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Influence of OprM Expression on Multiple Antibiotic Resistance in *Pseudomonas aeruginosa*

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MexA-MexB-OprM is an efflux system in *Pseudomonas aeruginosa*. OprM overproduced from the cloned gene was able to complement OprM-deficient mutants but did not alter the resistance of a wild-type *P. aeruginosa* strain to the different antimicrobial agents tested. This suggests that OprM cannot function by itself to efflux antibiotics, including β-lactams targeted to the periplasm.

Pseudomonas aeruginosa is well known for its intrinsic resistance to various structurally unrelated antimicrobial agents (29). This broad-spectrum resistance is largely due to the possession of an outer membrane with relatively low permeability (10, 22, 23, 32) coupled with secondary resistance mechanisms, such as efflux (14–16, 23, 25, 29). The efflux operon *mexA-mexB-oprM* has been identified in *P. aeruginosa*, and its products have been demonstrated to contribute to the high intrinsic antibiotic resistance of this organism as well as lead to multiple antibiotic resistance after overexpression in *nalB* mutants (7, 8, 18, 26–28). It has been suggested that the relatively hydrophilic and often negatively charged β-lactams, which have targets in the periplasm, can also be extruded directly from the periplasm or from the surface of cytoplasmic membrane through this system (16). Therefore, it was of interest whether the outer membrane component OprM could function independently. In this study, we overproduced OprM in various *P. aeruginosa* strains to investigate the role of OprM in efflux.

Two synthetic oligonucleotides were used to amplify *oprM* from plasmid pPV20 (27) and to incorporate *Nde*I and *Hin*dIII restriction sites at the 5′ and 3′ ends, respectively. The approximately 1.5-kb fragment was first cloned into plasmid pT7-7 (30), and the gene, together with the ribosome binding site on pT7-7, was then excised by *Xba*I and *Hin*dIII and ligated to plasmid pVLT31 (17) to create pKPM-2. DNA sequencing performed according to the protocols provided by Applied Biosystems Inc. (Foster City, Calif.) confirmed the published (27) sequence of the subcloned *oprM* gene. The control vector pVLT31 and the construct pKPM-2 were transformed into *Escherichia coli* DH5α, the *P. aeruginosa* wild-type strain H103 (laboratory collection), and two *P. aeruginosa* OprM-deficient ΩHg^r interposon mutants, K613 (27) and OCR03T (9). Expression of *oprM* from pKPM-2 was induced by isopropylthio-β-D-galactoside (IPTG) and confirmed by Western immunoblotting (20, 24, 31) with a murine monoclonal antibody against OprM. Surface exposure of OprM was also confirmed in all clones expressing *oprM* by indirect immunofluorescence by the method of Hofstra et al. (13). The fluorescence signal from cells carrying pKPM-2 and induced by IPTG was the strongest. The wild-type *P. aeruginosa* strain H103 and the vector control strain H103/pVLT31 gave weak signals due to OprM expressed from the chromosomal gene. However, excessive production of OprM from pKPM-2 seemed to be harmful to cells, as revealed by growth studies. Cell densities of strains carrying pKPM-2 started to decline after 2 h of induction with 0.1 or more mM IPTG (the results for strain K613/pKPM-2 are shown in Fig. 1), at which point OprM was already substantially overproduced, as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of outer membrane isolated as described previously (12). It is possible that excess OprM perturbed the outer membrane and led to cell lysis, as observed in the case of overexpression of a mutant OmpA precursor protein in *E. coli* (5). A concentration of 0.05 mM IPTG led to no change in growth rate for at least 3 h and a normal yield of cells after overnight growth at 37°C.

OprM was overproduced from cells carrying pKPM-2 and induced with IPTG (Fig. 2A, lane 5; Fig. 2B, lane 6). Strain H103/pKPM-2 produced significantly larger amounts of OprM (Fig. 2B, lane 6) than the wild-type strain, H103; the vector control strain H103/pVLT31 (Fig. 2B, lanes 1 through 4); and the *nalB* mutant OCR1 (Fig. 2B, lane 7). OprM was previously shown to be heat modifiable (6, 19). However, in this study we observed that heating the protein samples from strains carrying pKPM-2 in sample buffer alone did not give any noticeable change in the intensities of the 100-kDa oligomer band of OprM. Both the monomeric 50-kDa and the native oligomeric 100-kDa forms were associated with the outer membrane under such conditions (Fig. 2A, lane 5; Fig. 3, lanes 1 to 3). Only when β-mercaptoethanol was included did the 100-kDa band shift to the 50-kDa monomeric form (as confirmed by two-dimensional, unheated versus heated SDS-PAGE [data not shown]), and this occurred even when solubilization was performed at room temperature or 37°C (Fig. 3, lanes 4 and 5). Many porins exist as oligomers in the outer membrane (2, 3, 11, 21). OprM might also exist as an oligomer in its native form. Overproduction of OprM could have overwhelmed the ability of the cell to correctly form the oligomer, or most oligomers formed may have been less SDS stable. We presume that those oligomers which formed were stabilized by disulphide bridges. In this regard, it should be noted that there are three cysteine residues in the predicted amino acid sequence of OprM.

Antibiotic susceptibilities of the various clones were studied by broth microdilution assays in Mueller-Hinton broth by the method described by Amsterdam (1). MICs of different antimicrobial agents were determined after 20 to 22 h of incubation, and controls demonstrated that the growth of cells carrying pKPM-2 was not inhibited by 0.05 mM IPTG. As shown

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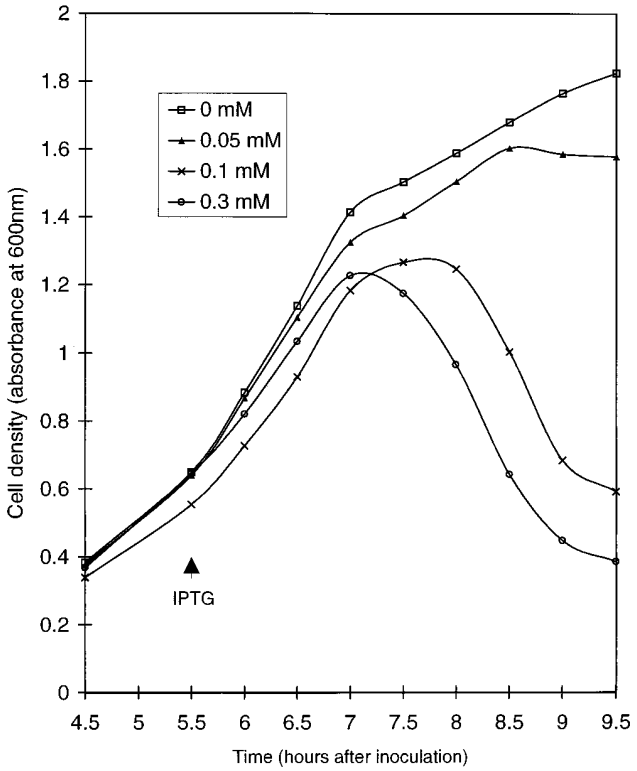

FIG. 1. Growth of *P. aeruginosa* strain K613/pKPM-2 in Luria-Bertani medium induced with different concentrations of IPTG.

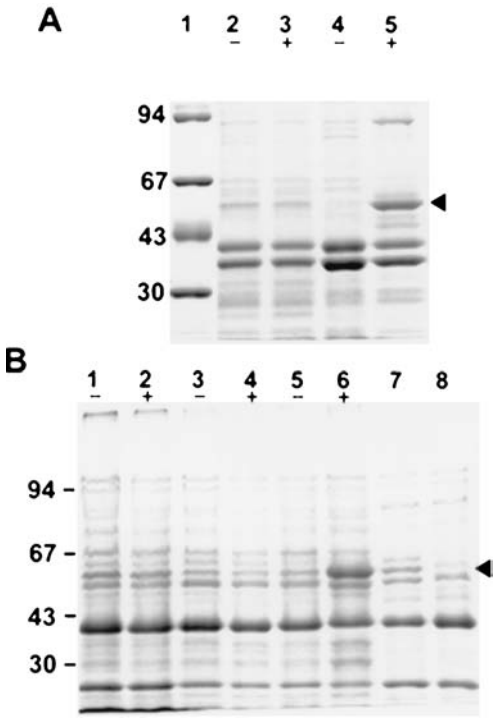

FIG. 2. SDS-PAGE of outer membrane proteins. (A) Samples from *E. coli* DH5α/pVLT31 (lanes 2 and 3) and DH5α/pKPM-2 (lanes 4 and 5) heated without β-mercaptoethanol in sample buffer. Molecular mass standards are shown in lane 1 and are as follows: phosphorylase B, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 30 kDa. (B) Samples from *P. aeruginosa* H103 (lanes 1 and 2), H103/pVLT31 (lanes 3 and 4), H103/pKPM-2 (lanes 5 and 6), OprM-overproducing strain OCR1 (lane 7), and OprM-deficient strain K613 (lane 8). β-Mercaptoethanol (10% [vol/vol]) was included in the sample buffer. Molecular masses are indicated on the left. −, samples from cultures without IPTG induction; +, samples from cultures with 0.05 mM IPTG induction. Position of OprM is shown by arrowheads on the right.

in Table 1, overproduction of OprM in the two OprM-deficient strains led to complementation of their mutations. Tetracycline resistance was the selective marker on pVLT31; thus, strains carrying pVLT31 or pKPM-2 were highly resistant to tetracycline. The MICs of some antibiotics for control vector strains K613/pVLT31 and OCR03T/pVLT31 were increased compared to the MICs of those antibiotics for the OprM-deficient parents of those strains. This is possibly due to the *tet* gene on pVLT31 or the requirement to include tetracycline to maintain the plasmids in growing bacteria to seed the MIC plates. Nevertheless, when comparing isogenic strains with the *oprM*-expressing plasmid or with the vector plasmid alone, complementation was observed. Most interestingly, overproducing OprM from the cloned gene in the wild-type *P. aeruginosa* PAO strain H103 did not alter the MICs of any of the antibiotics tested. Without IPTG induction, H103/pKPM-2 gave MIC results similar to those obtained by IPTG induction (data not shown). This indicated that OprM cannot function independently as an antibiotic efflux channel. In strains K613 and OCR03T, only the most distal gene, *oprM*, of the operon was interrupted and *mexA* and *mexB* could still be expressed. Thus, OprM produced from pKPM-2 could function with these MexA and MexB molecules to complement the OprM deficiency. The excess molecules of OprM produced in these pKPM-2-containing strains might not be able to function properly, since there would be too little MexA and MexB available to reconstruct additional complete efflux systems (assuming that the efflux systems involved stoichiometric amounts of the three components). Consistent with this view, there are small amounts of MexA, MexB, and OprM produced in the wild-type PAO strain H103 which assemble into an efflux apparatus and contribute to intrinsic antibiotic resistance (27). The lack of influence of OprM overexpression in strain H103 is consistent with the explanation that extra copies of OprM expressed from pKPM-2 would presumably not have any MexA and MexB molecules available to form additional efflux complexes. This would explain why, in this genetic background, there was no

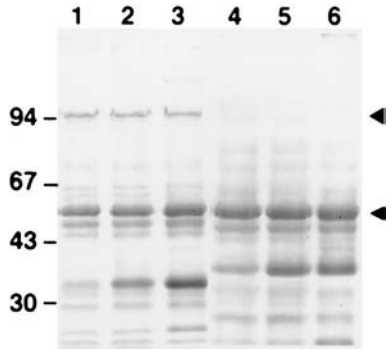

FIG. 3. SDS-PAGE of outer membrane proteins from strain H103/pKPM-2 induced with 0.05 mM IPTG. β-Mercaptoethanol (10% [vol/vol]) was included in the sample buffer in lanes 4, 5, and 6. After mixing with the sample buffer, the samples in lanes 1 and 4 were left at room temperature, the samples in lanes 2 and 5 were left at 37°C for 10 min, and the samples in lanes 3 and 6 were heated at 100°C for 10 min before being loaded onto the wells. Molecular masses are indicated on the left. The positions of the 50-kDa and 100-kDa OprM forms are shown by arrowheads on the right.

significant change in antibiotic susceptibility. OprM was also overproduced in an *E. coli tolC* mutant strain (4) and its parent strain AG100. There was no significant difference in their antibiotic susceptibilities (data not shown), indicating that OprM cannot replace TolC.

Our results do not provide concrete proof that OprM required MexA and MexB to function properly. However, these results indicated that OprM cannot function independently. Interestingly, a *P. aeruginosa tonB* homolog was recently cloned, and preliminary data indicated that drug resistance mediated by the *mexAB-oprM* operon might be TonB dependent (33). Perhaps the energy-dependent resistance to β-lactams mediated through this system is dependent on TonB as well. Alternatively, one of the other systems known to influence β-lactam susceptibility, including inducible β-lactamase and penicillin binding proteins, may be influential.

TABLE 1. Susceptibilities of *P. aeruginosa* strains to antibiotics

Strain[b]	OprM phenotype[c]	MIC (μg/ml)[a]												
		TET	CM	NA	NFX	KAN	GEN	CARB	IMI	MER	CTZD	CTAX	CFP	CFPM	CFSD
H103	+	3.13	6.25	25	0.2	50	0.39	25	0.78	0.39	0.5	4	0.5	1	0.78
H810 (H103/pVLT31)	+	>100	6.25	25	0.2	25	0.39	50	0.78	0.39	1	8	0.5	2	1.56
H811 (H103/pKPM-2)	+++++	>100	6.25	25	0.1	25	0.39	25	0.39	0.39	0.5	4	0.25	2	0.78
OCR1	+++	25	50	200	0.78	25	0.39	200	0.78	1.56	2	32	2	4	3.13
K372[d]	+	3.13	12.5	25	0.39	50	0.78	25	0.78	0.39	0.5	4	0.5	1	0.78
K613	−	0.098	0.78	1.56	≤0.05	25	0.39	0.39	0.78	0.2	0.5	1	0.063	0.25	0.2
H805 (K613/pVLT31)	−	100	3.1	6.25	0.2	25	0.195	0.39	0.78	0.1	0.25	1	0.25	0.5	0.2
H806 (K613/pKPM-2)	+++++	100	6.25	25	0.2	25	0.39	25	0.78	0.2	0.25	4	0.5	1	0.39
OCR03T[e]	−	0.195	0.78	0.78	≤0.05	12.5	0.195	0.39	0.78	0.05	0.5	1	0.063	0.125	0.2
H808 (OCR03T/pVLT31)	−	>100	1.56	6.25	0.1	3.13	0.1	0.39	0.39	0.1	0.25	1	0.125	0.5	0.39
H809 (OCR03T/pKPM-2)	+++++	>100	50	100	0.39	6.25	0.195	100	0.78	0.78	1	16	1	1	1.56

[a] The results were obtained from three repeated experiments. +, level comparable to that of the wild type; +++, level comparable to that of strain OCR1; +++++, increased level (by IPTG induction) compared with that of strain OCR1; −, undetectable level. Abbreviations: TET, tetracycline; CM, chloramphenicol; NA, nalidixic acid; NFX, norfloxacin; KAN, kanamycin; GEN, gentamycin; CARB, carbenicillin; IMI, imipenem; MER, meropenem; CTZD, ceftazidime; CTAX, ceftriaxone; CFP, cefepime; CFPM, cefpirome; CFSD, cefsulodin.

[b] 0.05 mM IPTG was added to the cells and included in the Mueller-Hinton medium in the assay.

[c] OprM expression, determined by SDS-PAGE and indirect immunofluorescence.

[d] Parent strain of K613 (26).

[e] Parent strain is OCR03, a multidrug-resistant mutant overproducing OprM and similar to strain OCR1 (9).

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