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Resistance to β-Lactam Antibiotics in *Pseudomonas aeruginosa* Due to Interplay between the MexAB-OprM Efflux Pump and β-Lactamase

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We evaluated the roles of the MexAB-OprM efflux pump and β-lactamase in β-lactam resistance in *Pseudomonas aeruginosa* by constructing OprM-deficient, OprM basal level, and OprM fully expressed mutants from β-lactamase-negative, -inducible, and -overexpressed strains. We conclude that, with the notable exception of imipenem, the MexAB-OprM pump contributes significantly to β-lactam resistance in both β-lactamase-negative and β-lactamase-inducible strains, while the contribution of the MexAB-OprM efflux system is negligible in strains with overexpressed β-lactamase. Overexpression of the efflux pump alone contributes to the high level of β-lactam resistance in the absence of β-lactamase.

A major problem in *Pseudomonas aeruginosa* infection is that this organism exhibits natural and acquired resistance to many structurally and functionally diverse antibiotics. The multiple antibiotic resistance of this organism is mainly caused by low outer membrane permeability (11) and the expression of efflux pumps. Three efflux pumps have been documented (4, 5, 8, 14, 15) so far, namely the MexAB-OprM (10, 13), the MexCD-OprJ (12), and the MexEF-OprN (6) pumps. In the wild-type strain only the MexAB-OprM pump is expressed and the others are silent (4, 5, 10, 13). The *nalB* mutant overexpresses the MexAB-OprM pump (10, 13), rendering the bacterium more resistant than the wild-type strain to certain antibiotics (15). *P. aeruginosa* also expresses a chromosomally encoded β-lactamase in the presence of an appropriate inducer and shows elevated resistance to β-lactam antibiotics (2, 3). An earlier study predicted a possible interplay between membrane permeability and β-lactamase in β-lactam resistance in *P. aeruginosa* (7). Thus, it is important to ask which factor contributes most to resistance under various conditions. We addressed this issue by constructing a series of mutants producing different levels of the MexAB-OprM efflux pump and of β-lactamase.

Table 1 lists the strains used, their relevant properties, and β-lactamase activities. The strains PAO1, PAO4096, and TNP001 produce inducible, undetectable, and fully expressed β-lactamase, respectively (2, 17). We mutagenized the *oprM* gene by inserting a Tet^r cassette as reported earlier (18). Manipulation of DNA has been described earlier (16). We confirmed the Tet^r marker insertion by amplification of the chromosomal *oprM* by PCR as described by Ausubel et al. (1) by using the primers 5′-CAGTTGCAGCTGACCAAGG and 5′-TCGCTGGCCTTGACCAGATCG (data not shown). We confirmed by the Western blotting method with an anti-OprM antibody (18) that the mutants carrying the Tet^r insertion in *oprM* showed no detectable OprM protein (data not shown).

We evaluated the role of the efflux pump without β-lactamase by constructing OprM-deficient (ΔOprM), OprM-constitutive (OprM$^+$), and OprM-overexpressed (OprM^{+++}) mutants from a β-lactamase-negative strain (Bla$^-$) which produces less than 0.9×10^{-3} U of β-lactamase (Table 1). The β-lactam

TABLE 1. Bacterial strains, relevant properties, and β-lactamase activities[a]

Strain	Parent	Relevant property	β-Lactamase activity (U)		Reference or study
			Uninduced	Induced[b]	
PAO1		Wild type	2.7×10^{-3}	0.65	15
TNP024	PAO1	*nalB*-type derivative	2.8×10^{-3}	0.67	This study
TNP025	PAO1	Δ*oprM* (Tet^r insertion)	2.6×10^{-3}	0.59	This study
PAO4096	PAO4069	*blaP9206 BlaI9407 met9020 pro9024*	0.8×10^{-3}	0.9×10^{-3}	2
TNP026	PAO4096	*nalB*-type derivative	0.7×10^{-3}	ND[c]	This study
TNP027	PAO4096	Δ*oprM* (Tet^r insertion)	0.5×10^{-3}	0.6×10^{-3}	This study
TNP001	PAO1	β-lactamase fully expressed	2.55	2.03	17
TNP028	TNP001	*nalB*-type derivative	2.33	2.32	This study
TNP029	TNP001	Δ*oprM* (Tet^r insertion)	2.40	2.82	This study

[a] The *nalB*-type mutants were isolated as previously reported (8). The β-lactamase assay used was also previously reported (17). One unit of β-lactamase hydrolyges 1 μmol of cephalothin per min per mg of protein.
[b] β-Lactamase was induced in the presence of 0.15 μg of imipenem/ml.
[c] ND, not detected.

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TABLE 2. MICs of antibiotics for strains with different levels of OprM expression and β-lactamase production[a]

Strain	MIC (μg/ml)										
	CAZ	CZOP	CFPM	CPR	CBPC	AZT	IPM	MPM	CPZ	CP	OFLX
PAO1	0.78	0.78	0.78	1.56	25	3.13	0.78	0.39	3.13	25	0.39
TNP024	3.13	1.56	3.13	3.13	100	12.5	0.78	0.78	12.5	200	1.56
TNP025	0.39	0.2	0.1	0.2	0.39	0.2	0.78	0.1	0.39	1.56	0.05
PAO4096	0.78	0.2	0.39	0.78	12.5	1.56	0.2	0.2	0.78	25	0.2
TNP026	3.13	0.78	1.56	3.13	50	12.5	0.2	0.78	6.25	200	1.56
TNP027	0.39	0.1	0.1	0.1	0.2	0.2	0.2	<0.013	0.2	1.56	0.05
TNP001	50	50	25	50	200	50	0.78	1.56	400	50	0.39
TNP028	50	50	25	50	400	50	0.78	3.13	400	>200	1.56
TNP029	50	25	12.5	50	100	25	0.78	0.78	400	1.56	0.05

[a] MICs were determined by the agar dilution method with Mueller-Hinton agar (Becton-Dickinson). Abbreviations: CAZ, ceftazidime; CZOP, cefozopran; CFPM, cefepime; CPR, cefpirome; CBPC, carbenicillin; AZT, aztreonam; IPM, imipenem; MPM, meropenem; CPZ, cefoperazone; CP, chloramphenicol; OFLX, ofloxacin.

MICs for the Bla$^-$ OprM^{+++} derivative (TNP026) were 8 to 250 times higher than those for the Bla$^-$ ΔOprM strain (TNP027). These increases in MICs are attributable to the *nalB* mutation, notably overexpression of the MexAB-OprM pump. This new finding clearly shows that overexpression of the efflux pump alone confers high β-lactam resistance without β-lactamase. The β-lactam MICs for the Bla$^-$ OprM$^+$ strain (PAO4096) were 2 to 64 times higher than those for the Bla$^-$ ΔOprM mutant (TNP027) except for meropenem. The higher MICs for PAO4096 than for TNP027 reflect the fraction that the basal level of the MexAB-OprM efflux pump contributes to the intrinsic β-lactam resistance. This result is consistent with recently reported conclusions (9).

Experiments using the strains with fully expressed β-lactamase (Bla^c OprM$^+$, TNP001), an ΔOprM derivative (TNP029), and an OprM^{+++} derivative (TNP028) showed entirely different MIC profiles. First of all, the β-lactam MICs for the Bla^c ΔOprM strain (TNP029) were 64 to 2,000 times higher than those for the Bla$^-$ ΔOprM mutant (TNP027). This large difference in MICs appears to be due solely to the contribution of the fully expressed β-lactamase (Table 2). The contributions of wild-type and elevated levels of MexAB-OprM expression in the TNP001 strain to the MICs of these β-lactams were nearly masked by high β-lactamase production, since the MICs of these antibiotics for the OprM^{+++} derivative, TNP028, were only one to four times higher than those for TNP029. Based on these new findings, we conclude that in the β-lactamase fully expressed strain, the β-lactamase predominates in causing β-lactam resistance and the role of the efflux pump is secondary.

In the next experiment, we designed an experiment taking a wild-type laboratory strain (PAO1) and constructing ΔOprM (TNP025) and *nalB* (TNP024) mutants. The β-lactamase activities of these strains in the presence and absence of the inducer were 0.59 to 0.67 U and 2.6×10^{-3} to 2.8×10^{-3} U, respectively (Table 1). The β-lactam MICs for the wild-type strain, PAO1, were 0.39 to 25 μg/ml, and these values were unexpectedly only one to four times higher than the MICs of these antibiotics for the Bla$^-$ counterpart (PAO4096). These results clearly indicate that the contribution of β-lactamase to the MICs of these β-lactams was marginal. This is probably due to poor β-lactamase inducibility of the β-lactams used, since the MICs of these antibiotics for the Bla^c strain (TNP001) were very high (Table 2).

To determine the role of the efflux pump in β-lactam resistance, we compared the MICs of antibiotics for the Bla^i OprM$^+$ (PAO1) and the Bla^i ΔOprM (TNP025) strains. The β-lactams MICs for PAO1 were 2 to 64 times higher than those for TNP025, indicating that the low-level expression of the efflux pump mainly contributes to the intrinsic resistance. This result is consistent with that of a recent report (9). In addition, the MICs of these antibiotics for the Bla^i OprM^{+++} strain were 8- to 256-fold higher than those for the Bla^i ΔOprM strain (TNP025). These results showed that the efflux pump alone can confer very high β-lactam resistance with a negligible contribution of β-lactamase. To ascertain the contribution of inducible β-lactamase to β-lactam resistance in the MexAB-OprM-overexpressed environment, we compared the MICs of β-lactams for TNP024 and TNP026 and found that the MICs for TNP024 were only one to two times higher than those for TNP026, indicating again that the contribution of inducible β-lactamase was small compared with that of the efflux pump under these conditions. After this paper was submitted for publication, Masuda et al. reported on the interplay between β-lactamase and the efflux pump (9). Our results concur in part with theirs and add additional results.

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