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Interplay between Chromosomal β-Lactamase and the MexAB-OprM Efflux System in Intrinsic Resistance to β-Lactams in *Pseudomonas aeruginosa*

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We investigated the role of chromosomal β-lactamase and the MexAB-OprM efflux system in intrinsic resistance to β-lactams in *Pseudomonas aeruginosa*. Determination of the susceptibilities of a series of isogenic mutants with impaired production of the β-lactamase and the efflux system to 16 β-lactams including penicillins, cephems, oxacephems, carbapenems, and a monobactam demonstrated that the intrinsic resistance of *P. aeruginosa* to most of the β-lactams is due to the interplay of both factors.

Pseudomonas aeruginosa is a clinically significant pathogen with intrinsic resistance to various antimicrobial agents. Although this organism has an outer membrane with low permeability (1, 26), this alone does not adequately explain its intrinsic resistance (19, 20). An additional mechanism that interferes with access of the agents to their targets is suspected to exist. MexA-MexB-OprM (3, 14, 24), MexC-MexD-OprJ (23), and MexE-MexF-OprN (12) are multidrug efflux systems of *P. aeruginosa*. Each of these systems consists of three components. The MexAB-OprM system expressed in wild-type strains (5) contributes to the intrinsic resistance of *P. aeruginosa* to most β-lactams and many other structurally unrelated antimicrobial agents by actively extruding them out of the cells (21, 24). In fact, inactivation of this system by an artificial mutation causes hypersusceptibility to the agents in this bacterium (2, 6, 24). The chromosomal AmpC β-lactamase can also contribute to the intrinsic resistance of *P. aeruginosa* to β-lactams by inactivating the agents via hydrolysis (19, 20). Although the respective contributions of the efflux system and β-lactamase have been well characterized, there is little information on the interplay between the two. Therefore, we determined the alterations in the susceptibility of *P. aeruginosa* that accompany defects in the production of AmpC β-lactamase and the MexAB-OprM system using isogenic mutants from a laboratory strain, PAO1, and we discuss the interplay between the two resistance factors.

After constructing KG2504 (*ampC*::Ω Sm^r [22]) from PAO1 by an allelic exchange technique (8), KG2507 (Δ*mexRAB-oprM ampC*::Ω Sm^r [22]) was constructed from KG2504 by the same technique and was used as an *ampC* and *mexRAB-oprM* double mutant. KG2239 (Δ*mexRAB-oprM* [7]) was also used as a MexAB-OprM-deficient mutant from PAO1. The AmpC β-lactamase activity in crude cell extracts was examined by spectrophotometric assay with 100 μM cephaloridine as a substrate as described previously (16). No production of AmpC β-lactamase was detected in the cultures of any of the strains tested. When the cells were treated with cefmetazole as an inducer at a concentration of one-fourth the MIC for 1 h, PAO1 and KG2239 produced 0.3 to 0.6 U of β-lactamase per mg of protein, respectively, whereas KG2504 and KG2507 produced amounts of β-lactamase that were smaller than the detection limit. The production of MexAB-OprM was tested by an immunoblotting assay with TM001 (4), a murine monoclonal antibody specific to OprM encoded on the third gene of this operon. No OprM production was detected in KG2239 or KG2507 in the assay, although its production was apparent in PAO1 and KG2504 (data not shown). Thus, disruption of the *ampC* gene and/or deletion of the *mexAB-oprM* operon in KG2239, KG2504, and KG2507 were confirmed.

MICs were determined by the usual twofold agar dilution technique with Mueller-Hinton II agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.) with an inoculum of 10^4 cells. Cefpodoxime, panipenem, and S-4661 (10) were synthesized at Sankyo Co., Ltd., Tokyo, Japan. The other antimicrobial agents used in this study were obtained from commercial sources. Table 1 presents the MICs of 16 β-lactams for PAO1, KG2239, KG2504, and KG2507. The β-lactams included penicillins (carbenicillin, piperacillin, and amoxicillin), cephems (cefoperazone, cefsulodin, cefmetazole, cefpodoxime, cefuroxime, and ceftriaxone), oxacephems (flomoxef and moxalactam), carbapenems (panipenem, imipenem, meropenem, and S-4661), and a monobactam (aztreonam). All the β-lactams tested had potent activities against the AmpC- and MexAB-OprM-deficient double mutant KG2507, suggesting that they have high affinities for the penicillin-binding proteins of *P. aeruginosa*. In fact, although *P. aeruginosa* exhibits a higher level of resistance to carbenicillin, cefpodoxime, and moxalactam than *Escherichia coli*, the penicillin-binding proteins of *P. aeruginosa*, like those of *E. coli*, are inhibited by these agents at low concentrations (13, 25).

The 16 β-lactams were grouped into the following three categories on the basis of the qualitative pattern of change in susceptibilities accompanying the deficiency of AmpC and/or MexAB-OprM. Group I consists of carbenicillin, piperacillin, cefoperazone, aztreonam, and cefsulodin. The loss of AmpC from PAO1 caused less than 2-fold increases in susceptibility to these agents, whereas the loss of MexAB-OprM from PAO1 caused 4- to 64-fold increases in susceptibility. Group II consists of amoxicillin, cefmetazole, flomoxef, panipenem, and imipenem. The loss of MexAB-OprM from PAO1 caused less than 2-fold increases in susceptibility to these agents, whereas

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TABLE 1. Susceptibilities of PAO1 and its β-lactamase- and/or efflux system-deficient mutants to various β-lactams

Group and antimicrobial agent	MIC (μg/ml)[a]			
	PAO1	KG2239	KG2504	KG2507
Group I				
Carbenicillin	25	0.39	25	0.10
Piperacillin	1.56	0.20	1.56	0.05
Cefoperazone	3.13	0.39	1.56	0.10
Aztreonam	3.13	0.10	1.56	0.05
Cefsulodin	0.78	0.20	0.78	0.20
Group II				
Amoxicillin	3,200	1,600	6.25	0.10
Cefmetazole	1,600	1,600	100	0.20
Flomoxef	6,400	6,400	12.5	0.20
Panipenem	3.13	3.13	0.20	0.10
Imipenem	0.78	0.78	0.20	0.20
Group III				
Cefpodoxime	400	400	100	0.20
Cefuroxime	800	400	400	0.78
Ceftriaxone	6.25	12.5	3.13	0.20
Moxalactam	6.25	3.13	6.25	0.39
Meropenem	0.39	0.20	0.20	0.012
S-4661	0.20	0.39	0.10	0.025

[a] PAO1 produced AmpC and MexAB-OprM, KG2239 produced AmpC but was MexAB-OprM deficient, KG2504 was AmpC deficient but produced MexAB-OprM, and KG2507 was AmpC and MexAB-OprM deficient.

the loss of AmpC from PAO1 caused 4- to 512-fold increases in susceptibility. Thus, in PAO1 the efflux system removes the group I agents from the periplasm more effectively than the β-lactamase does and the β-lactamase removes the group II agents from the periplasm more effectively than the efflux system does. Group III consists of cefpodoxime, cefuroxime, ceftriaxone, moxalactam, meropenem, and S-4661. The loss of either AmpC or MexAB-OprM alone from PAO1 did not cause any substantial change in susceptibilities to these agents, whereas the loss of both AmpC and MexAB-OprM from PAO1 caused remarkable increases in susceptibility to these agents. Thus, group III agents are equally removed from the periplasm by both the β-lactamase and the efflux system. The results obtained in this study are consistent with those obtained in another investigation with another series of OprM-deficient isogenic mutants derived from the AmpC-deficient strain PAO4089 (*met-9020 pro-9024 blaJ9111 blaP9202*; H. Matsumoto, Shinshu University) (17).

Most of the agents tested are known to be stabler against chromosomal β-lactamase of *P. aeruginosa* than the old cephems, e.g., cephaloridine (3, 18). However, the loss of AmpC from MexAB-OprM-deficient strain KG2239 caused a drastic increase in susceptibility to group II and III agents. Thus, a synergistic effect between the slow inactivation of these agents by the β-lactamase and low level of permeability of the outer membrane for these agents might contribute to this resistance. Moreover, a spontaneous mutant that constitutively produced the AmpC β-lactamase from PAO1 was 4 to 128 times more resistant to group I agents than its parent (17). Thus, the absence of any effect of the loss of AmpC on the susceptibilities to the group I agents may be due to the lower levels of AmpC inducibility by these agents rather than their higher AmpC stabilities.

The loss of either AmpC or MexAB-OprM from PAO1 caused little change in the susceptibilities to group III agents, suggesting that each removal mechanism contributes little to the intrinsic resistance to these agents individually. However, the loss of both AmpC and MexAB-OprM from PAO1 caused remarkable increases in the susceptibilities to these agents. These results show that either mechanism alone is almost sufficient to provide the wild-type level of resistance to group III agents, and both mechanisms contribute equally and powerfully to the removal of these agents from the periplasm. Thus, *P. aeruginosa* possesses redundant mechanisms for the removal of these agents.

We can explain the limitation in the change in susceptibilities to imipenem caused by the loss of AmpC and/or MexAB-OprM, although we cannot explain the limited change in susceptibilities to cefsulodin. To elucidate the former limitation we also isolated by the same technique a series of OprD-deficient isogenic mutants with impaired production of AmpC and/or MexAB-OprM (17). The loss of AmpC from PAO1 and MexAB-OprM-deficient strain KG2239 caused only fourfold increases in susceptibilities to imipenem, whereas the loss of AmpC from OprD-deficient strains caused 64-fold increases. Thus, in PAO1, the higher permeation of imipenem through the OprD outer membrane channel contributes to the limited change in susceptibilities to imipenem by the loss of AmpC.

Homologues of the MexAB-OprM efflux system are found in many species of gram-negative bacteria such as *Neisseria gonorrhoeae* (9), *E. coli* (15), *Pseudomonas putida* (11), *Burkholderia cepacia* (2), and *Haemophilus influenzae* (4). Thus, the interplay of double removal systems might be at work in a number of gram-negative bacteria.

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