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ROLE OF PORINS IN THE ANTIBIOTIC SUSCEPTIBILITY OF *PSEUDOMONAS AERUGINOSA* : CONSTRUCTION OF MUTANTS WITH DELETIONS IN THE MULTIPLE PORIN GENES

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Summary We inserted deletions in the chromosomal genes of *Pseudomonas aeruginosa* coded for the outer membrane porins, proteins C, D2, or E1, and all possible combinations of these proteins by the gene replacement technique and selecting for imipenem-resistance. Determination of the minimum inhibitory concentrations of ß-lactams, fluoroquinolones, chloramphenicol and gentamicin in these mutants revealed that most mutants showed equal susceptibility to the porin-sufficient strain. The only exception was that imipenem and meropenem showed increased minimum inhibitory concentrations in all of the mutants lacking protein D2. These results firmly established that the *P. aeruginosa* porins identified so far form the pores do not accommodate the passage of most antipseudomonal antibiotics, with the exception of carbapenems.

Pseudomonas aeruginosa is a major pathogen of immunocompromised patients and shows high and broad resistance to many structurally unrelated antibiotics (1). A cause of this resistance is thought to be low antibiotic permeability across the outer membrane (1). Predominance of porins forming small-sized pores explains this low antibiotic permeability (2,3). Outer membrane proteins (Opr) C, D2, and E1 have been identified as the porins that hardly allow penetration of disaccharide (4). Protein F is reportedly a porin forming a very large, but inefficient pore (5), although this conclusion remains a matter of debate.

Imipenem is a potent antipseudomonal ß-lactam antibiotic (6) that seems to penetrate specifically through the OprD2 pore, since most imipenem-resistant strains lack OprD2 (7). Cloning and expression of the *oprD* gene established unequivocally that OprD2 functions as a route for imipenem penetration (8). On the other hand, the contribution of other porins such as OprC

Abbreviations: Opr, outer membrane protein; AP, calf intestine alkaline phospatase; MIC, minimum inhibitory concentration; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TET, tetracycline.

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and OprE1 to antibiotic penetration still remains unclear. In this report, we addressed this issue by constructing a series of deletion mutants of *oprC*, *oprD* and *oprE*, known porin genes by the gene replacement technique and evaluated the role of the porins in the antibiotic susceptibility.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used are listed in Table 1. We manipulated cloned DNA in *Escherichia coli* XL1-Blue (Stratagene) and the DNA was transferred from *E. coli* S17-1 (9) to *P. aeruginosa* by conjugation. Bacteria were grown in L-broth containing 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl per liter (pH 7.4), with or without 5 mM $MgCl_2$ at 37°C overnight under aeration.

Construction of plasmid-borne *oprC* or *oprE* deletion. These are described in the legends to Figs. 1 and 2. Most of the recombinant DNA techniques used have been described previously (8,10).

Transfer of the TET^r-marked *oprC* or *oprE* deletion to the PAO1 chromosome. pTN103 or pOPRE3211 (15) was transformed into the mobilizer strain *E. coli* S17-1 (9). Transfer of the plasmids into *P. aeruginosa* PAO1 was carried out by the filter mating method as described previously (11). Colonies grown were spread on an L-agar plate containing 5% (w/v) sucrose and sucrose-resistant colonies were obtained.

Southern blot analysis. Chromosomal DNA from *P. aeruginosa* was isolated using the procedure described by Ausbel *et al.* (12). After electrophoresis, DNA fragments were transferred to nylon membranes by capillary blotting overnight. The membrane was treated and probed with digoxigenin-labeled DNA fragment according to the manufacturer's instructions.

Table 1. Strains and plasmids

Strains and Plasmids	Relevant properties	Reference
Strains		
P. aeruginosa PAO1	wild type	8
TNP064	Δ*oprC*	This study
TNP004	Δ*oprD*	14
YY100	Δ*oprE*	15
TNP065	Δ*oprC*, Δ*oprD*	This study
TNP066	Δ*oprC*, Δ*oprE*	This study
YY200	Δ*oprD*, Δ*oprE*	15
TNP067	Δ*oprC*, Δ*oprD*, Δ*oprE*	This study
PAO2354	*oprF*$^+$, *oru-325*, *puuC10*, *tyu-9018*, *ben-9010*	13
KG1079	Δ*oprF*, *oru-325*, *puuC10*, *tyu-9018*, *ben-9010*	13
Plasmids		
pTN100	ABPC^r, *oprC* fragment (5.7 kb) in pBluscript II SK+	in preparation
pTN102	ABPC^{r}ABPC^r, *oprC* fragment (8.0 kb) in pBluscript II SK+	in preparation
pTN100ΔAPA	ABPC^r, *Apa*I fragment (1.7 kb) deletion in *oprC*	This study
pNOT322ΔoprC	ABPC^r, TET^r, TET gene (1.4 kb) from pBR322 was inserted into *Apa*I site of pTN100ΔAPA	This study
pTN103	ABPC^r, MOB cassette (5.8 kb) was inserted into NotI site of pNOT322ΔoprC	This study
pNOT19ΔoprC	ABPC^r, *Sal* I fragment (4.3 kb) of pTN100ΔAPA was inserted into Sal I site of pNOT19	This study
pTN104	ABPC^r, MOB cassette (5.8 kb) was inserted into *Not*I site of pNOT19ΔoprC	This study

Only relevant strains and plasmids are listed.

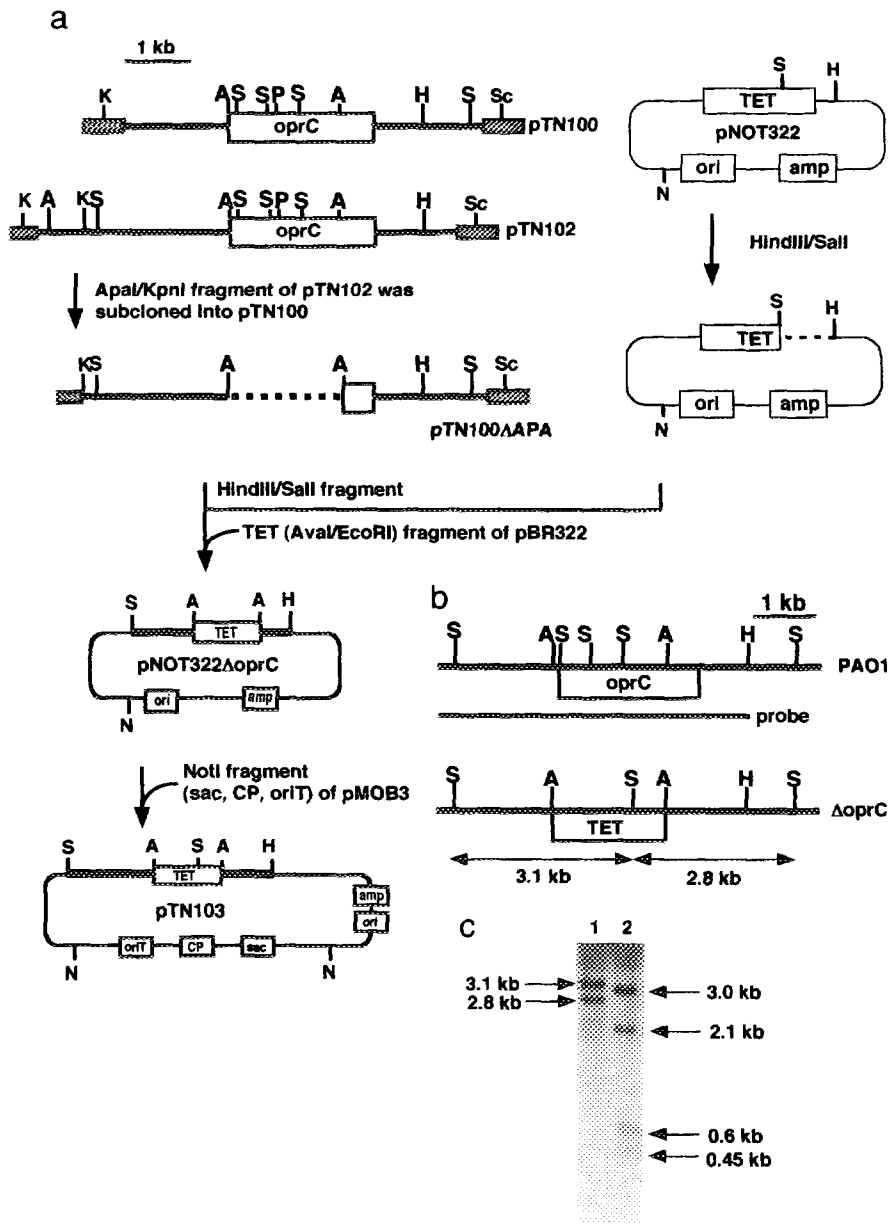

Fig. 1. Construction of *oprC* deletion mutant. (a) Plasmid-borne *oprC* deletion was constructed as follows. A 2.1 kb *Apa*I-*Kpn*I fragment of pTN102 was ligated to the *Apa*I- and *Kpn*I-digest of pTN100 yielding pTN100ΔAPA. In the next step, a *Hind*III-*Sal*I fragment (about 3.3 kb) of pTN100ΔAPA was subcloned into pNOT322 (11) pretreated with *Hind*III, *Sal*I, and AP. To the blunt-ended *Apa*I site of this plasmid, the TET^r marker in the blunt-ended *Ava*I-*Eco*RI fragment of pBR322 was ligated yielding pNOT322ΔoprC. Finally, a 5.8 kb *Not*I fragment containing *oriT*, CP^r and *sac* was isolated from pMOB3 (11) and inserted at the *Not*I site of the pNOT322ΔoprC yielding pTN103. Hatched boxes represent vector DNA (pBluescript SKII+). Restriction enzymes: A, *Apa*I; H, *Hind*III; K, *Kpn*I; N, *Not*I; P, *Pst*I; S, *Sal*I; Sc, *Sac*I. (b) This plasmid-borne *oprC* deletion was transferred to PAO1 as described in Materials and Methods. (c) Southern blot analysis of the chromosomal DNA (10 μg). Lanes 1, TNP064; Lane 2, PAO1.

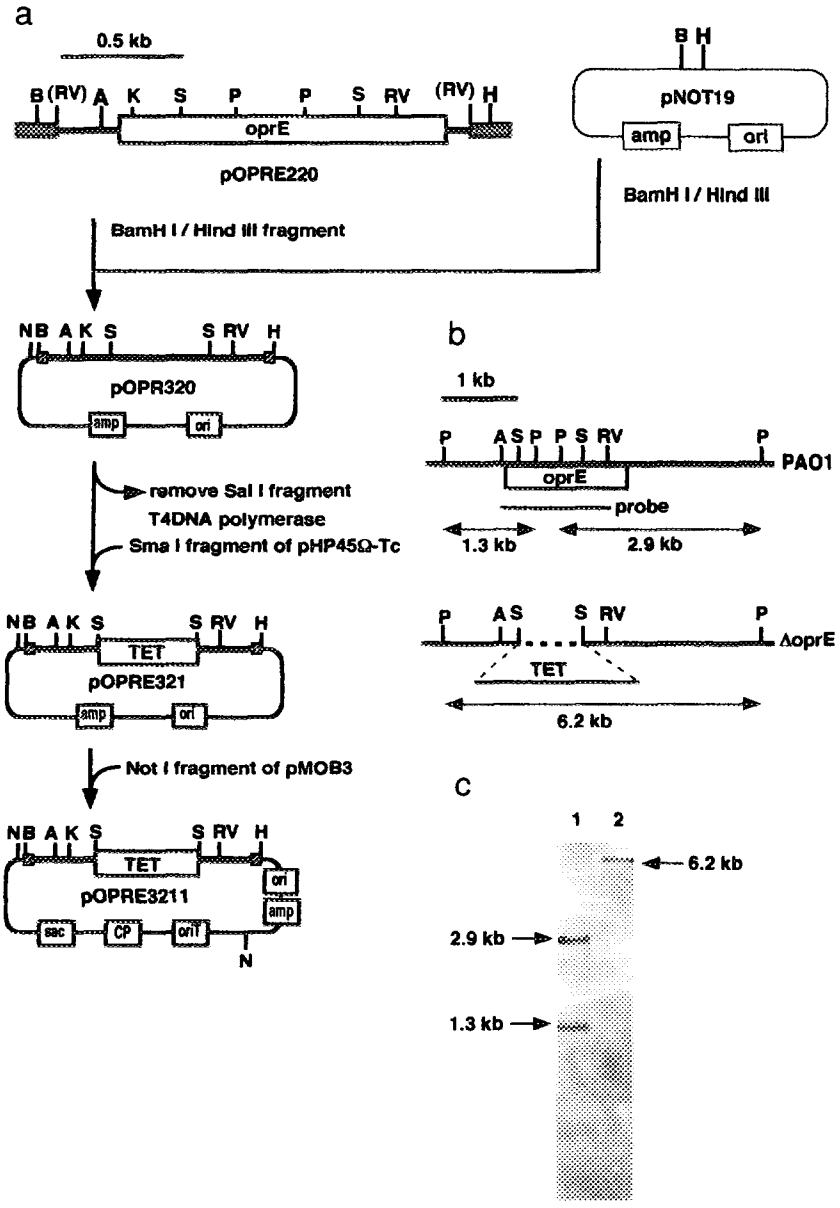

Fig. 2. Construction of *oprE* deletion. (a) Plasmid-borne *oprE* deletion (pOPRE3211) was constructed as follows. A 1.7 kb *Bam*HI-*Hind*III fragment of pOPR220 (15) was ligated to pNOT19 pretreated with *Bam*HI, *Hind*III, and AP to yield pOPRE320 (15). Next, the 0.65 kb *Sal*I fragment in *oprE* was removed and blunt-ended with T4 DNA polymerase. A 2.4 kb *Sma*I fragment of pHP45Ω-Tc (16) covering the TET^r gene was ligated to this blunt-ended site to yield pOPRE321. Finally, a MOB cassette from pMOB3 was inserted into the *Not*I site of the pOPRE321 to yield pOPRE3211 (15). Hatched boxes represent vector DNA (pBluescript SKII-). (b) This plasmid-borne *oprE* deletion was transferred to PAO1 as described in Materials and Methods. Restriction enzymes: Rv, *Eco*RV; see the legend to Fig. 1 for others. (c) Southern blot analysis of the chromosomal DNA. A 258 bp *Pst*I fragment was not seen. Lane 1, PAO1 1.3 μg DNA; Lane 2, YY100, 5 μg DNA.

Other techniques. Determination of the MIC of the antibiotics, isolation of the outer membrane, method for SDS-PAGE and quantification of protein have been described before (8).

RESULTS AND DISCUSSION

Replacement of the *oprC* or *oprE* gene of the PAO1 chromosome. Figure 1a shows a partial restriction map of cloned *oprC* encoding the OprC porin (submitted for publication). We constructed a plasmid-borne *oprC* deletion on pTN103; this was transferred to PAO1 by conjugation and the plasmid integrants were screened on Vogel-Bonner agar containing 100 μg/ml of TET. Next, the TET- and CBPC-resistant colonies were screened on L-agar containing 5% (w/v) sucrose and 100 μg/ml of TET (TNP064) to eliminate the strains with integrated pTN103. SDS-PAGE analysis of the outer membrane of TNP064 showed no detectable protein band corresponding to OprC (Fig. 3). The replacement of the *oprC* gene was confirmed by Southern blot analysis (Fig. 1b, 1c), which showed that the *Sal*I digest of TNP064 DNA exhibited 2.8 and 3.1 kb fragments due to the presence of a *Sal*I site in the TET marker gene and the deletion of three *Sal*I sites in *oprC* (Fig 1).

A plasmid-borne *oprE* deletion was introduced into the chromosome as described above using pOPRE3211. Southern blot analysis of the YY100 chromosomal DNA treated with *Pst*I produced a 6.2 kb fragment due to loss of the *Pst*I sites in the *oprE* gene (Fig. 2b, 2c), confirming that the *oprE* gene was replaced with the TET marker gene.

Construction of deletions in both *oprC* and *oprE* genes. Since the *oprE* gene in pTN103 was replaced by the TET marker, it was inappropriate to use this plasmid to introduce the *oprC* deletion into the YY100 chromosome. We designed a new plasmid without any positive selective marker as suggested by Schweizer (11) (Fig. 4). Gene replacement at the *oprC* region in YY100 was confirmed by Southern blot analysis (Fig. 4c, strain TNP066).

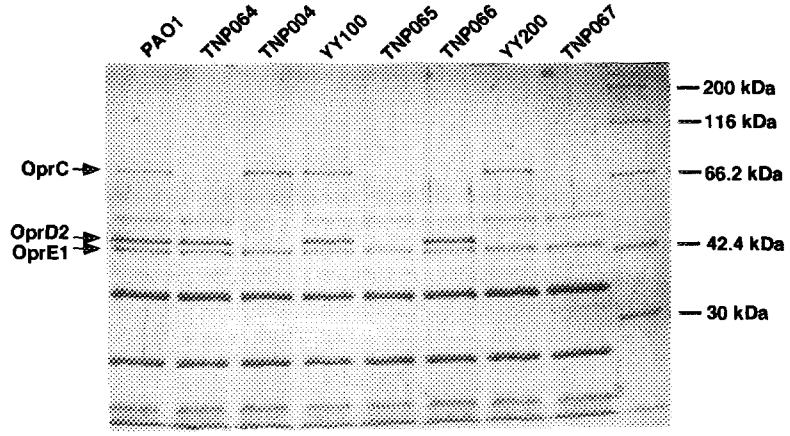

Fig. 3. SDS-PAGE analysis of the outer membrane proteins. Purified outer membrane (20 μg protein) was subjected to electrophoresis in 10 %-polyacrylamide gel and stained with coomassie blue.

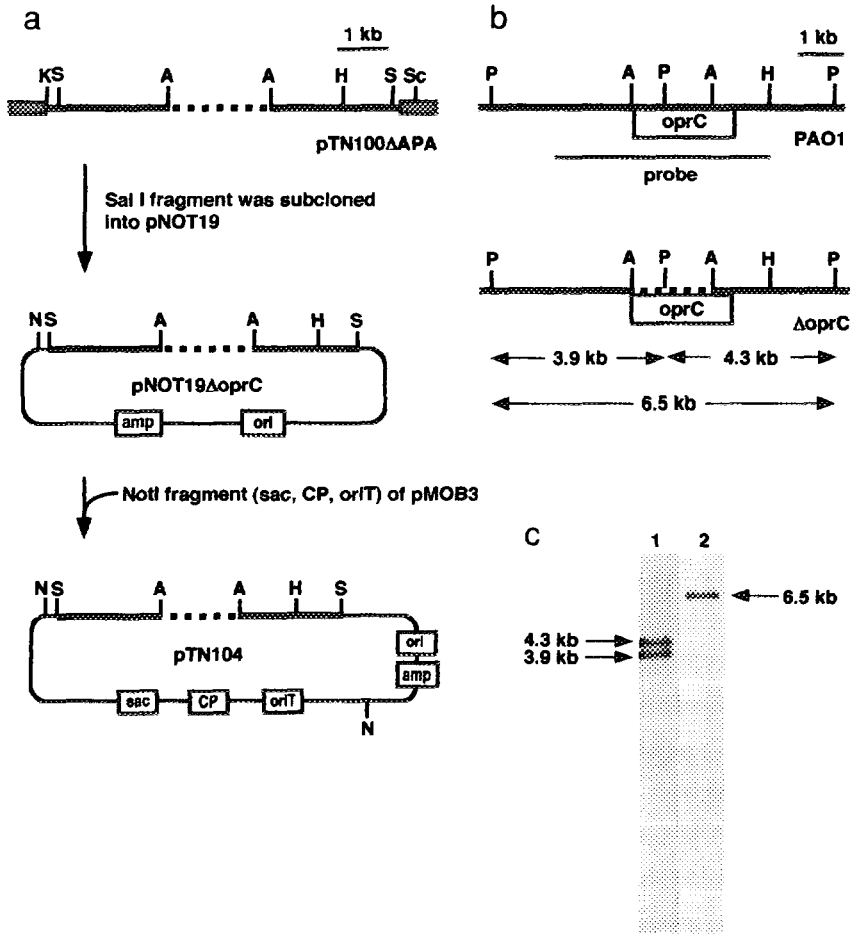

Fig. 4. Construction of a double deletion mutant of *oprC* and *oprE* genes.
(a) Construction of plasmid-borne *oprC* and *oprE* double mutant. The *Sal*I fragment (about 4.4 kb) of pTN100ΔAPA lacking the *Apa*I fragment was subcloned into pNOT19 pretreated with *Sal*I and AP to yield pNOT19Δ*oprC*. Next, the *Not*I fragment (MOB cassette) from pMOB3 was inserted at the *Not*I site of the pNOT19Δ*oprC* generating pTN104. (b) Transfer of pTN104 to YY100 was carried out by conjugation via *E. coli* S17-1 as described above and conjugants were selected on Vogel-Bonner agar containing 25 μg/ml of carbenicillin (plasmid marker, *amp*). Next, the second screening was carried out on an L-agar plate containing 5% (w/v) of sucrose. Restriction enzymes: see the legend to Fig. 1. (c) Southern blotting analysis of the chromosomal DNA (3 μg DNA). Lane1, PAO1; Lane 2, TNP066.

Isolation of the protein D2 deficient mutants. To isolate *oprD* mutants we selected imipenem-resistant mutants TNP065, YY200, and TNP067 from TNP064 (ΔoprC), YY100 (ΔoprE), and TNP066 (ΔoprC, ΔoprE), respectively. We analyzed the DNAs of both the *oprC* and *oprE* regions of these mutants by Southern blot analysis and confirmed the deletion (data not shown). Analysis of the outer membrane proteins by SDS-PAGE (Fig. 3) exhibited the presence of protein bands corresponding to the location of protein E1 in YY100, YY200, TNP066, and TNP067. These protein bands were protein E2 and E3, which had co-migrated to the location of

Table 2. MICs (μg/ml) of antibiotics against porinless mutants

Strains	Phenotype	CAZ	LMOX	IPM	CBPC	OFLX	CP
PAO1	wild	0.78	12.5	0.78	25	0.78	25
TNP064	ΔOprC	1.56	12.5	0.78	50	0.78	50
TNP004	ΔOprD2	1.56	12.5	12.5	50	0.78	50
YY100	ΔOprE1	1.56	12.5	0.78	25	0.78	25
TNP065	ΔOprC, ΔOprD2	1.56	12.5	12.5	25	0.78	50
TNP066	ΔOprC, ΔOprE1	1.56	12.5	0.78	50	0.78	25
YY200	ΔOprD2,ΔOprE1	1.56	12.5	12.5	50	0.78	25
TNP067	ΔOprC, ΔOprD2, ΔOprE1	1.56	12.5	12.5	50	0.78	25
PAO2354	OprF+	1.56	12.5	0.39	50	0.78	100
KG1079	ΔOprF	0.78	6.25	0.39	25	0.39	50

Abbreviations: CAZ, ceftazidime; LMOX, latamoxef; IPM, imipenem; CBPC, carbenicillin; OFLX, ofloxacin; CP, chloramphenicol. Other antibiotics tested were cefsulodin, meropenem, tosfloxacin and gentamicin. MICs of these antibiotics were unchanged among the strains.

protein E1. Thus, we confirmed that all the mutants constructed had the desired genotype and phenotype.

Antibiotic susceptibility of the porin-deficient mutants. We inserted a deletion into *oprC*, *oprD* or *oprE* individually and to all possible combinations of *oprC*, *oprD* and *oprE*, and determined the MICs of several antibiotics in these mutants to assess the contribution of each porin to the antibiotic susceptibility (Table 2). All the mutants carrying a protein D2-deficiency, TNP004 (Δ*oprD*), TNP065 (Δ*oprC*, Δ*oprD*), YY200 (Δ*oprD*, Δ*oprE*), and TNP067 (Δ*oprC*, Δ*oprD*, Δ*oprE*) showed 16-fold higher MICs of imipenem, confirming the previous results (8). Neither protein C nor protein E1 contributed to imipenem permeability, since all the protein C- or protein E1-deficient mutants concomitant with the protein D2 deficiency showed indistinguishable MICs (12.5 μg/ml) from that of the protein D2 single deletion mutant. Mutants deficient in protein C or/and protein E1 (TNP064, YY100, and TNP066) showed identical imipenem susceptibility to the wild strain, PAO1.

On the other hand, MICs of penicillin derivatives, cephalosporins, quinolones, and chloramphenicol in the protein C, D2 or E1-deficient strains appeared identical with the MICs of these in PAO1. Furthermore, the mutants lacking both OprC and OprD2 (TNP065), OprC and OprE1 (TNP066), OprD2 and OprE1 (YY200) or all three porins (TNP067) showed identical susceptibility to these antibiotics with their parent strain PAO1. These results suggest that porins OprC, OprD2 and OprE1 may play little role in the diffusion of these antibiotics. The diffusion pathway(s) of these antibiotics in *P. aeruginosa* remain to be uncovered.

Protein F was reportedly the porin forming a large, but inefficient pore (5). To test the role of protein F in antibiotic diffusion, we compared the MICs of the antibiotics in protein F-deficient mutant, KG1079 and that of protein F-sufficient parent strain, PAO2354 (Table 2). We found no detectable difference between the MICs of the tested antibiotics, confirming the previous result (13). Thus, we concluded that protein F contributes little to the penetration of antibiotics.

This study unexpectedly showed that protein C, D2, and E1 made little contribution to the penetration of antibiotics across the outer membrane of *Pseudomonas aeruginosa* , excepting protein D2 in imipenem penetration. Thus, it is possible that most antibiotics cross the outer membrane dissolving in the lipid bilayer or through specific carrier protein(s). The results reported here provided important information in designing antipseudomonal antibiotics. We believe that the genetically defined porin-deficient mutants constructed in this study will serve as a powerful strategy for future analysis of the antibiotic diffusion pathway and resistance.

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REFERENCES

(1) Brown, M. R. W. (1975) In Resistance of Pseudomonas aeruginosa (Brown, M. R. W., Ed.) pp. 71-107. John Wiley and Sons, London.
(2) Caulcott, C. A., Brown, M. R. W., and Gonda, I. (1984) FEMS Microbiol. Lett. 21, 119-123.
(3) Yoneyama, H., and Nakae, T. (1986) Eur. J. Biochem. 157, 33-38.
(4) Yoshihara, E., and Nakae, T. (1989) J. Biol. Chem. 264, 6297-6301.
(5) Nikaido, H., and Hancock, R. E. W. (1986) In The bacteria (Sokatch, J. R., Ed.) pp. 145-193. Academic Press. Inc., Orlando, Fla.
(6) Neu, H. C., and Labthavikul, P. (1982) Antimicrob. Agents Chemother. 21, 180-187.
(7) Büscher, K. H., Cullann, W., Dick, W., Wendt, S., and Opferkuch, W. (1987) J. Infect. Dis. 156, 681- 684.
(8) Yoneyama, H., and Nakae, T. (1991) FEBS Lett. 283, 177-179.
(9) Simon, R., Priefer, U., and Pühler, A. (1983) Bio/Technology 1, 784-791.
(10) Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular cloning; A laboratory manual, 2nd ed. Cold Spring Harbor Press. Cold Spring Harbor.
(11) Schweizer, H. P. (1992) Mol. Microbiol. 6, 1195-1204.
(12) Ausbel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. , and Struhl, K. (1987) Current protocols in molecular biology. John Wiley and Sons.
(13) Gotoh, N., Wakebe, H., Yoshihara, E., Nakae, T., and Nishino, T. (1989) J. Bac. 171, 983-990.
(14) Satake, S., Yoneyama, H., and Nakae, T. (1991) J. Antimicrob. Chemother. 28, 199-207.
(15) Yamano, Y. (1994) Ph. D. Thesis, Kyoto University.
(16) Felley, R., Frey, J., and Krisch, H. (1987) Gene 52, 147-154.