome of this strain (13). When *mexB* primers were used, no fragment was observed in either KG2114 or K636, although a ca. 3.2-kb fragment corresponding to *mexB* was amplified in all other strains. The failure to amplify the expected 3.2-kb fragment in K636 is consistent with the known insertion of a mini-Tn*10*-Kan element into the *mexB* gene of this strain (13). The lack of the expected fragment in the OprM-deficient strain KG2114 suggests that this mutant carries a Tn*5* insertion in *mexB* and, further, that the resultant *mexB*::Tn*5* was not in this instance readily amplifiable, because of its greater size. One explanation for the loss of OprM in a *mexB*::Tn*5* insertion mutant is a polar effect on downstream *oprM* expression. This would suggest that the third gene, *oprK*, of the *mex* operon actually encodes the OprM protein and that OprK and OprM are the same protein. The failure of *oprK*-specific primers to amplify the expected 1.4-kb fragment in the OprM-deficient mutants KG2109 and KG2110 is certainly consistent with the Tn*5* element residing in the chromosomal *oprK* gene in these mutants, as this fragment was similarly not amplified in K636 carrying a gene replacement *oprK*::ΩHg.

To determine whether the loss of OprM production in KG2114, KG2109, and KG2110 was caused by insertion of Tn*5* into *mexB* (KG2114) and *oprK* (KG2109 and KG2110), the cloned *mexA-mexB-oprK* operon on plasmid pPV20 (a derivative of an *Escherichia coli-P. aeruginosa* shuttle vector, pAK1900, carrying a carbenicillin resistance gene [8]) was introduced into these strains, and antibiotic susceptibility and OprM expression were assessed. Transformants were selected by incubation at 37°C on L agar plates containing 100 μg of carbenicillin per ml and purified on the same medium. Susceptibility of the transformants to antimicrobial agents was determined by the agar dilution method (2). OprM production was detected by the Western immunoblot assay, using a murine polyclonal antiserum directed against OprM as reported previously (2). Introduction of pPV20 into OprM-deficient strains KG2109 and KG2110 restored production of OprM (Fig. 2) and decreased susceptibility to all tested antimicrobial agents except carbenicillin (Table 1), demonstrating that the cloned operon carries the gene encoding OprM production. The OprM-specific antiserum did not, however, react with the OprK outer membrane protein overproduced in the multiply antibiotic resistant strain K385 (data not shown), indicating that OprM and OprK are distinct polypeptides. Moreover, these data indicate that *oprK* actually encodes OprM and not OprK, as originally concluded. The identification of OprK as the product of the third gene of the *mex* operon was based on agreement between the amino acid sequence of a CNBr-de-

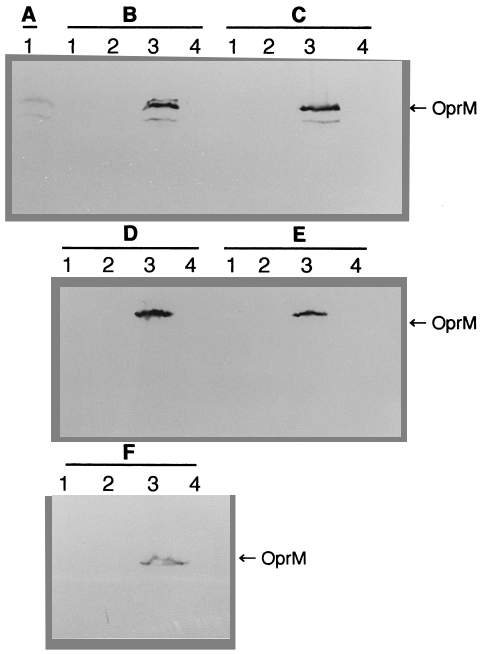

FIG. 2. Detection of OprM by Western immunoblot assay. Crude outer membrane proteins were prepared from transformants of PAO4141 (A), KG2109 (B), KG2110 (C), K590 (D), K613 (E), and K636 (F) by the brief sonication technique described by Trias et al. (16). Twenty micrograms of the proteins was solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (5) at 95°C for 5 min, electrophoresed, and analyzed by the assays, using murine polyclonal antiserum directed to OprM as described previously (2). Lanes: 1, untransformed cells; 2, pAK1900-transformed cells; 3, pPV20-transformed cells; 4, pPV20-cured cells. Established procedures (1) were employed for preparation of plasmid DNA and transformation. Protein assay and analytical SDS-PAGE were performed according to the method of Lowry et al. (9) and Laemmli (6), respectively.

TABLE 1. Susceptibility of PAO derivatives carrying cloned resistance determinants to antimicrobial agents

Strain[a]	MIC (μg/ml)									
	Imipenem	Meropenem	Carbenicillin	Ceftazidime	Norfloxacin	Ofloxacin	Sparfloxacin	Kanamycin	Tetracycline	Chloramphenicol
PAO4141	0.39	0.2	25	1.56	0.39	0.39	0.39	1.56	6.25	6.25
KG2109	0.39	0.025	0.39	0.39	0.1	0.05	0.05	>400	0.39	0.39
KG2109(pPV20)	0.39	0.78	>200	3.13	3.13	3.13	1.56	>400	25	50
KG2110	0.39	0.025	0.39	0.39	0.1	0.05	0.05	>400	0.39	0.78
KG2110(pPV20)	0.39	1.56	>200	6.25	3.13	3.13	3.13	>400	25	100
K372	1.56	0.39	100	1.56	1.56	1.56	3.13	200	12.5	25
K590	1.56	0.2	1.56	0.78	1.56	1.56	1.56	200	200	6.25
K590(pPV20)	1.56	3.13	>200	6.25	6.25	6.25	3.13	100	200	200
K613	1.56	0.1	0.78	0.78	0.2	0.2	0.2	100	0.78	3.13
K613(pPV20)	1.56	3.13	>200	6.25	6.25	6.25	3.13	100	25	200
K635	3.13	1.56	100	6.25	3.13	6.25	1.56	1.56	25	200
K636	3.13	0.39	0.78	1.56	0.78	0.78	0.78	400	6.25	50
K636(pPV20)	3.13	6.25	>200	6.25	6.25	6.25	3.13	400	50	200

[a] PAO1141 is the parent strain of KG2109 and KG2110, K372 is the parent strain of K590 and K613, and K635 is the parent strain of K636. The presence of pAK1900 alone in the tested strains did not change the susceptibility to any agents except carbenicillin.

rived fragment of OprK and the predicted amino acid sequence of the third gene of the operon. This is explained, however, by the recent discovery that the OprK and OprM proteins are highly homologous (4).

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