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Cross-Resistance to Meropenem, Cephems, and Quinolones in *Pseudomonas aeruginosa*

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Multiple-drug-resistant mutants were isolated from *Pseudomonas aeruginosa* PAO1 on agar plates containing ofloxacin and cefsulodin. These mutants were four to eight times more resistant to meropenem, cephems, carbenicillin, quinolones, tetracycline, and chloramphenicol than the parent strain was. In contrast, these mutants showed no significant changes in their susceptibilities to all carbapenems except meropenem. In these mutants, the amounts of an outer membrane protein with an apparent molecular weight of 49,000 (designated OprM) were increased compared with the amount in PAO1. Multiple-drug-resistant mutants of this type were also isolated from PAO1 on agar plates containing meropenem. Approximately 5% of clinical isolates showed cross-resistance to meropenem, cephems, and quinolones, concomitant with overproduction of OprM. Moreover, these two phenotypes, i.e., multiple-drug resistance and overproduction of OprM, were cotransferable by transduction. These data suggest that overproduction of OprM is associated with cross-resistance to meropenem, cephems, and quinolones in *P. aeruginosa*. The ofloxacin-cefsulodin-resistant mutant required higher concentrations of meropenem to induce β-lactamase than PAO1 did, indicating the possibility that this mutation involves decreased outer membrane permeability to meropenem.

Pseudomonas aeruginosa is a clinically important pathogen because of its natural resistance to many antimicrobial agents. Only a few antimicrobial agents, such as carbapenems and quinolones, have potent antibacterial activity against a broad spectrum of bacteria including *P. aeruginosa*. The main reason for this bacterial resistance is thought to be the organism's low outer membrane permeability to antimicrobial agents (1, 22). In recent years, the number of *P. aeruginosa* clinical isolates that are resistant to some of these antibiotics, especially to quinolones, has increased.

Carbapenems, such as imipenem, panipenem, and meropenem, have potent antibacterial activities against *P. aeruginosa*. However, carbapenem-resistant *P. aeruginosa* strains have already been isolated clinically. Most of them lack the outer membrane protein D2 (2, 15, 20), which forms a specific channel for basic amino acids and carbapenems (7, 19, 21). This is the reason that these strains do not show cross-resistance to other antimicrobial agents, including cephems and penicillins. However, we recently isolated meropenem-resistant *P. aeruginosa* strains from clinical sources, and these strains show cross-resistance to cephems, quinolones, and some other antibiotics but not to imipenem and panipenem.

In this article, we describe the association of this cross-resistance with overproduction of an outer membrane protein with an apparent molecular weight of 49,000.

TABLE 1. Susceptibilities of *P. aeruginosa* clinical isolates to antibiotics

Strain	MIC (μg/ml)[a]												
	IPM	PAPM	MEPM	L-627	CFS	CAZ	CPZ	CBPC	OFLX	CPFX	TC	CP	GM
—[b]	3.13[c]	6.25[c]	0.78[c]	1.56[c]	1.56[c]	1.56[c]	3.13[c]	100[c]	0.39[c]	0.025[c]	25[c]	200[c]	0.78[c]
8510	1.56	6.25	3.13	0.78	12.5	6.25	50	400	6.25	0.39	100	400	1.56
8555	3.13	6.25	6.25	0.78	6.25	3.13	25	200	3.13	0.39	50	200	3.13
8480	1.56	12.5	6.25	1.56	>200	100	>200	>400	50	6.25	100	>400	>100
3719	0.78	6.25	12.5	0.39	12.5	3.13	25	200	25	6.25	100	>400	1.56
1573	6.25	25	50	3.13	100	6.25	100	>400	12.5	3.13	50	>400	1.56
1584	12.5	50	50	3.13	>200	6.25	200	>400	25	3.13	>200	>400	>100

[a] Abbreviations: IPM, imipenem; PAPM, panipenem; MEPM, meropenem; CFS, cefsulodin; CAZ, ceftazidime; CPZ, cefoperazone; CBPC, carbenicillin; OFLX, ofloxacin; CPFX, ciprofloxacin; TC, tetracycline; CP, chloramphenicol; GM, gentamicin.
[b] 54 randomly selected clinical isolates.
[c] MIC for 50% of isolates tested.

MATERIALS AND METHODS

Chemicals. Imipenem, panipenem, meropenem, and L-627 (LJC 10,627) were synthesized at 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: cefsulodin, Takeda Chemical Industries, Osaka, Japan; ceftazidime,

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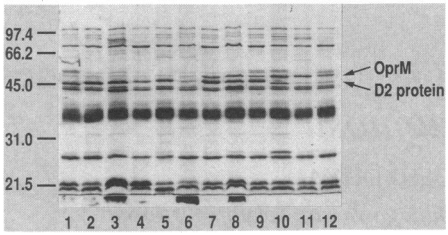

FIG. 1. SDS-PAGE showing the outer membrane proteins of *P. aeruginosa* PAO1 (lane 1), meropenem-susceptible clinical isolates 1882 (lane 2), 2394 (lane 3), 1596 (lane 4), 3498 (lane 5), and 3449 (lane 6), and meropenem-resistant clinical isolates 8510 (lane 7), 8555 (lane 8), 8480 (lane 9), 3719 (lane 10), 1573 (lane 11), and 1584 (lane 12). Numbers on the left are molecular weights (in thousands).

Glaxo Japan, Tokyo, Japan; cefoperazone, Toyama Chemical Co., Tokyo, Japan; carbenicillin, Fujisawa Pharmaceutical Co., Osaka, Japan; ofloxacin, Daiichi Pharmaceutical Co., Tokyo, Japan; ciprofloxacin, Bayer Pharmaceutical Co., Osaka, Japan; tetracycline, Lederle Japan, Tokyo, Japan; chloramphenicol, Sankyo Co. Ltd.; and gentamicin and cephaloridine, Shionogi Pharmaceutical Co., Osaka, Japan.

Bacterial strains and bacteriophage. *P. aeruginosa* clinical isolates were randomly selected from our collection at Biological Research Laboratories. *P. aeruginosa* PAO1, its isogenic mutants, and PAO969 (*proC130*) were also used. Phage F116L was used for transduction.

Isolation of mutants. Meropenem-resistant mutants were isolated by plating PAO1 on L-agar plates containing 3 μg of meropenem per ml. Ofloxacin-cefsulodin-resistant mutants were isolated by plating PAO1 on L-agar plates that contained 1 μg (each) of ofloxacin and cefsulodin per ml.

Susceptibility testing. MICs were determined by the usual twofold agar dilution technique with Mueller-Hinton II agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.) and an inoculum size of 10^4 cells.

Assay of outer membrane proteins. Outer membrane proteins of *P. aeruginosa* grown in L broth were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (6).

Assay of β-lactamase. Cells were grown overnight in Mueller-Hinton broth and then diluted with a 20-fold volume of the same prewarmed medium and incubated for 2 h at 37°C with shaking. A 1/20th volume of meropenem solution was added, and the incubation was continued for 1 h at 37°C. Samples were withdrawn and mixed with an equal volume of ice-cold saline containing 800 μg of chloramphenicol per ml to inhibit protein synthesis. Cells were harvested by centrifugation at 7,000 × *g* for 10 min at 4°C, suspended in ice-cold 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (pH 7.5), and then broken with a sonicator (Branson Sonic Power Co., Danbury, Conn.) for 20 s. Unbroken cells were removed by centrifugation. The enzyme activity in the supernatant was assayed spectrophotometrically at 260 nm with 50 μM cephaloridine in 10 mM MOPS buffer (pH 7.5) as a substrate at 37°C.

Transduction. Transductions were performed as described by Haas and Holloway (8).

RESULTS

Susceptibilities and outer membrane proteins of meropenem-resistant clinical isolates. The majority of *P. aeruginosa* clinical isolates showed higher susceptibilities to meropenem than to imipenem, as reported previously by Edwards et al. (4). However, approximately 5% of them showed lower susceptibilities to meropenem than to imipenem. The susceptibilities of six *P. aeruginosa* clinical isolates that showed lower susceptibilities to meropenem than to imipenem are shown in Table 1, along with minimal concentrations required to inhibit 50% of randomly selected clinical isolates.

TABLE 2. Susceptibilities of *P. aeruginosa* PAO1 and its mutants to antibiotics

Strain	MIC (μg/ml)[a]												
	IPM	PAPM	MEPM	L-627	CFS	CAZ	CPZ	CBPC	OFLX	CPFX	TC	CP	GM
Parent (PAO1)	0.78	6.25	0.20	0.20	0.78	0.39	1.56	25	0.39	0.05	12.5	25	1.56
Weakly meropenem resistant													
MR02	12.5	25	3.13	6.25	0.78	0.78	3.13	25	0.39	0.05	12.5	25	3.13
MR08	12.5	25	3.13	6.25	1.56	0.78	3.13	25	0.39	0.05	12.5	25	1.56
MR39	12.5	25	3.13	6.25	1.56	0.78	3.13	25	0.39	0.05	12.5	25	1.56
Highly meropenem resistant													
MR9	12.5	12.5	12.5	3.13	3.13	1.56	12.5	200	1.56	0.20	25	100	1.56
MR27	12.5	12.5	12.5	3.13	3.13	1.56	12.5	200	3.13	0.39	50	200	1.56
MR36	12.5	25	12.5	12.5	6.25	3.13	12.5	200	3.13	0.39	50	200	1.56
Ofloxacin-cefsulodin resistant													
OCR1	0.78	6.25	1.56	0.20	6.25	3.13	12.5	200	3.13	0.39	50	200	0.78
OCR3	0.78	6.25	1.56	0.20	6.25	1.56	12.5	200	3.13	0.39	50	200	0.78
OCR4	0.78	6.25	1.56	0.20	6.25	1.56	12.5	200	3.13	0.39	50	200	0.78

[a] For abbreviations, see Table 1.

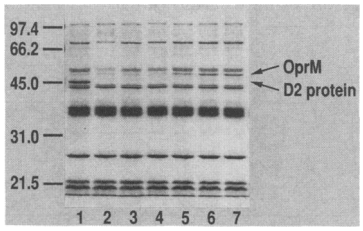

FIG. 2. SDS-PAGE showing the outer membrane proteins of *P. aeruginosa* PAO1 (lane 1) and its meropenem-resistant mutants MR02 (lane 2), MR08 (lane 3), MR39 (lane 4), MR9 (lane 5), MR27 (lane 6), and MR36 (lane 7). Numbers on the left are molecular weights (in thousands).

These strains also showed reduced susceptibilities to cephems (i.e., cefsulodin, ceftazidime, and cefoperazone), carbenicillin, quinolones (i.e., ofloxacin and ciprofloxacin), tetracycline, and chloramphenicol.

The amount of an outer membrane protein with an apparent molecular weight of 49,000 (designated OprM [outer membrane protein responsible for multiple-drug resistance]) was increased in each of the six *P. aeruginosa* strains (Fig. 1, lanes 7 through 12) compared with the amounts in strains that showed higher susceptibilities to meropenem than to imipenem (Fig. 1, lanes 2 through 6). There were also some differences in the amounts of other proteins. The amounts of D2 protein tended to be decreased in the strains that showed lower susceptibilities to all carbapenems. However, the differences in the amount of OprM were the most remarkable.

Isolation of meropenem-resistant mutants from PAO1. To study the mechanism of resistance to meropenem in *P. aeruginosa*, we isolated meropenem-resistant mutants from *P. aeruginosa* PAO1 on L-agar plates containing 3 μg of meropenem per ml. They were isolated at a frequency of 10^{-9} to 10^{-8}. These mutants fell into two types according to their susceptibilities to meropenem and other antimicrobial agents. Table 2 shows the susceptibilities of representative strains of each type and their parent strain, PAO1, to antimicrobial agents. One type (weakly meropenem resistant) of mutant, comprising MR02, MR08, and MR39, was 4 to 32 times more resistant to all carbapenems tested than its parent strain. The weakly meropenem-resistant mutants showed no significant changes in susceptibilities to the other antimicrobial agents. The other type (highly meropenem-resistant) of mutant, comprising MR9, MR27, and MR36, showed the same level of resistance to the carbapenems,

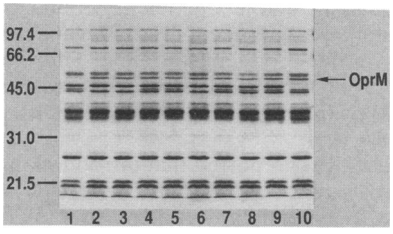

FIG. 3. SDS-PAGE showing the outer membrane proteins of *P. aeruginosa* PAO1 (lane 1) and its ofloxacin-cefsulodin-resistant mutants OCR1 through OCR8 (lanes 2 through 9) and the meropenem-resistant mutant MR27 (lane 10). Numbers on the left are molecular weights (in thousands).

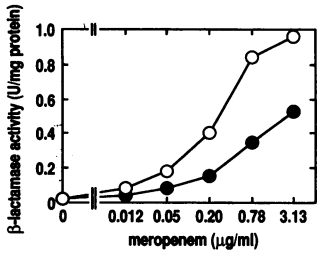

FIG. 4. Induction of β-lactamase in *P. aeruginosa* PAO1 (○) and the ofloxacin–cefsulodin-resistant mutant OCR1 (●) by meropenem.

with the exception of meropenem, as the weakly meropenem-resistant mutants. The highly meropenem-resistant mutants were fourfold less susceptible to meropenem than the weakly meropenem-resistant mutants. The highly meropenem-resistant mutants also had reduced susceptibilities to cephems, carbenicillin, quinolones, tetracycline, and chloramphenicol. There were no significant changes in the susceptibilities of the highly meropenem-resistant mutants to gentamicin.

Figure 2 shows outer membrane protein profiles of six meropenem-resistant mutants compared with that of their parent strain, PAO1. None of the mutants produced D2 protein. The amounts of OprM were increased in the highly meropenem-resistant mutants, MR9, MR27, and MR36, compared with the amounts in PAO1 and the weakly meropenem-resistant mutants, MR02, MR08, and MR39. The amounts of all other proteins were comparable to those of PAO1.

Isolation of ofloxacin-cefsulodin-resistant mutants from PAO1. To study the mechanism of cross-resistance to meropenem, cephems, and quinolones in *P. aeruginosa*, we isolated ofloxacin-cefsulodin-resistant mutants from PAO1 on L-agar plates that contained 1 μg (each) of ofloxacin and cefsulodin per ml. The ofloxacin-cefsulodin-resistant mutants were obtained at a frequency of 10^{-8} to 10^{-7}. The susceptibilities of three mutants, OCR1, OCR3, and OCR4, to antimicrobial agents are shown in Table 2. The ofloxacin-cefsulodin-resistant mutants were four to eight times more resistant to meropenem, cephems, carbenicillin, quinolones, tetracycline, and chloramphenicol than the parent strain was. In contrast, these mutants showed no significant changes in their susceptibilities to all the carbapenems except meropenem. There were no significant changes in the susceptibilities of these mutants to gentamicin.

Figure 3 shows outer membrane protein profiles of eight ofloxacin-cefsulodin-resistant mutants together with those of PAO1 and a highly meropenem-resistant mutant, MR27. The amounts of OprM were increased in all of the mutants compared with the amount in PAO1. There were no significant changes in the production of other proteins.

β-Lactamase inducibility of the ofloxacin-cefsulodin-resistant mutant. PAO1 and its ofloxacin-cefsulodin-resistant mutant, OCR1, were tested for their β-lactamase inducibility to check for the possibility of a change in the permeability of the mutant to meropenem. They were incubated with various concentrations (0.012 to 3.13 μg/ml) of meropenem for 1 h, and the induced β-lactamase was quantified. There was a progressive induction of β-lactamase in both PAO1 and OCR1 as the concentration of meropenem was increased (Fig. 4). OCR1 required higher concentrations of meropenem than PAO1 to show the same levels of induction. OCR1 required almost the same concentrations of imi-

TABLE 3. Susceptibilities of *P. aeruginosa* PAO969 and transductants to antibiotics

Strain	MIC (μg/ml)[a]												
	IPM	PAPM	MEPM	L-627	CFS	CAZ	CPZ	CBPC	OFLX	CPFX	TC	CP	GM
PAO969	0.78	6.25	0.10	0.10	0.78	0.78	6.25	50	0.20	0.012	12.5	50	0.20
T9	0.39	6.25	0.78	0.10	6.25	6.25	25	400	3.13	0.20	50	400	0.20
T27	0.39	6.25	0.78	0.10	6.25	6.25	25	400	3.13	0.20	50	400	0.20
T36	0.39	6.25	0.78	0.10	6.25	6.25	50	400	3.13	0.20	100	400	0.20

[a] For abbreviations, see Table 1.

penem, panipenem, and L-627 as PAO1 to induce the same levels of β-lactamase (data not shown).

Transduction of multiple-drug resistance. Cell-free lysate of the generalized transducing phage F116L was prepared with OCR1 and used for transduction to a recipient, PAO969 (*proC130*). The transfer of amino acid prototrophy was used as the primary criterion of selection for transductants. Eighty-seven transductants were obtained on minimal medium containing glucose as the only carbon source. Twenty-one (24%) of 87 transductants isolated grew on L-agar plates that contained 1 μg (each) of ofloxacin and cefsulodin per ml. Table 3 shows the susceptibilities of three representative transductants that were resistant to ofloxacin and cefsulodin. They show the same phenotype of resistance to the donor, OCR1. Figure 5 shows outer membrane protein profiles of nine representative transductants that showed multiple-drug resistance. Each of them produced a higher amount of OprM than the recipient strain, PAO969.

DISCUSSION

Ofloxacin-cefsulodin-resistant mutants probably have a single mutation, because such mutants could be isolated at a frequency of 10^{-8} to 10^{-7}. The cross-resistance to antimicrobial agents of several different categories suggests that this resistance involves a change in outer membrane permeability. The results of β-lactamase induction are consistent with reduced drug permeation, although an alternative possibility—that the cell membrane component(s) that interacts with β-lactams to initiate induction has reduced affinity for meropenem—cannot be excluded.

An association of overproduction of OprM with cross-resistance to meropenem, cephems, carbenicillin, quinolones, tetracycline, and chloramphenicol in *P. aeruginosa* was demonstrated in both clinical isolates and PAO1 mutants isolated with meropenem or ofloxacin-cefsulodin. A significant difference between the ofloxacin-cefsulodin-resistant mutants and a wild-type strain could be found only in OprM. Moreover, two phenotypes, multiple-drug resistance and overproduction of OprM, were cotransferable by transduction for all strains tested. Therefore, overproduction of OprM in these mutants is probably associated with an alteration of outer membrane permeability to meropenem, cephems, carbenicillin, quinolones, tetracycline, and chloramphenicol. Although a reduction in the amount of a 55-kDa band was seen in some strains (Fig. 1, 2, and 5), it was independent of their susceptibilities to antibiotics. This protein was probably an esterase (14), and it seemed not to be related to multiple-drug resistance.

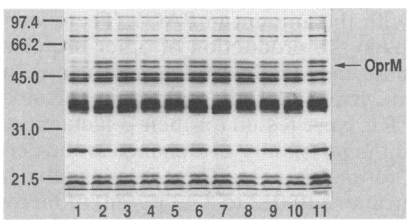

FIG. 5. SDS-PAGE showing the outer membrane proteins of *P. aeruginosa* PAO969 (lane 1), transductants T9 (lane 2), T16 (lane 3), T27 (lane 4), T28 (lane 5), T36 (lane 6), T44 (lane 7), T164 (lane 8), T166 (lane 9), and T173 (lane 10), and the ofloxacin-cefsulodin-resistant mutant OCR1 (lane 11). Numbers on the left are molecular weights (in thousands).

Two types of quinolone resistance in *P. aeruginosa* are well known: (i) alterations in DNA gyrase (9, 10, 17, 18) and (ii) alterations in outer membrane permeability (3, 5, 9, 17, 18). *nalB* mutants are known to show resistance to quinolones, β-lactams, tetracycline, and chloramphenicol which is due to decreased outer membrane permeability (5, 17). *nalB* and *proC* are reported to be cotransducible at a frequency of 2.5% with phage G101 (17). We showed that the mutation for multiple-drug resistance and *proC* in OCR1 were cotransducible at a frequency of 24% with phage F116L. These results strongly suggest that our isolated ofloxacin-cefsulodin-resistant mutants probably have mutations at the *nalB* locus.

Livermore and Yang (12) reported that carbenicillin-resistant mutants of *P. aeruginosa* had decreased susceptibilities to meropenem, cephems, penicillins, nalidixic acid, tetracycline, and chloramphenicol but not to imipenem. This phenotype of resistance is similar to that of our isolated mutants. However, those authors did not mention changes in the outer membrane protein profiles, except for a decreased expression of D2 protein. Multiple-drug-resistant mutants that show cross-resistance to quinolones and imipenem have also been reported (5, 16). In these strains, resistance to imipenem seems to result from decreased production of D2 protein. These strains have an *nfxC* mutation, which is different from the *nalB* mutation (5).

Decreases in susceptibility to meropenem, associated with overproduction of OprM in our PAO1 mutants, were seen in both D2-producing and D2-deficient strains, whereas the level of resistance to meropenem was higher in D2-deficient strains than in D2-producing strains. This indicates that permeation through the D2 channel is not responsible for the mechanism(s) of the resistance associated with overproduction of OprM.

Komatsu et al. (11) reported that introduction of the *romA* gene of *Enterobacter cloacae* into *Escherichia coli* resulted in resistance to ampicillin, cephems, quinolones, tetracyclines, and chloramphenicol, with the concomitant appearance of a new outer membrane protein. Nicas and Hancock (13) also reported that overexpression of outer membrane protein H1 in *P. aeruginosa* resulted in decreased susceptibilities to gentamicin, polymyxin B, and EDTA. They hy-

pothesized that H1 protein replaces divalent cations at negatively charged sites on lipopolysaccharides and blocks penetration of these antibiotics. The function of OprM in *P. aeruginosa* is unclear. It is, however, possible that overproduced OprM interacts with either lipopolysaccharides or phospholipids and thus alters outer membrane permeability to meropenem, cephems, carbenicillin, quinolones, tetracycline, and chloramphenicol through an unknown penetration pathway(s).

Our data suggest that overproduction of OprM, with an associated lack of D2 protein, leads to higher resistance to meropenem in *P. aeruginosa*. Meropenem may select this type of multiple-drug-resistant strains.

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