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Hypersusceptibility of the *Pseudomonas aeruginosa nfxB* Mutant to β-Lactams Due to Reduced Expression of the AmpC β-Lactamase

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The *Pseudomonas aeruginosa nfxB* mutant lacking *mexAB-oprM* showed hypersusceptibility to 9 out of 24 β-lactams tested. This hypersusceptibility was found for the *nfxB* mutant lacking *mexAB-oprM-mexXY* (N108) but not for the *nfxB* mutant lacking both *mexAB-oprM-mexXY* and *ampC*. The level of the AmpC β-lactamase induction was reduced in N108. Thus, the reduced AmpC induction must be the cause of the hypersusceptibility.

A series of multicomponent efflux systems, each made up of three components, play important roles in the intrinsic and acquired resistance of gram-negative bacteria (20, 21, 22). Four of these efflux systems, MexAB-OprM (11, 24), MexCD-OprJ (25), MexEF-OprN (9), and MexXY-OprM (1, 19, 26), have been characterized for *Pseudomonas aeruginosa*. MexAB-OprM and MexXY-OprM contribute to both intrinsic and acquired resistance, whereas MexCD-OprJ and MexEF-OprN contribute to only acquired resistance. MexAB-OprM is slightly expressed in wild-type strains, and a *nalB* mutation causes an overexpression of the efflux system. The expression of MexCD-OprJ and MexEF-OprN is strictly suppressed in wild-type strains, and mutations in *nfxB* and *nfxC* cause overexpression of MexCD-OprJ and MexEF-OprN, respectively. The expression of MexXY, which is not detectable in the wild-type strain, is induced by several antimicrobial agents such as tetracycline, erythromycin, and gentamicin (17). MexXY is associated with OprM and contributes to the intrinsic resistance to these agents. While *nfxB* mutants show resistance to quinolones, tetracycline, erythromycin, chloramphenicol, and expanded-spectrum cephems such as cefpirome, they show hypersusceptibility to penicillins, carbapenems, and aminoglycosides (15). A characterization of mutants lacking the *mexAB-oprM* region demonstrated that the hypersusceptibility to β-lactams such as carbenicillin and aztreonam is caused by the reduced expression of MexAB-OprM in the *nfxB* mutants (4).

To investigate whether the hypersusceptibility of the *nfxB* mutants to β-lactams is generally attributable to this mechanism, in this study, we compared the susceptibilities of the isogenic mutants, i.e., the MexCD-OprJ-producing KG2259 (ΔMexAB-OprM of COR6 [4]) and the non-MexCD-OprJ-producing KG2239 (ΔMexAB-OprM of PAO1 [4]), to the 24 β-lactams. Table 1 shows the MICs determined by the usual twofold agar dilution technique with Mueller-Hinton II agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.). The results indicated that these β-lactams can be classified into three groups. The first group, which consists of piperacillin, cloxacillin, nafcillin, cefpirome, cefepime, cefozopran, and cefoselis, showed 8- to 64-fold-lower activities against KG2259 than against KG2239. The second group, which consists of penicillin G, cefuroxime, cefmetazole, ceftazidime, cefsulodin, meropenem, S-4661 (7), and aztreonam, showed almost the same activities against KG2239 and KG2259. The third group, which consists of sulbenicillin, cefpodoxime, ceftriaxone, moxalactam, flomoxef, imipenem, panipenem, biapenem, and R-95867 (an active form of a new oral carbapenem, CS-834 [3]), showed 4- to 32-fold-higher activities when MexCD-OprJ was expressed and MexAB-OprM was not. These results suggest that there is at least one other mechanism responsible for the hypersusceptibility to the third group of β-lactams independent of the decreased expression of MexAB-OprM with accompanying expression of MexCD-OprJ.

The deletion of *mexXY* from KG2239 (N103 [17]) and KG2259 (N108 [18]) did not affect their susceptibilities to the third group of β-lactams, whereas the deletion of *mexCD-oprJ* from N108 (KG4507 [N. Gotoh, unpublished data]) eliminated these hypersusceptibilities (Table 2). These results suggest that MexCD-OprJ expression is directly related to hypersusceptibility.

Given that OprJ and other outer membrane components of the multicomponent efflux systems of *P. aeruginosa* are assumed to form a channel, expression of OprJ might enhance the permeability of the *P. aeruginosa* outer membrane to the agents. To examine this hypothesis, we introduced an OprJ expression plasmid into N103. Although we confirmed the expression of OprJ by immunoblot assay using an OprJ-specific antibody (5), the susceptibilities of these strains to the agents were not affected by this expression (data not shown).

We previously demonstrated that the chromosomal AmpC β-lactamase acts as one of the factors causing the intrinsic resistance of *P. aeruginosa* to β-lactams via its interplay with the efflux system (16). To examine the possibility that the AmpC β-lactamase is involved in hypersusceptibility, we compared the susceptibilities of N119 (*ampC*::Ω of N108 [18]) and N106 (*ampC*::Ω of N103 [18]). N108 was 8- to 32-fold more

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TABLE 1. Susceptibilities of isogenic MexAB-OprM mutants with or without MexCD-OprJ expression

Group and antimicrobial agent	MIC (μg/ml) for strain[a]:	
	KG2239	KG2259
Group I		
Piperacillin	0.25	2
Cloxacillin	256	>4,096
Nafcillin	32	256
Cefpirome	1	16
Cefepime	0.06	4
Cefozopran	0.13	8
Cefoselis	0.5	4
Group II		
Penicillin G	2,048	2,048
Cefuroxime	512	256
Cefmetazole	2,048	1,024
Ceftazidime	0.25	0.5
Cefsulodin	0.25	0.5
Meropenem	0.13	0.25
S-4661	0.25	0.13
Aztreonam	0.06	0.13
Group III		
Sulbenicillin	2	0.25
Cefpodoxime	1,024	64
Ceftriaxone	64	8
Moxalactam	8	1
Flomoxef	8,192	1,024
Imipenem	1	0.13
Panipenem	4	1
Biapenem	0.25	0.03
R-95867	16	0.5

[a] KG2239 was MexAB-OprM deficient. KG2259 produced MexCD-OprJ but was MexAB-OprM deficient.

susceptible than was N103 to R-95867, cefpodoxime, ceftriaxone, and flomoxef, whereas N119 was 4- to 128-fold less susceptible than N106 to these agents (Table 2). We also evaluated the susceptibilities of N103 and N108 to R-95867 in the presence of various concentrations of Syn2190 (23), an AmpC β-lactamase inhibitor. MICs of Syn2190 were >4,096, 8, and 128 μg/ml against PAO1, N103, and N108, respectively. Figure 1 shows the effect of subinhibitory concentrations of Syn2190 on the susceptibilities of N103 and N108. The susceptibility of N103 to R-95867 increased as the concentration of Syn2190 increased, while the subinhibitory concentrations of Syn2190 had little effect on the susceptibility of N108 to R-95867.

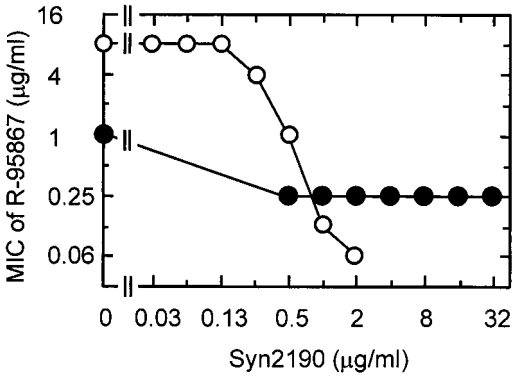

FIG. 1. Effect of Syn2190 on susceptibilities of *P. aeruginosa* N103 (○) and N108 (●) to R-95867. MICs were determined by the microdilution broth method.

Syn2190 did not induce β-lactamase activity at 0.03 to 2 μg/ml in N103 and at 0.5 to 128 μg/ml in N108, although it did induce slight β-lactamase activity at the higher concentrations in N103 (data not shown). The presence of the intact *ampC* gene imparted R-95867 resistance to N103 (compared with N106) but not to N108 (compared with N119) (Table 2). In addition, the inhibition of AmpC β-lactamase had little effect on the susceptibility of N108, a strain that produces MexCD-OprJ (Fig. 1). Since these results suggested a defect in the AmpC expression of N108, we examined the β-lactamase activity induced by R-95867 in N103 and N108. These strains were incubated with various concentrations (0.25 to 64 μg/ml) of R-95867 for 1 h, and the induced β-lactamase was quantified by a spectrophotometric assay with 50 μM cephaloridine used as a substrate, as described previously (14). N108 produced a lower amount of β-lactamase than did N103 (Fig. 2), suggesting that the decreased level of AmpC expression is the cause of the hypersusceptibility to R-95867 in the MexCD-OprJ-producing strain.

Although R-95867 is stable in response to hydrolysis by AmpC (3), a synergistic effect between the slow inactivation of the agent by AmpC and the low level of permeability of the outer membrane might contribute to the resistance in *P. aeruginosa*, imparting the same mode of resistance seen when this organism is exposed to imipenem and meropenem (12, 16). The *nfxB* mutant showed hypersusceptibility to only certain kinds of β-lactams (15) (Table 2). A balance between the

TABLE 2. Susceptibilities of isogenic strains to β-lactams

Strain	Genotype[a]					MIC (μg/ml) of drug[b]:								
	nfxB	ABM	XY	CDJ	*ampC*	SBPC	CPD	CRO	MOX	FMOX	IPM	PAPM	BIPM	R-95867
KG2239	W	−	+	+	+	2	1,024	64	8	8,192	1	4	0.25	16
KG2259	M	−	+	+	+	0.25	64	8	1	1,024	0.13	1	0.03	0.5
N103	W	−	−	+	+	2	1,024	64	8	8,192	1	4	0.25	16
N108	M	−	−	+	+	0.25	128	8	1	1,024	0.25	1	0.03	0.5
KG4507	M	−	−	−	+	2	1,024	64	8	8,192	1	4	0.25	16
N106	W	−	−	+	−	0.25	0.25	0.25	0.5	0.13	0.13	0.13	0.06	0.06
N119	M	−	−	+	−	0.25	32	1	0.5	2	0.06	0.25	0.03	0.25

[a] *nfxB*, wild type (W) or mutated (M); ABM, *mexAB-oprM* possessing (+) or deficient (−); XY, *mexXY* possessing (+) or deficient (−); CDJ, *mexCD-oprJ* possessing (+) or deficient (−); *ampC*, *ampC* possessing (+) or deficient (−).
[b] SBPC, sulbenicillin; CPD, cefpodoxime; CRO, ceftriaxone; MOX, moxalactam; FMOX, flomoxef; IPM, imipenem; PAPM, panipenem; BIPM, biapenem.

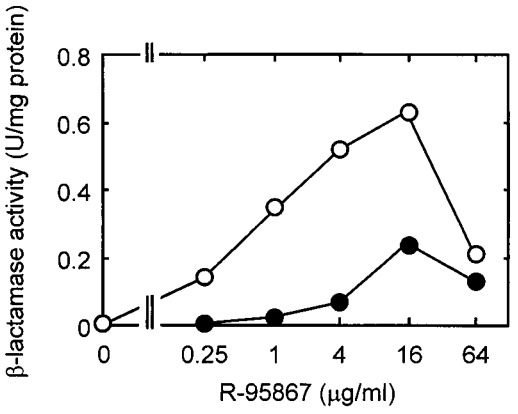

FIG. 2. Induction of β-lactamase in *P. aeruginosa* N103 (○) and N108 (●) by R-95867.

reduced expression of AmpC and the extrusion of each β-lactam by MexCD-OprJ might determine the phenotype, i.e., hypersusceptibility or resistance. The induction mechanism of AmpC β-lactamase has been well studied for *Enterobacter cloacae* (2, 6, 8). The inhibition of cell wall synthesis by β-lactam results in the accumulation of precursors, *N*-acetylglucosamyl-1,6-anhydromuropeptides, in the periplasm. These precursors are transported via AmpG into the cytoplasm, where they are converted into 1,6-anhydromuropeptides by a cytosolic β-*N*-acetylglucosaminidase. The 1,6-anhydromuropeptides convert the transcriptional regulator AmpR into an activator of AmpC expression. Given that AmpG and AmpR were also reported for *P. aeruginosa* (10, 13), a similar induction mechanism must be present in *P. aeruginosa*. MexCD-OprJ might extrude some of the *N*-acetylglucosamyl-1,6-anhydromuropeptides or 1,6-anhydromuropeptides and reduce the β-lactamase expression. In a previous paper (15), we reported that *nfxB* mutants isolated from β-lactamase-deficient strains were also more susceptible than were their parent strains to carbenicillin, imipenem, moxalactam, and aztreonam. This discrepancy can be explained by the MexAB-OprM expression of the β-lactamase-deficient strains. The reduced level of MexAB-OprM must have caused the hypersusceptibility of the *nfxB* mutants isolated from the AmpC-deficient strains.

Our results suggest that the reduction of AmpC induction is the cause of the hypersusceptibility, although further experiments are needed to elucidate the mechanism of the reduction of AmpC induction.

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