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Increase in Susceptibility of *Pseudomonas aeruginosa* to Carbapenem Antibiotics in Low-Amino-Acid Media

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The in vitro susceptibility of *Pseudomonas aeruginosa* PAO1 to carbapenem antibiotics, such as CS-533, was influenced by various concentrations of basic amino acids, i.e., L-lysine, L-histidine, and L-arginine, in agar media. *P. aeruginosa* PAO1 showed higher susceptibility to carbapenems in minimal medium than it did in rich media such as Mueller-Hinton II agar. The susceptibility was decreased by the addition of a basic amino acid to the minimal medium, whereas it was influenced less by other amino acids. The susceptibility of PAO1 to cephalosporins, piperacillin, quinolones, and gentamicin was not influenced by the addition of a basic amino acid to the minimal medium. A significant change in susceptibility to carbapenems by the addition of a basic amino acid was not observed with D2 protein-deficient mutants of PAO1. Clinical isolates of *P. aeruginosa* also showed an increase in susceptibility in minimal medium. L-Lysine in minimal medium did not have any influence on the production of D2 protein, β-lactamases, or penicillin-binding proteins of PAO1 or on the chemical degradation of CS-533. These results strongly indicate that the increase in susceptibility of *P. aeruginosa* to carbapenems relates to less competition with basic amino acids for permeation through the D2 protein channel of *P. aeruginosa*.

Pseudomonas aeruginosa is a clinically important pathogen. There are only a few antibiotics, however, that show potent activity against this species. One main reason is considered to be the low rate of permeation of antibiotics through the outer membrane of this species. Imipenem, a carbapenem antibiotic, is one of the few antibiotics that have potent antipseudomonal activity (8). It is known that cephem- and penicillin-resistant *P. aeruginosa* strains usually do not show cross-resistance to imipenem. However, imipenem-resistant clinical isolates have been reported in recent years. A number of reports have shown that the resistance to imipenem is associated with a change in outer membrane proteins (1, 2, 4, 10, 12). Some of imipenem-resistant strains were shown to be D2 protein-deficient mutants. Trias and Nikaido (14) reported that some hydrophilic antibiotics with a dipolar ion and of small molecular size, such as imipenem, permeate the outer membrane of *P. aeruginosa* through a channel formed by the D2 protein. Generally, cephalosporins and penicillins cannot use this channel. Satake et al. (11) also reported the preferential permeation of carbapenems through the D2 protein channel of *P. aeruginosa*. Trias and Nikaido (15) recently showed that the D2 channel has a binding site for basic amino acids and small peptides containing basic amino acids, and permeation of imipenem through the D2 protein into the periplasm could be competitively inhibited by basic amino acids.

Another characteristic feature of imipenem is its bactericidal activity against slow-growing or nongrowing cells. β-Lactam antibiotics are considered to be bactericidal only against exponentially growing cells. Tuomanen (16) has reported, however, that imipenem shows bactericidal activity not only against exponentially growing cells but also against slow-growing or nongrowing cells. We tested whether this unique activity of imipenem is characteristic for carbapenems using another carbapenem antibiotic, CS-533 {formerly RS-533; (5*R*,6*S*)-3-[(*S*)-1-(acetimidoylpyrrolidin-3-yl)thio]-6-[(*R*)-1-hydroxyethyl]-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid}, which has been reported to have antimicrobial activity comparable to that of imipenem against both gram-positive and gram-negative organisms (7). CS-533 also showed bactericidal activity against slow-growing cells (these results will be reported elsewhere). We investigated this mechanism by using *P. aeruginosa* strains grown in a minimal medium as a model of slow-growing cells. Unexpectedly, we found that in the minimal medium *P. aeruginosa* was more susceptible to CS-533 than it was in rich media, such as Mueller-Hinton II agar. Such an increase in susceptibility was also observed for imipenem, but not for cephalosporins and penicillins.

We investigated this mechanism in this study and postulate that this increase in susceptibility of *P. aeruginosa* to carbapenems is related to a change in the permeation of the antibiotics through the D2 protein channel of the outer membrane.

MATERIALS AND METHODS

Chemicals. CS-533, imipenem, meropenem, and cefpirome were synthesized in the Research Laboratories, Sankyo Co., Ltd., Tokyo, Japan. Other antibiotics used in this study were all commercially available products. The antibiotics and their sources were as follows: ceftazidime, Glaxo Japan, Tokyo, Japan; piperacillin, Sankyo Co.; cefsulodin, Takeda Chemical Industries, Osaka, Japan; norfloxacin, Kyorin Pharmaceutical Co., Tokyo, Japan; ofloxacin, Daiichi Pharmaceutical Co., Tokyo, Japan; ciprofloxacin, Bayer Pharmaceutical Co., Osaka, Japan; and gentamicin and cephaloridine, Shionogi Pharmaceutical Co., Osaka, Japan.

Strains and susceptibility testing. *P. aeruginosa* PAO1, 2 D2 protein-deficient mutants of PAO1, and 19 clinical isolates of *P. aeruginosa* were used in this study. The D2 protein-deficient mutants were isolated by plating PAO1 on L-agar plates containing 10 μg of imipenem per ml. The

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clinical isolates were randomly selected from the collection at our Research Laboratories. MICs were determined by the usual twofold agar dilution technique by using Mueller-Hinton II agar (MHA; BBL Microbiology Systems) or other agar media and an inoculum size of 10^4 cells. The effect of amino acids on susceptibility of *P. aeruginosa* to CS-533 was tested by using minimal medium (MM) supplemented with an amino acid at 50 mM. One liter of MM contained 10.5 g of K_2HPO_4, 4.5 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.1 g of $MgSO_4 \cdot 7H_2O$, and 4.36 g of sodium gluconate. MM was solidified with 1.5% agar (Difco) to make agar plates (MMA) for the determination of MICs.

Assay of outer membrane proteins. Exponentially growing cells were harvested by centrifugation at 6,000 × *g* for 20 min, washed with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 8), and then broken with a sonicator (Cho-onpa Kogyo, Tokyo, Japan) for 3 min. Unbroken cells were removed by centrifugation at 6,000 × *g* for 20 min. Membranes were pelleted by centrifugation at 100,000 × *g* for 1 h and washed once with the same buffer. The pellet was suspended in the buffer. Its inner membrane was solubilized by adding Sarkosyl NL-97 to the suspension, to make a final concentration of 1%, followed by incubation for 30 min at 30°C. The outer membrane was pelleted by centrifugation at 100,000 × *g* for 30 min, washed once with the buffer, and finally suspended in the buffer. The outer membrane fractions were assayed by sodium dodesyl sulfate (SDS)–11% polyacrylamide gel electrophoresis (PAGE), as reported by Laemmli (6). Samples for SDS-PAGE were treated with 1% SDS–3% 2-mercaptoethanol for 5 min at 100°C, and then they were subjected to electrophoresis at a constant current of 25 mA for 4 h with the following five proteins with known molecular sizes ranging from 21,500 to 97,400 daltons: soybean trypsin inhibitor, 21,500; carbonic anhydrase, 31,000; ovalbumin, 42,700; bovine serum albumin, 66,200; and phosphorylase *b*, 97,400.

RESULTS

Effect of amino acids in minimal medium on susceptibility of *P. aeruginosa*. The susceptibilities of *P. aeruginosa* PAO1 and its D2 protein-deficient mutants DD-13 and DD-62 to CS-533 were determined by using MMA and MMA supplemented with an amino acid and were compared with those obtained with other agar media (Table 1). *P. aeruginosa* PAO1 was 8 to 16 times more susceptible to CS-533 in MMA than it was in MHA, Trypticase soy agar, heart infusion agar, and L agar. The susceptibility of PAO1 to CS-533 was decreased 8 to 16 times by the addition of either 2% Casamino Acids or 50 mM basic amino acid, i.e., L-lysine, L-arginine, or L-histidine. Other amino acids were less effective in decreasing the susceptibility of PAO1 to CS-533. As shown in Fig. 1, DD-13 and DD-62 were D2 protein-deficient mutants. They were cultivated in Luria broth and in MM supplemented with sodium gluconate as the carbon source. D1 protein is not produced in these media (5). This protein makes it difficult to identify D2 protein by its migration in comparison with the migration of proteins with similar apparent molecular weights on SDS-PAGE (14). These strains also showed higher susceptibilities to CS-533 in MMA than they did in MHA, Trypticase soy agar, heart infusion agar, and L agar. Their susceptibilities, however, were influenced less by the addition of a basic amino acid in MMA.

Figure 2 shows the effect of the L-lysine concentration in MMA on the susceptibilities of PAO1 and DD-13 to CS-533. The susceptibility of PAO1 to CS-533 increased as the concentration of L-lysine in MMA decreased. PAO1 showed the highest susceptibility in the absence of L-lysine. In

TABLE 1. Susceptibilities of *P. aeruginosa* PAO1 and its D2 protein-deficient mutants to CS-533 in various media

Medium	MIC (μg/ml)		
	PAO1	DD-13	DD-62
MHA	3.13	25	25
Trypticase soy agar	3.13	12.5	12.5
Heart infusion agar	6.25	25	25
L agar	6.25	25	25
MMA[a]	0.39	6.25	6.25
MMA + 2% Casamino Acids	3.13	6.25	6.25
MMA + 50 mM L-lysine	6.25	6.25	12.5
MMA + 50 mM L-histidine	3.13	12.5	12.5
MMA + 50 mM L-arginine	6.25	12.5	12.5
MMA + 50 mM glycine	0.39	6.25	6.25
MMA + 50 mM L-serine	0.78	6.25	6.25
MMA + 50 mM L-asparagine	0.78	6.25	6.25
MMA + 50 mM L-glutamine	0.78	6.25	6.25
MMA + 50 mM L-glutamic acid	0.78	6.25	12.5
MMA + 50 mM L-phenylalanine	0.78	12.5	12.5
MMA + 50 mM L-methionine	0.78	12.5	12.5
MMA + 50 mM L-proline	0.78	6.25	6.25

[a] MM was solidified with 1.5% agar. The contents of MM are described in the text.

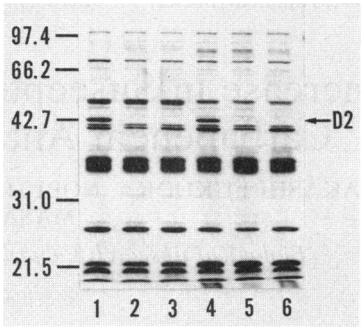

FIG. 1. SDS-PAGE showing the outer membrane proteins of *P. aeruginosa* PAO1 (lanes 1 and 4) and its D2 protein-deficient mutants DD-62 (lanes 2 and 5) and DD-13 (lanes 3 and 6) grown in Luria broth (lanes 1 through 3) and MM (lanes 4 through 6).

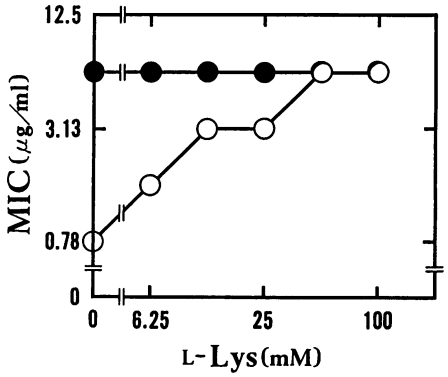

FIG. 2. Effect of L-lysine concentration in MM on the susceptibilities of *P. aeruginosa* PAO1 (○) and its D2 protein-deficient mutant DD-13 (●) to CS-533.

TABLE 2. Susceptibilities of *P. aeruginosa* PAO1 and its D2 protein-deficient mutant to various antibiotics

Antibiotic	MIC (μg/ml) for:			
	PAO1		DD-13	
	MMA[a]	MMA + Lys[b]	MMA	MMA + Lys
CS-533	0.39	3.13	6.25	6.25
Imipenem	0.20	1.56	6.25	12.5
Meropenem	0.012	0.20	0.78	1.56
Cefsulodin	0.39	0.39	0.39	0.20
Ceftazidime	0.20	0.20	0.39	0.20
Cefpirome	0.78	0.78	0.78	0.78
Piperacillin	0.39	0.78	0.78	0.78
Norfloxacin	0.20	0.20	0.20	0.20
Ofloxacin	0.39	0.39	0.39	0.39
Ciprofloxacin	0.05	0.05	0.05	0.05
Gentamicin	0.78	0.78	1.56	1.56

[a] Minimal medium was solidified with 1.5% agar. The contents of MM are described in the text.
[b] MMA supplemented with 50 mM L-lysine.

contrast, the susceptibility of DD-13 to CS-533 was not influenced by the concentration of L-lysine in MMA.

Table 2 shows the effect of 50 mM L-lysine in MMA on the susceptibilities of PAO1 and DD-13 to various antibiotics, including carbapenems, cephalosporins, a penicillin, new quinolones, and an aminoglycoside. The susceptibility of PAO1 to the carbapenems tested was 8 to 16 times higher in MMA than it was in MMA with 50 mM L-lysine. The susceptibility of PAO1 to other antibiotics was not influenced by the addition of 50 mM L-lysine in MMA, except for a twofold increase in the MIC of piperacillin. In contrast, the susceptibility of DD-13 to the carbapenems as well as other antibiotics was not influenced by the addition of 50 mM L-lysine in MMA. The susceptibilities of 19 clinical isolates to CS-533 were also influenced with the addition of 50 mM L-lysine in MMA (Table 3). All strains except strain 2400 showed 4 to 16 times higher susceptibilities in MMA than they did in MMA with 50 mM L-lysine. *P. aeruginosa* 2400 was also a D2 protein-deficient strain.

TABLE 3. Susceptibilities of *P. aeruginosa* clinical isolates to CS-533 in MMA and MMA with 50 mM L-lysine

Strain no.	MIC (μg/ml)		Change in MIC (fold)
	MMA[a]	MMA + Lys[b]	
1872	0.20	1.56	8
2287	0.20	1.56	8
2396	0.20	1.56	8
1611	0.20	1.56	8
1594	0.20	3.13	16
2394	0.20	3.13	16
3725	0.20	3.13	16
1601	0.39	6.25	16
1602	0.39	6.25	16
1615	0.39	6.25	16
1599	0.78	6.25	8
1874	0.78	6.25	8
1875	0.78	6.25	8
3498	0.78	12.5	16
3449	1.56	6.25	4
1573	3.13	12.5	4
1584	3.13	12.5	4
1583	3.13	12.5	4
2400	6.25	12.5	2

[a] Minimal medium was solidified with 1.5% agar. The contents of MM are described in the text.
[b] MMA supplemented with 50 mM L-lysine.

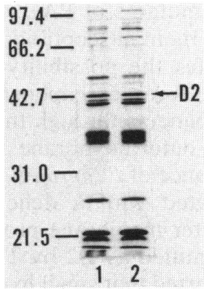

FIG. 3. SDS-PAGE showing the outer membrane proteins of *P. aeruginosa* PAO1 grown in MM (lane 1) and MM with 50 mM L-lysine (lane 2).

Outer membrane protein profiles. We examined the effect of 50 mM L-lysine on the production of D2 protein in *P. aeruginosa* PAO1 using SDS-PAGE (Fig. 3). The amount of D2 protein was quantified by densitometry of a dried gel after staining with Coomassie brilliant blue. There were no significant differences in the amount of D2 proteins of PAO1 cultivated in MM and MM with 50 mM L-lysine.

Effect of L-lysine on β-lactamase and penicillin-binding protein production. β-Lactamase activity in sonic extracts of PAO1 cultivated in MM and MM with 50 mM L-lysine was determined by the UV method reported previously (9), using 70 μM cephaloridine as the substrate. There were no significant differences in the β-lactamase activity of PAO1 cultivated in MM and MM with 50 mM L-lysine. We also tested the ability of CS-533, imipenem, and ceftazidime to induce β-lactamase from PAO1. Both CS-533 and imipenem induced β-lactamase from *P. aeruginosa* PAO1. However, there were no significant differences in the amounts of induced β-lactamase in MM and in MM with 50 mM L-lysine. Both CS-533 and imipenem were quite stable against hydrolysis by the β-lactamase and showed a relative V_{max} of less than 0.1% of that of cephaloridine. The amount of penicillin-binding proteins of PAO1 was also examined as reported previously (9). There were no significant differences in the amounts of penicillin-binding proteins between the cells cultivated in MM and those cultivated in MM with 50 mM L-lysine.

Possibility of chemical degradation of CS-533 by basic amino acids. We examined the possibility of chemical degradation of CS-533 by L-lysine using high-performance liquid chromatography. The amount of free CS-533 in MM and MM with 50 mM L-lysine was determined at 1, 2, 3, and 21 h after incubation at 37°C. No significant differences were detected in the remaining amounts of CS-533 in the two media.

DISCUSSION

We demonstrated that basic amino acids in minimal medium have a marked influence on the susceptibility of *P. aeruginosa* PAO1 and clinical isolates to carbapenem antibiotics, but not to cephalosporins, piperacillin, new quinolones, or gentamicin. Other amino acids had little influence on the susceptibility of PAO1 to carbapenems. Moreover,

D2 protein-deficient mutants of PAO1 did not show such a marked increase in their susceptibilities to carbapenems. This strongly indicates the possibility that the increase in susceptibility of PAO1 to carbapenems is related to the permeation of carbapenems through the D2 protein channel of the *P. aeruginosa* outer membrane. Several reports have shown that the resistance of *P. aeruginosa* clinical isolates to imipenem is associated with a deficiency or diminished expression of the outer membrane protein(s) (1, 2, 10, 12). The protein was identified as D2 by Trias et al. (13). Trias and Nikaido (14) reported that small hydrophilic solutes with a dipolar ion, such as carbapenems, permeate the outer membrane of *P. aeruginosa* through a D2 protein channel. This channel facilitates the transport of basic amino acids and small peptides that contain basic amino acids. In consideration of this information, we assume that the most probable mechanism for the increase in susceptibility of *P. aeruginosa* to carbapenems in minimal medium is related to a change in the permeability of the outer membrane to carbapenems through the D2 protein channel. If this is the case, there could be two possibilities. One is that an overproduction of D2 protein in amino-acid-starved medium enhances the rate of permeation of carbapenems through the D2 protein channel. The other is that a decreased competition of basic amino acids with carbapenems for permeation through the D2 protein channel enhances the rate of permeation of carbapenems. We examined the production of D2 protein in MM and MM with 50 mM L-lysine, but no significant changes were detected in the amounts of the D2 protein of PAO1 cultivated in the two media. Therefore, the latter possibility is more probable. Gotoh and Nishino (4) reported that CS-533 was able to permeate the D2 protein channel of *P. aeruginosa* PAO1 and that decreased susceptibilities to CS-533 were found in D2 protein-deficient mutants of PAO1. In this report we did not show the competition of CS-533 permeation through the D2 protein channel with basic amino acids. However, Trias and Nikaido (15) recently showed that permeation of imipenem through the D2 protein channel could be competitively inhibited by basic amino acids. This also supports the latter possibility.

There may be other factors that increase the susceptibility of *P. aeruginosa* to carbapenems, since both PAO1 and its D2 protein-deficient mutants showed higher susceptibilities in MMA than they did in MHA and other rich media. β-Lactamases, penicillin-binding proteins, and chemical degradation of carbapenems by basic amino acids did not seem to be involved in this mechanism.

Gitlitz et al. (3) reported the concentrations of free amino acids in the plasma of healthy men. The concentrations of lysine, arginine, and histidine were 172 ± 35, 95 ± 18, and 86 ± 11 μM, respectively. These concentrations are 40 times or more lower than those in MHA. Therefore, *P. aeruginosa* could be expected to be more susceptible to carbapenems in human serum than in MHA. Thus, the bactericidal activities of carbapenems in the human body are probably stronger than those expected from MICs determined with MHA, which is recommended as the standard susceptibility test medium. We stress that the in vitro antipseudomonal activity of carbapenems is probably underestimated when it is evaluated with amino-acid-rich medium, such as MHA.

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