

## Outer Membrane Proteins Responsible for Multiple Drug Resistance in *Pseudomonas aeruginosa*

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Received 18 August 1994/Returned for modification 22 November 1994/Accepted 22 December 1994

Three types of multiple-drug-resistant mutants which were phenotypically similar to previously described *nalB*, *nfxB*, and *nfxC* mutants were isolated from *Pseudomonas aeruginosa* PAO1 and two clinical isolates. Type 1 (*nalB*-type) mutants showed cross-resistance to meropenem, cepheims, and quinolones. They overproduced an outer membrane protein with an apparent molecular mass of 50 kDa (OprM). Type 2 (*nfxB*-type) mutants showed cross-resistance to quinolones and new cepheims, i.e., ceftiofime and ceftiofopran, concomitant with overproduction of an outer membrane protein with an apparent molecular mass of 54 kDa (OprJ). Type 3 (*nfxC*-type) mutants showed cross-resistance to carbapenems and quinolones. They produced decreased amounts of OprD and increased amounts of a 50-kDa protein (OprN), which was almost the same molecular weight as that of OprM, but it was distinguishable from OprM by its heat modifiability on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In the presence of salicylate, the parent strains showed an increased level of resistance to carbapenems and quinolones and produced decreased amounts of OprD and increased amounts of OprN. Salicylate caused the repression of OprJ production and the loss of resistance to ceftiofime and ceftiofopran in two of the three OprJ-overproducing mutants, although salicylate slightly increased the level of resistance in the parent strains. The changes in susceptibilities were transient in the presence of salicylate. These data suggest that at least three different outer membrane proteins, OprM, OprJ, and OprN, are associated with multiple drug resistance in *P. aeruginosa*.

*Pseudomonas aeruginosa* is a clinically significant opportunistic pathogen that infects compromised hosts in hospitals. This organism has an outer membrane with a low level of permeability and is thereby intrinsically resistant to a wide variety of commonly used antibiotics (1, 22). Only a few antimicrobial agents, such as carbapenems and quinolones, show potent antibacterial activity against this species. In recent years, a number of clinical *P. aeruginosa* isolates have been reported to be resistant to some of these antibiotics, especially quinolones. Several mutations conferring quinolone resistance have been identified and mapped on chromosomes in *P. aeruginosa*. The *gyrA* (*nfxA*, *nalA*, or *cfxA*) mutation causes an alteration in the subunit A of DNA gyrase (5, 7, 16, 17). The *nalB* (*cfxB*) (12, 16, 17), *nfxB* (5), and *nfxC* (3) mutations cause a decrease in the level of accumulation of norfloxacin, and strains with these mutations show cross-resistance to structurally unrelated antimicrobial agents. The *nalB* mutant shows cross-resistance to quinolones, cepheims, carbenicillin, meropenem, tetracycline, chloramphenicol, and novobiocin and overproduces an outer membrane protein with an apparent molecular mass of 49 kDa (OprM). The *nfxB* mutant shows cross-resistance to quinolones and hypersusceptibility to  $\beta$ -lactams and aminoglycosides and overproduces an outer membrane protein with an apparent molecular mass of 54 kDa. The *nfxC* mutant shows cross-resistance to quinolones, imipenem, and chloramphenicol and hypersusceptibility to cepheims, carbenicillin, and aminoglycosides and overproduces an outer membrane protein with an apparent molecular mass of 50 kDa.

In the present study, we used different selection schemes to isolate three types of multiple-drug-resistant mutants showing different resistance spectra and showed that each type is char-

acterized by overproduction of a different outer membrane protein. We designate them type 1, 2, and 3 mutants (*nalB*-, *nfxB*-, and *nfxC*-type mutants, respectively) on the basis of their phenotypic characteristics.

### MATERIALS AND METHODS

**Chemicals.** Imipenem, panipenem, meropenem, biapenem (L-627), and ceftiofopran (SCE-2787) (8) were synthesized at the Research Laboratories, Sankyo Co., Ltd., Tokyo, Japan. The other antibiotics used in the study were all commercially available products. The antibiotics and their sources were as follows: cefsulodin, Takeda Chemical Industries, Osaka, Japan; ceftazidime, Glaxo Japan, Tokyo, Japan; cefoperazone and tosylfloxacin, Toyama Chemical Co., Tokyo, Japan; ceftiofime, Chugai Pharmaceutical Co., Tokyo, Japan; aztreonam, Eisai Co., Ltd., Tokyo, Japan; ofloxacin, Daiichi Pharmaceutical Co., Tokyo, Japan; norfloxacin and feroxacin, Kyorin Pharmaceutical Co., Tokyo, Japan; ciprofloxacin, Bayer Pharmaceutical Co., Osaka, Japan; lomefloxacin, Hokuriku Pharmaceutical Co., Fukui, Japan; sparfloxacin, Dainippon Pharmaceutical Co., Osaka, Japan; tetracycline, Lederle Japan, Tokyo, Japan; chloramphenicol, Sankyo Co. Ltd.; and gentamicin, Shionogi Pharmaceutical Co., Osaka, Japan.

**Bacterial strains.** *P. aeruginosa* clinical isolates 1008 and 8380 and 25 other strains were randomly selected from our collection at the Research Laboratories. *P. aeruginosa* PAO1 was also used.

**Isolation of multiple-drug-resistant mutants.** Type 1 (*nalB*-type) mutants (i.e., PAO1OCR03, 1008OCR01, and 8380OCR02) were isolated by plating PAO1, 1008, and 8380 on Mueller-Hinton II agar (MHA; Becton Dickinson Microbiology Systems, Cockeysville, Md.) plates that each contained 0.8 to 1.5  $\mu$ g of ofloxacin and 1.5 to 3  $\mu$ g of cefsulodin per ml. They were isolated at a frequency of  $3 \times 10^{-8}$  to  $3 \times 10^{-6}$ . Type 2 (*nfxB*-type) mutants (i.e., PAO1OR01, 1008OR01, and 8380OR01) were isolated by plating PAO1, 1008, and 8380 on MHA plates containing 1.5  $\mu$ g of ofloxacin per ml. The largest colony in each plate was picked up and was designated a type 2 mutant. They were isolated at a frequency of  $3 \times 10^{-8}$  to  $2 \times 10^{-7}$ . Type 3 (*nfxC*-type) mutants (i.e., 1008OIR01 and 8380OIR01) were isolated by plating 1008 and 8380 on MHA plates that each contained 1.5  $\mu$ g of ofloxacin and 1.5  $\mu$ g of imipenem per ml. They were isolated at a frequency of  $5 \times 10^{-9}$  to  $2 \times 10^{-7}$ . OprD-deficient mutants (i.e., PAO1IR01, 1008IR01, and 8380IR01) were also isolated by plating PAO1, 1008, and 8380 on MHA plates containing 3  $\mu$ g of imipenem per ml. They were obtained at a frequency of  $1 \times 10^{-7}$  to  $5 \times 10^{-7}$ . Double mutants that showed the phenotypes of both the type 1 and the OprD-deficient mutants were isolated by plating PAO1 on MHA plates that each contained ofloxacin and imipenem. No type 3 mutant could be isolated from PAO1.

**Susceptibility testing.** MICs were determined by the usual twofold agar dilution technique with MHA and an inoculum size of  $10^4$  cells. MHA was supple-

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TABLE 1. Susceptibilities of *P. aeruginosa* PAO1, 1008, and 8380 and their mutants to antibiotics

Strain	Type	MIC ( $\mu\text{g/ml}$ ) <sup>a</sup>										
		CFS	CPR	CZOP	AZT	IPM	PAPM	MEPM	OFLX	TC	CP	GM
PAO1		1.56	0.78	0.78	1.56	0.78	6.25	0.39	0.39	25	50	1.56
PAO1OCR03	1 ( <i>nalB</i> )	12.5	6.25	6.25	50	0.78	3.13	3.13	3.13	100	400	1.56
PAO1OR01	2 ( <i>nfxB</i> )	1.56	12.5	12.5	3.13	0.78	3.13	0.39	6.25	100	400	0.39
PAO1IR01	OprD	0.78	0.78	0.78	1.56	12.5	12.5	6.25	0.39	25	100	1.56
1008		1.56	1.56	1.56	6.25	0.78	6.25	0.39	0.39	50	400	1.56
1008OCR01	1 ( <i>nalB</i> )	12.5	6.25	6.25	50	0.78	6.25	1.56	3.13	200	800	1.56
1008OR01	2 ( <i>nfxB</i> )	1.56	12.5	25	3.13	0.78	6.25	0.39	3.13	50	400	1.56
1008OIR01	3 ( <i>nfxC</i> )	1.56	1.56	1.56	6.25	3.13	12.5	0.78	3.13	100	800	0.78
1008IR01	OprD	1.56	1.56	1.56	6.25	12.5	12.5	6.25	0.39	50	400	1.56
8380		1.56	1.56	1.56	3.13	0.78	6.25	0.20	0.78	50	400	1.56
8380OCR02	1 ( <i>nalB</i> )	12.5	12.5	6.25	50	0.78	6.25	1.56	12.5	100	800	1.56
8380OR01	2 ( <i>nfxB</i> )	1.56	12.5	12.5	3.13	0.78	6.25	0.20	6.25	100	400	1.56
8380OIR01	3 ( <i>nfxC</i> )	1.56	1.56	0.78	3.13	6.25	25	0.78	6.25	25	800	0.78
8380IR01	OprD	1.56	1.56	1.56	3.13	12.5	25	6.25	0.78	50	200	3.13

<sup>a</sup> Abbreviations: CFS, cefsulodin; CPR, ceftiofime; CZOP, ceftazidime; AZT, aztreonam; IPM, imipenem; PAPM, panipenem; MEPM, meropenem; OFLX, ofloxacin; TC, tetracycline; CP, chloramphenicol; GM, gentamicin.

mented with 32 mM sodium salicylate when the effect of salicylate was determined.

**Assay of outer membrane proteins.** Exponentially growing cells in Mueller-Hinton broth (MHB; Becton Dickinson Microbiology Systems) or MHB containing 32 mM sodium salicylate were harvested by centrifugation at  $7,000 \times g$  for 10 min at 4°C, suspended in 30 mM Tris-HCl (pH 8.0), and then broken with a sonicator (Cho-onpa Kogyo, Tokyo, Japan) for 2 min. Unbroken cells were removed by centrifugation at 4°C. Membranes were pelleted by centrifugation at  $100,000 \times g$  for 1 h at 4°C and were suspended in the same buffer. The inner membrane was solubilized by adding sodium *N*-lauroylsarcosinate to the suspension at a final concentration of 1%; this was followed by incubation for 30 min at 30°C. The outer membrane was pelleted by centrifugation at  $18,000 \times g$  for 40 min at 30°C and was suspended in the buffer. The outer membrane fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), as reported by Laemmli (10), with 10.7% (wt/vol) acrylamide and 0.3% (wt/vol) *N,N'*-methylenebisacrylamide in the running gel. Samples for SDS-PAGE were treated with 2% SDS–5% 2-mercaptoethanol at 100°C for 5 min or at 37°C for 10 min, and then they were subjected to electrophoresis at a constant current of 25 mA at 4°C.

## RESULTS

**Susceptibilities of multiple-drug-resistant mutants.** We examined the susceptibilities of the multiple-drug-resistant mutants of each type and their parent strains to the antimicrobial agents listed in Materials and Methods. Table 1 provides the susceptibilities of the organisms to 11 representatives out of

the 20 antimicrobial agents tested. The type 1 mutants were 2 to 16 times more resistant than the parent strains to cepheims (i.e., cefsulodin, ceftazidime, cefoperazone, ceftiofime, and ceftazopran), aztreonam, meropenem, quinolones (i.e., norfloxacin, ofloxacin, ciprofloxacin, fleroxacin, lomefloxacin, tosufloxacin, and sparfloxacin), tetracycline, and chloramphenicol. In contrast, these mutants showed no significant changes in their susceptibilities to imipenem, panipenem, biapenem, and gentamicin. The type 2 mutants were 8 to 16 times more resistant than the parent strains to new cepheims, i.e., ceftiofime and ceftazopran, as well as quinolones. There were some changes in the susceptibilities of these mutants to tetracycline, chloramphenicol, and gentamicin. However, these changes were not consistent in all of the type 2 mutants isolated. The type 3 mutants were two to eight times more resistant than their parents to carbapenems and quinolones. The type 3 mutants showed a lower level of resistance to the carbapenems than the OprD-deficient mutants of their parents. There were no significant changes in the susceptibilities of the type 3 mutants to other agents.

**Outer membrane protein profiles of multiple-drug-resistant mutants.** Figure 1 shows the outer membrane protein profiles of the multiple-drug-resistant mutants together with those of

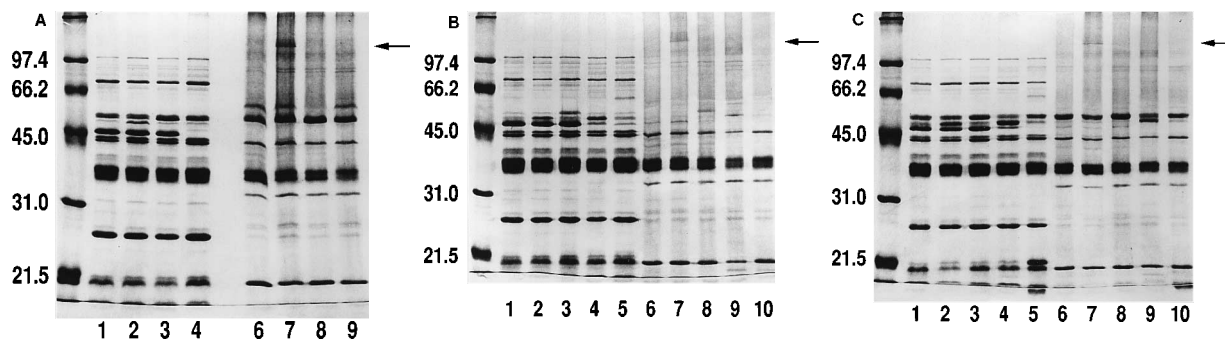


FIG. 1. SDS-polyacrylamide gels showing the outer membrane proteins of *P. aeruginosa*. Cells were grown in MHB, and outer membranes were prepared and solubilized in 2% SDS–5% 2-mercaptoethanol at 100°C for 5 min (lanes 1 through 5) or at 37°C for 10 min (lanes 6 through 10). (A) PAO1 (lanes 1 and 6), PAO1OCR03 (lanes 2 and 7), PAO1OR01 (lanes 3 and 8), and PAO1IR01 (lanes 4 and 9). (B) Isolates 1008 (lanes 1 and 6), 1008OCR01 (lanes 2 and 7), 1008OR01 (lanes 3 and 8), 1008OIR01 (lanes 4 and 9), and 1008IR01 (lanes 5 and 10). (C) Isolates 8380 (lanes 1 and 6), 8380OCR02 (lanes 2 and 7), 8380OR01 (lanes 3 and 8), 8380OIR01 (lanes 4 and 9), and 8380IR01 (lanes 5 and 10). Numbers on the left indicate molecular weights (in thousands). The arrows indicate the position of OprM when the outer membranes were solubilized at 37°C for 10 min.

TABLE 2. Numbers of *P. aeruginosa* clinical isolates whose susceptibilities to carbapenems were decreased by salicylate<sup>a</sup>

Increase in MIC (fold)	No. of isolates			
	IPM	PAPM	MEPM	BIPM
2			5	1
4		8	10	5
8	10	12	8	15
16	14	5	2	4
32	1			

<sup>a</sup> Abbreviations: IPM, imipenem; PAPM, panipenem; MEPM, meropenem; BIPM, biapenem.

the corresponding parent strains. The amounts of an outer membrane protein with an apparent molecular mass of 50 kDa (OprM) were increased in each of the type 1 mutants (Fig. 1A, lane 2; Fig. 1B, lane 2; and Fig. 1C, lane 2) compared with those in their parent strains (Fig. 1A, lane 1; Fig. 1B, lane 1; and Fig. 1C, lane 1). The amounts of all other proteins were comparable to those in the parent strains. The amounts of an outer membrane protein with an apparent molecular mass of 54 kDa (designated OprJ) were increased in each of the type 2 mutants (Fig. 1A, lane 3; Fig. 1B, lane 3; and Fig. 1C, lane 3) compared with those in the parent strains. There were no significant changes in the levels of production of other proteins. The amounts of OprD were decreased in the type 3 mutants (Fig. 1B, lane 4, and Fig. 1C, lane 4) compared with those in their parent strains. The amounts of an outer membrane protein with an apparent molecular mass of 50 kDa were also increased in each of the type 3 mutants compared with those in their parent strains, as was the case in the type 1 mutants. When outer membrane proteins were solubilized in SDS-2-mercaptoethanol at 37°C for 10 min instead of at 100°C for 5 min prior to loading onto SDS-polyacrylamide gels (4), a change in the mobility of OprM was observed, from an apparent molecular mass of 50 kDa to one of more than 100 kDa (Fig. 1A, lanes 2 and 7; Fig. 1B, lanes 2 and 7; and Fig. 1C, lanes 2 and 7). The 50-kDa protein overproduced in the type 3 mutants was, however, not heat modifiable (Fig. 1B, lanes 4 and 9, and Fig. 1C, lanes 4 and 9) and it could be distinguished from OprM. The overproduced protein in the type 3 mutants was designated OprN. The 49-kDa protein (designated OprM before) overproduced in the *nalB* mutant OCR1 (12) was also heat modifiable (data not shown). These results suggest that the overproduced proteins in the type 1 mutants are identical to those in the *nalB* mutant. Therefore, we used the same designation, OprM. The OprJ overproduced in the type 2 mutants was not heat modifiable (Fig. 1A, lanes 3 and 8; Fig. 1B, lanes 3 and 8; and Fig. 1C, lanes 3 and 8), like OprN. The overproduction of OprH in the OprD-deficient mutant of 8380 occurred because it was cultured in L broth instead of MHB, and OprH was also overproduced in the parent strains if they were cultured in L broth (data not shown). OprH is induced in media that contain low Mg<sup>2+</sup> concentrations (13).

**Effects of salicylate on phenotypes of clinical isolates and multiple-drug-resistant mutants.** Salicylate is known to suppress the synthesis of OprD in *P. aeruginosa* (20) and porins in *Escherichia coli* (18), *Klebsiella pneumoniae*, *Serratia marcescens* (19), and *Pseudomonas cepacia* (2). It induces low-level resistance to some antibiotics in such organisms. We also examined the effects of salicylate on the antipseudomonal activities and outer membrane protein profiles in *P. aeruginosa*. Table 2 shows the changes in susceptibilities to four carbap-

TABLE 3. Changes in susceptibilities of *P. aeruginosa* PAO1, 1008, and 8380 and their mutants by salicylate

Strain	Type	Fold change in MIC caused by salicylate <sup>a</sup>				
		CPR	CZOP	AZT	IPM	OFLX
PAO1		2	2	1	8	4
PAO1OCR03	1 ( <i>nalB</i> )	1	2	0.25	8	2
PAO1OR01	2 ( <i>nfxB</i> )	0.125	0.125	0.5	8	0.25
PAO1IR01	OprD	2	2	2	2	4
1008		2	2	0.5	16	4
1008OCR01	1 ( <i>nalB</i> )	1	4	0.25	8	2
1008OR01	2 ( <i>nfxB</i> )	1	0.5	0.5	16	4
1008OIR01	3 ( <i>nfxC</i> )	2	2	0.5	4	4
1008IR01	OprD	2	2	0.5	4	4
8380		2	4	1	8	2
8380OCR02	1 ( <i>nalB</i> )	1	2	0.25	8	1
8380OR01	2 ( <i>nfxB</i> )	0.25	0.25	1	8	0.25
8380OIR01	3 ( <i>nfxC</i> )	2	4	1	4	4
8380IR01	OprD	2	2	1	2	2

<sup>a</sup> For definitions of abbreviations, see footnote<sup>a</sup> of Table 1.

enems of clinical isolates that produce a normal amount of OprD in the presence of 32 mM sodium salicylate. The susceptibilities to all carbapenems tested decreased in the presence of salicylate. Table 3 shows the changes in the susceptibilities of the mutants of each type and their parent strains to the antimicrobial agents in the presence of 32 mM sodium salicylate. The susceptibilities of the parent strains to ofloxacin were two to four times lower in the presence of salicylate than in its absence. Figure 2 shows the outer membrane protein profiles of PAO1 and its mutants grown in the absence and presence of 32 mM sodium salicylate. In PAO1 the amount of OprD decreased in the presence of salicylate (Fig. 2, lanes 1 and 2). Moreover, the amount of the 50-kDa protein increased in the presence of salicylate. This 50-kDa protein was not heat modifiable on SDS-PAGE (data not shown), and it was thought to be OprN. The decreased amount of OprD and the overproduction of OprN were also observed in strains 1008 and 8380 (data not shown). Although some minor changes existed, decreased amounts of OprD and the overproduction

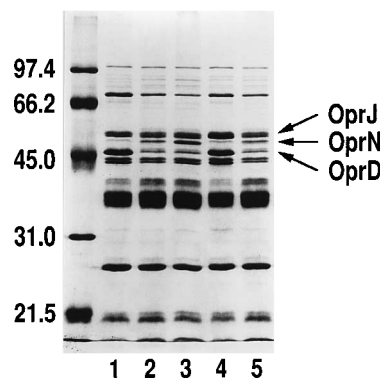


FIG. 2. Effect of salicylate on the outer membrane proteins of *P. aeruginosa*. PAO1 (lanes 1 and 2), PAO1OCR03 (lane 3), and PAO1OR01 (lanes 4 and 5) were grown in MHB (lanes 1 and 4) or MHB containing 32 mM sodium salicylate (lanes 2, 3, and 5) to the late-logarithmic growth phase, and the outer membranes were prepared and solubilized in 2% SDS-5% 2-mercaptoethanol at 100°C for 5 min. The numbers on the left indicate molecular weights (in thousands).



of OprN were significant in all of the strains. The susceptibilities of the OprJ-overproducing (type 2) mutants PAO1OR01 and 8380OR01 to cefpirome and ceftazidime increased in the presence of salicylate, although those of the parent strains decreased slightly in the presence of salicylate (Table 3). The amount of OprJ in PAO1OR01 and 8380OR01 decreased in the presence of salicylate, as was seen in the parent strains (Fig. 2, lanes 4 and 5). In the OprJ-overproducing mutant 1008OR01, such changes in susceptibility and the amount of OprJ were not shown. The overproduction of OprJ corresponded to resistance to the new cepheims, although the effects of salicylate on the expression of OprJ were different among the strains. Cells were precultured in MHB containing 32 mM sodium salicylate. Subsequently, the susceptibilities of these cells to each antibiotic were examined on salicylate-free MHA. They showed susceptibilities that were the same as those determined with preincubation in MHB without salicylate, summarized in Table 1, which indicates that the changes in susceptibilities are not heritable.

### DISCUSSION

We isolated three types of multiple-drug-resistant mutants which were phenotypically similar to previously described *nalB* (12), *nfxB* (5), and *nfxC* (3) mutants from *P. aeruginosa* PAO1 and two clinical isolates. The changes in the susceptibilities to the antimicrobial agents tested and the outer membrane proteins in the type 1, 2, and 3 mutants were consistent with those in the *nalB*, *nfxB*, and *nfxC* mutants, respectively. This is, however, the first report that has shown a correlation between the amount of OprJ and the antipseudomonal activities of new cepheims, i.e., cefpirome and ceftazidime. An association of overproduction of OprJ with cross-resistance to new cepheims in *P. aeruginosa* was demonstrated in both the absence and the presence of salicylate. These new cepheims possess a positively charged substitution at position C-3 and a negatively charged carboxyl group at position C-2. In contrast, old cepheims (i.e., cefsulodin, ceftazidime, and cefoperazone) possess an additional negative charge in the substitution at position C-7. This may be the reason for the difference in the susceptibilities of the OprJ-overproducing mutant to old and new cepheims. The function of OprJ in *P. aeruginosa* is unclear. However, OprJ in the mutants is probably associated with an alteration in efflux transport or outer membrane permeability to new cepheims and quinolones, because an overproduction of this protein caused cross-resistance to these agents.

Recently, Poole et al. (15) cloned an operon, *mexA-mexB-oprK*, which is believed to function in the export of the siderophore pyoverdine and to confer resistance to a broad range of antimicrobial agents in *P. aeruginosa*. If one of the *mexA*, *mexB*, or *oprK* genes was inactivated by a mutation, the *P. aeruginosa* strain tested became hypersusceptible to many antimicrobial agents. Thus, the intrinsic resistance to a wide variety of antibiotics observed in *P. aeruginosa* is indeed due to a combination of a multiple drug efflux transporter and an effective permeability barrier (11, 14). The OprK-overproducing strain isolated by Poole et al. (15) showed cross-resistance to quinolones, tetracycline, and chloramphenicol. OprK had almost the same molecular weight as OprM. We tried to determine the N-terminal amino acid sequence of the OprM that had been purified from an OprM-overproducing mutant. However, it was unsuccessful. The reason was probably because the N terminus was blocked. Poole et al. (15) also reported that amino acid sequencing of the N terminus of OprK was unsuccessful, as might be expected for a possible lipoprotein. These results suggest that OprM and OprK are the same protein.

However, we cannot judge whether OprM and OprK are the same protein or not, because the susceptibility of the OprK-overproducing strain to cepheims and carbapenems is unclear. An outer membrane protein with an apparent molecular mass of 50 kDa was also overproduced when the cells were grown in the presence of zinc ions (6, 15, 21). Poole et al. (15) thought that this protein was identical to OprK. We also confirmed the fact that a 50-kDa protein was overproduced in the presence of zinc ion (data not shown). This protein was, however, not heat modifiable, and it was thought to probably be OprN, not OprM.

Salicylate is a membrane-permeable weak acid and is known to suppress the synthesis of some outer membrane proteins by an unknown mechanism(s) (2, 18–20). In the presence of salicylate, the amount of OprD and the susceptibilities of *P. aeruginosa* isolates to all carbapenems tested decreased in the presence of salicylate, as reported by Sumita and Fukasawa (20). For PAO1 (Table 3), the addition of salicylate affected the activity of meropenem less than it did those of the other carbapenems, as reported by Sumita and Fukasawa (20). They also reported that the addition of salicylate had less of an effect on the activity of meropenem than it did on those of other carbapenems in clinical isolates, and that there was only a twofold increase in the meropenem MIC at which 50 and 90% of the clinical isolates are inhibited in the presence of 32 mM sodium salicylate. The addition of salicylate, however, had a significant effect on the activity of meropenem against clinical isolates (Table 2). The reason for this discrepancy was unclear. The clinical isolates used in the study of Sumita and Fukasawa (20) might have included OprD-deficient strains.

The susceptibilities of the OprM-overproducing (type 1) mutants to aztreonam were four times greater in the presence of salicylate than in its absence (Table 3). When outer membranes of the OprM-overproducing mutants were solubilized at 37°C for 10 min and analyzed by SDS-PAGE, the amounts of OprM in the presence of salicylate were comparable to the amounts without salicylate (data not shown). The decreased amount of OprD and the overproduction of OprN (a heat-unmodifiable 50-kDa protein) were also seen in the OprM-overproducing mutants. The reason for the changes in susceptibilities to aztreonam is unknown. Salicylate suppressed the expression of OprJ and induced the expression of OprN in the OprJ-overproducing mutants (Fig. 2). If OprM, OprJ, and OprN act as independent efflux transporters, the expression of OprJ and OprN may be regulated cooperatively, like OmpF and OmpC in *E. coli* (9).

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