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Conservation of the Multidrug Resistance Efflux Gene *oprM* in *Pseudomonas aeruginosa*

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An intragenic probe derived from the multidrug resistance gene *oprM* hybridized with genomic DNA from all 20 serotypes of *Pseudomonas aeruginosa* and from all 34 environmental and clinical isolates tested, indicating that the MexA-MexB-OprM multidrug efflux system is highly conserved in this organism. The *oprM* probe also hybridized with genomic DNA from *Pseudomonas aureofaciens*, *Pseudomonas chlororaphis*, *Pseudomonas syringae*, *Burkholderia pseudomallei*, and *Pseudomonas putida*, suggesting that efflux-mediated multidrug resistance mechanisms may be somewhat broadly distributed.

Pseudomonas aeruginosa is an opportunistic human pathogen characterized by an innate resistance to a wide variety of antibiotics (intrinsic multidrug resistance). Although this resistance has most often been attributable to the known low outer membrane permeability of the organism (18), recent evidence implicates drug efflux as a contributing factor. Indeed, efflux of tetracycline, quinolones, chloramphenicol, and β-lactams has been demonstrated in wild-type cells (10, 11) and is attributed to the operation of an efflux pump encoded by the *mexA-mexB-oprM* operon (4, 12, 24, 25). Mutants defective in the efflux genes display a decrease in drug efflux and an increase in antibiotic susceptibility (12). Moreover, overexpression of this efflux operon in *nalB* strains is responsible for the enhanced multidrug resistance (16, 26) and drug efflux (12) seen in these strains. A second efflux system, encoded by the *mexC-mexD-oprJ* operon, is highly homologous to MexA-MexB-OprM (23), although it fails to contribute to intrinsic drug resistance (15). Overexpressed in *nfxB* mutants (16, 23), the MexC-MexD-OprJ pump facilitates resistance to tetracycline, chloramphenicol, quinolones, and some of the newer cephems but not most β-lactams (15, 16, 23). Interestingly, and despite the contribution of these efflux systems to resistance to a variety of drugs, *nfxB* and *nalB* strains are preferentially, although not exclusively (16, 25), selected by fluoroquinolones (3, 5, 6, 9, 17, 20, 21, 29). The current study was undertaken to assess the conservation of *oprM* (and, by association, *mexA-mexB-oprM*) in *P. aeruginosa* and related organisms.

The bacterial strains used in this study include *P. aeruginosa* serotype strains 1 to 20 (according to the International Antigenic Typing System) (13, 14) and those listed in Table 1. Streptomycin-resistant and OprM-deficient derivatives of these are also listed in Table 1, and their isolation is described below. Most of the bacteria were routinely cultivated in Luria (L) broth (27) with shaking (200 rpm) at 37°C; however, *Pseudomonas chlororaphis*, *Stenotrophomonas maltophilia*, *Burkholderia solanacearum*, *Pseudomonas stutzeri*, *Pseudomonas syringae*, and *Pseudomonas putida* WCS358 and BN7 were cultivated at 30°C. Spontaneous streptomycin-resistant derivatives of *P. aeruginosa*, for use as targets for gene replacement mutagenesis (see below), were isolated by plating the equivalent of 3 ml of an overnight culture of bacteria grown in L broth onto L agar supplemented with 1,000 μg of streptomycin per ml. The colonies appearing after 24 to 48 h of growth at 37°C were then recovered.

A digoxigenin (DIG; Boehringer Mannheim)-labelled 848-bp internal fragment of the *oprM* gene for use in Southern hybridizations with bacterial genomic DNA was prepared by PCR with *Taq* DNA polymerase and primers *oprM*1 (5′-CTG AACGTCGAGGCCTTCC-3′), which anneals 264 bp upstream of the 3′ end of *oprM*, and *oprM*2 (5′-CTGGATCTTC GCGTAGTCC-3′), which anneals 342 bp downstream of the 5′ end of the gene. Reaction mixtures (100 μl) were formulated as described previously (23) and were treated for 90 s at 94°C, followed by 35 cycles of 30 s at 94°C, 1 min at 58°C, and 90 s at 72°C, before finishing with 10 min at 72°C.

For Southern hybridizations, genomic DNA (prepared by using the Puregene DNA Isolation Kit [Gentra Systems, Minneapolis, Minn.] by a protocol provided by the manufacturer) was digested with 50 U of *Pst*I (Life Technologies Inc.) in the appropriate buffer supplemented with bovine serum albumin (2% [wt/vol]) for 3 to 18 h at 37°C and was electrophoresed on agarose gels (0.8% [wt/vol]) at 75 mV (28). Genomic DNA was subsequently transferred to nitrocellulose membranes as described by Sambrook et al. (28) with the Turbo blotter system (Schleicher & Schuell, Keene, N.H.). Hybridization with the DIG-labelled *oprM* probe was carried out by using a protocol supplied with the DIG DNA labelling and detection kit (Boehringer Mannheim). Hybridization signals were detected by using a chemiluminescent substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate (CSPD; Boehringer Mannheim) and a 1- to 5-min exposure to Kodak X-Omat AR film (Picker International Canada, Brampton, Ontario, Canada).

Insertional inactivation of chromosomal *oprM* with the $HgCl_2$ resistance interposon ΩHg was carried out exactly as described previously (25) by using streptomycin-resistant *P. aeruginosa* strains as recipients in the mutagenesis (to permit counterselection of the donor *Escherichia coli* strain harboring the plasmid-borne *oprM*::ΩHg). The MICs of various antibiotics were determined by the broth dilution assay as described previously (25), except that L broth was used as the culture medium.

To assess the distribution of the MexA-MexB-OprM efflux system among different strains of *P. aeruginosa*, genomic DNA from serotype, environmental, and clinical strains was hybridized with an *oprM* gene probe. Sequence data indicated that *oprM* is present on a 4.3-kb *Pst*I fragment in wild-type strain PAO1, and indeed, the *oprM* probe hybridized with a 4.3-kb

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TABLE 1. Bacterial strains

Strain[a]	Description	Source or reference[b]
Clinical strains		
DU160-1 (K568)	CF isolate[c]	W. Paranchych
DU1295-1 (K569)	CF isolate	W. Paranchych
K961	Spontaneous streptomycin-resistant derivative of K569	This study
K964	K961 *oprM*::ΩHg (OprM deficient)	This study
DU3980-2 (K570)	CF isolate	W. Paranchych
DU4104-2 (K571)	CF isolate	W. Paranchych
DU4556-1 (K572)	CF isolate	W. Paranchych
DU4823-1 (K573)	CF isolate	W. Paranchych
SP2231-1 (K574)	CF isolate (from sputum)	W. Paranchych
SP8598-1 (K575)	CF isolate (from sputum)	W. Paranchych
5D10 (K576)	Burn patient isolate (University of Alberta Provincial Laboratory)	W. Paranchych
PS62 (K577)	Burn isolate (University of Alberta Provincial Laboratory)	W. Paranchych
C27M (K257)	CF isolate (serotype: O6, monoclonal; O6, polyclonal)[d]	J. Lam[e]
C589M (K258)	CF isolate (serotype: nontypeable, monoclonal; 9/10, polyclonal)	7
C517M (K259)	CF isolate (serotype: nontypeable, monoclonal; 9/10, polyclonal)	7
C510M (K260)	CF isolate (serotype: nontypeable, monoclonal; nontypeable, polyclonal)	7
C260D (K261)	CF isolate (serotype: nontypeable, monoclonal; nontypeable, polyclonal)	7
C63C (K262)	CF isolate (serotype: nontypeable, monoclonal; polytypeable, polyclonal)	7
C49C (K263)	CF isolate (serotype: nontypeable, monoclonal; polytypeable, polyclonal)	7
K959	Spontaneous streptomycin-resistant derivative of K263	This study
K962	K959 *oprM*::ΩHg (OprM deficient)	This study
C13M (K264)	CF isolate (serotype: nontypeable, monoclonal; polytypeable, polyclonal)	7
C11M (K265)	CF isolate (serotype: nontypeable, monoclonal; 9/10, polyclonal)	7
C9M (K266)	CF isolate (serotype: nontypeable, monoclonal; 9/10, polyclonal)	7
C40C (K267)	CF isolate (serotype: O16, monoclonal; polytypeable, polyclonal)	7
C69C (K268)	CF isolate (serotype: O5, monoclonal; polytypeable, polyclonal)	J. Lam
C690M (K269)	CF isolate (serotype: O1, monoclonal; nontypeable, polyclonal)	7
C312C (K270)	CF isolate (serotype: O6, monoclonal; nontypeable, polyclonal)	J. Lam
C253C (K271)	CF isolate (serotype: O7, monoclonal; O7, polyclonal)	J. Lam
C180C (K272)	CF isolate (serotype: O5, monoclonal; polytypeable, polyclonal)	7
C127C (K273)	CF isolate (serotype: O9, monoclonal; O9, polyclonal)	J. Lam
C81C (K274)	CF isolate (serotype: O5, monoclonal; nontypeable, polyclonal)	J. Lam
Environmental strains		
Env1 (K558)		W. Paranchych
K960	Spontaneous streptomycin-resistant derivative of K558	This study
K963	K960 *oprM*::ΩHg (OprM deficient)	This study
Env2 (K559)		W. Paranchych
Env3 (K560)		W. Paranchych
Env4 (K561)		W. Paranchych
W28665 (K562)	Water isolate (University of Alberta Provincial Laboratory)	W. Paranchych
W30525 (K563)	Water isolate (University of Alberta Provincial Laboratory)	W. Paranchych
W31448 (K564)	Water isolate (University of Alberta Provincial Laboratory)	W. Paranchych
W33985 (K565)	Water isolate (University of Alberta Provincial Laboratory)	W. Paranchych
ATCC 34362 (K566)		W. Paranchych
ST1383 (K567)		W. Paranchych
Additional strains		
Pseudomonas fluorescens ATCC 13525		
Pseudomonas aureofaciens ATCC 13985		
Burkholderia cepacia ATCC 25416		
Pseudomonas chlororaphis ATCC 9446		
Stenotrophomonas maltophilia ATCC 13637		
Burkholderia solanacearum ATCC 11696		
Pseudomonas stutzeri ATCC 17588		
Pseudomonas syringae ATCC 19310		
Burkholderia pseudomallei ATCC 23343		
Pseudomonas putida BN7		1
Pseudomonas putida WCS358		1

[a] The laboratory designation for the strains is indicated in parentheses.
[b] W. Paranchych (deceased), University of Alberta; J. Lam, University of Guelph.
[c] CF isolate, isolate from a patient with cystic fibrosis.
[d] A number of the clinical isolates were previously serotyped with International Antigenic Typing Scheme antiserum (polyclonal) or monoclonal antibodies, as outlined previously (7).
[e] These strains were not previously reported but were typed as described previously (7).

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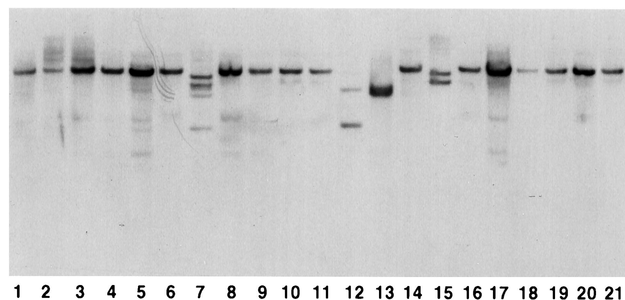

FIG. 1. Southern hybridization of *Pst*I-restricted genomic DNA from *P. aeruginosa* serotype strains O1 to O20 (lanes 1 to 20, respectively) with an 848-bp *oprM*-specific DNA probe. Lane 21, PAO1.

band of *Pst*I-restricted PAO1 genomic DNA (Fig. 1, lane 21). *oprM* also hybridized with a 4.3-kb *Pst*I fragment of genomic DNA for 16 of 20 serotype strains (Fig. 1). The four exceptions, serotype strains O7, O12, O13, and O15, also hybridized with the *oprM* probe, although the hybridizing fragments were smaller in all cases and multiple hybridization signals were observed for serotypes O7 and O15 (Fig. 1). This variation in hybridization pattern suggested some variation or divergence in *oprM* in these strains. The presence of multiple bands in O7 and O15 further suggested either the presence of multiple copies of *oprM* in these strains or the existence of a *Pst*I site within the region of the *oprM* gene encompassed by the *oprM* probe. Nonetheless, these data indicated that *oprM* is highly conserved among all serotype strains of *P. aeruginosa*, including, perhaps most importantly, serotypes O3 and O6, which appear to be the most relevant clinically (8, 22).

Probing of a number of environmental and clinical strains of *P. aeruginosa* (Table 1) similarly revealed the conservation of *oprM* on a 4.3-kb *Pst*I fragment (data not shown). Thus, *oprM* is highly conserved within the species and the *mexA-mexB-oprM* efflux operon is thus likely a significant contributor to intrinsic multidrug resistance in most, if not all, strains of *P. aeruginosa.*

To assess directly the role of the efflux system in the intrinsic resistance of clinical, environmental, and serotype strains, a number of these strains, chosen at random, were mutagenized by insertional inactivation of *oprM* with the mercury resistance interposon ΩHg. Insertional inactivation of *oprM* was achieved in one environmental and two clinical strains, and despite some variability in the antibiotic susceptibilities of the wild-type strains, the loss of *oprM* in each case correlated with hypersensitivity to chloramphenicol, tetracycline, ciprofloxacin (as a representative of the quinolone family), and carbenicillin (as a representative of the β-lactam family) (Table 2), a pattern reminiscent of the effects of an *oprM* knockout on the drug susceptibilities of previous *P. aeruginosa* strains (12, 25). This represents the first demonstration that *oprM*, and by association, *mexA-mexB-oprM*, plays a role in the intrinsic antibiotic resistance of clinical isolates.

In an effort to assess the distribution of *oprM* homologs in related organisms, a number of so-called pseudomonads were also probed with *oprM*. Interestingly, hybridization signals were obtained for *P. aureofaciens* and *P. chlororaphis* under high-stringency conditions (65°C annealing temperature) and for *P. syringae*, *P. putida* WCS358 and BN7, and *Burkholderia pseudomallei* under lower-stringency conditions (55°C annealing temperature) (data not shown). *P. chlororaphis*, *Pseudomonas aureofaciens*, and *P. putida*, like *P. aeruginosa*, are members of the RNA homology group I (19) and are thus closely related evolutionarily. The presence of an *oprM* homolog in these organisms is therefore not surprising. The failure, however, to see hybridization with *Burkholderia cepacia*, despite the existence of an OprM homolog (OpcM) in this organism (2), suggests that the reported homology at the amino acid level is probably not reflected at the nucleotide level. Thus, the lack of a hybridization signal in the remaining organisms is not a guarantee that they lack an *oprM*-like gene or related efflux system. Still, the data here indicate that *oprM* homologs are present in a number of organisms and that efflux systems at least have the potential to contribute to multidrug resistance in a variety of bacteria.

TABLE 2. Influence of *oprM* on drug susceptibility of clinical and environmental strains of *P. aeruginosa*

Strain[a]	MIC (μg/ml)[b]			
	TET	CAM	CIP	CAR
K960 (OprM+)	25	200	1	>200
K963 (OprM−)	≤3.125	100	0.5	12.5
K961 (OprM+)	25	200	>2	>100
K964 (OprM−)	12.5	50	0.5	12.5
K959 (OprM+)	25	50	1	50
K962 (OprM−)	12.5	6.25	0.25	≤3.125

[a] OprM+, OprM positive; OprM−, OprM negative.
[b] TET, tetracycline; CAM, chloramphenicol; CIP, ciprofloxacin; CAR, carbenicillin.

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