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Mechanism of Efficient Elimination of Protein D2 in Outer Membrane of Imipenem-Resistant *Pseudomonas aeruginosa*

HIROSHI YONEYAMA* AND TAIJI NAKAE

Department of Molecular Life Science, School of Medicine, Tokai University, Isehara 259-11, Japan

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Most imipenem-resistant *Pseudomonas aeruginosa* isolates produce an immunologically undetectable level of protein D2 (OprD2). To study the efficient elimination of the protein, we selected 23 independent imipenem-resistant mutants from a strain harboring the plasmid carrying cloned *oprD* and having a mutation in chromosomal *oprD*. All these *oprD*/*oprD* (plasmid/chromosomal) mutants expressed undetectable levels of OprD2, as shown from an assay by the immunoblotting method. Restriction maps of the DNAs from all 23 mutant plasmids could be divided into two groups. Restriction mapping and sequencing analysis of DNA from one representative plasmid from each group showed that both mutant *oprD* genes had a deletion. One had an 11-bp deletion in the coding region generating a frameshift mutation and a premature termination codon. Another had a large deletion encompassing the upstream site of its putative promoter region through the coding region. Northern blotting analysis showed that the gene with the 11-bp deletion was transcribed to about 1.5 kb of mRNA, but the gene with the large deletion produced undetectable RNA complementary to the *oprD* DNA probe. Since we analyzed only plasmid-borne *oprD*, we cannot exclude the possibility that the imipenem resistance caused by the chromosomal mutation is by a different mechanism(s). It is suggested, yet, that clear elimination of OprD2 from most imipenem-resistant *P. aeruginosa* isolates is due to efficient selection of the *oprD* deletion mutants.

The outer membrane of gram-negative bacteria possesses water-filled channels formed by outer membrane proteins termed porins (16, 18). Well-characterized *Escherichia coli* porin channels allow the diffusion of saccharides with M_rs of less than about 600 (15). *Pseudomonas aeruginosa* produces several porins, which are proteins C, D2, and E1 (OprC, OprD2, and OprE1, respectively) that form channels; they allow the diffusion of saccharides with M_rs of less than about 350 to 400 (29). Protein F was reported to be the porin that forms a large, but extremely inefficient pore, but that remains a matter of debate (5, 6, 17, 29). Diffusion of antibiotics through the outer membrane of *P. aeruginosa* is very low, at a level of 1/100 to 1/200 of that through the *E. coli* outer membrane (2, 31). This low outer membrane permeability might confer high-level and broad-spectrum antibiotic resistance on this organism.

Imipenem, a carbapenem antibiotic with an M_r of 299, has been developed. Treatment of *P. aeruginosa*-infected patients with imipenem has often allowed for the emergence of mutants that are selectively resistant to imipenem but that have unchanged susceptibilities to other antibiotics (9, 19, 23). Most of these mutants lack OprD2, suggesting that this protein facilitates passage of imipenem across the outer membrane.

We cloned *oprD* and tested to see whether the imipenem-resistant OprD2-deficient strains recovered imipenem susceptibility when the plasmid carrying cloned *oprD* was transferred to them. We found, as expected, that the transformants became imipenem susceptible and expressed OprD2 in the outer membrane (27). These results provided assurance that, in vivo, OprD2 forms imipenem-permeable channels (27). A question raised by these findings is what would be the mechanism of efficient deletion of OprD2 from the outer membrane of imipenem-resistant *P. aeruginosa*? We approached this problem by selecting imipenem-resistant strains from a partially diploid strain carrying the cloned *oprD* plasmid and the chromosomal *oprD* mutation. We report here that such mutants have a deletion in the *oprD* gene.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *P. aeruginosa* strains and plasmids used in the present study are listed in Table 1. *P. aeruginosa* PAO2003 (*recA2 argH32* FP^-) was obtained from B. W. Holloway, Monash University, Clayton, Australia. *P. aeruginosa* TNP031 is the OprD2-deficient imipenem-resistant mutant derived from PAO2003. TNP038 is a derivative of TNP031 harboring pTN003 into which *oprD* was cloned (27). *E. coli* XL1-BLUE (Stratagene) was used to manipulate cloned DNA. Bacteria were grown in L broth containing 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter (pH 7.2) at 37°C overnight under aeration. The outer membrane was purified by the method of Mizuno and Kageyama (14).

Isolation of the imipenem-resistant mutants from the strain harboring the plasmid carrying cloned *oprD*. Fully grown TNP038 was streaked onto L agar containing 6.25 μg of imipenem per ml and 300 μg of carbenicillin per ml (pTN003 carries *amp*) and was incubated at 37°C overnight. Colonies were purified on L agar containing 200 μg of carbenicillin per ml.

DNA techniques and nucleotide sequence analysis. Most of the recombinant DNA techniques that we used have been described previously (21, 27, 28). A restriction map of the cloned *oprD* gene was constructed by treating the plasmid DNA with several restriction enzymes independently or in combination. The oligonucleotides used for the determination of the nucleotide sequence of a coding strand were the T3 primer (Stratagene), AATGAATACAGCGCGACG, CA AACAGAATAGCCTCGC, AAAGAATCCGTCGCTTCG,

TABLE 1. Strains and plasmids used in the present study

Strain or plasmid	Genotype	Phenotype	Reference
P. aeruginosa			
PAO2003	*recA2 argH32* FP$^-$	OprD2$^+$	27
TNP031	*oprD*	OprD2$^-$	27
TNP038	*oprD*$^+$ (pTN003)/*oprD* (chromosome)	OprD2$^+$	27
TNP042	*oprD* (pTN004)/*oprD* (chromosome)	OprD2$^-$	This study
TNP043	*oprD* (pTN005)/*oprD* (chromosome)	OprD2$^-$	This study
TNP048	*oprD*$^+$ (pTN006)/*oprD* (chromosome)	OprD2$^+$	This study
TNP049	*oprD* (pTN007)/*oprD* (chromosome)	OprD2$^-$	This study
TNP050	*oprD* (pTN008)/*oprD* (chromosome)	OprD2$^-$	This study
Plasmid			
pTN003	*oprD*$^+$	OprD2$^+$	27
pTN004	*oprD*	OprD2$^-$	This study
pTN005	*oprD*	OprD2$^-$	This study
pTN006	*oprD*$^+$ (*Bam*HI-*Kpn*I fragment from pTN003)	OprD2$^+$	This study
pTN007	*oprD* (*Bam*HI-*Kpn*I fragment from pTN004)	OprD2$^-$	This study
pTN008	*oprD* (*Bam*HI-*Kpn*I fragment from pTN005)	OprD2$^-$	This study

TTCCGCAGGTAGCACTCAGT, CTGGGCCTGCAGCTC GACG, CGAACTCTATGCCACCTA, CACTTTCACCTT GGCCTA, and TTCATGGTCCGCTATATC. Those used for an anticoding strand were the T7 primer (Stratagene), AATCAAACGGCTGATCGC, CAATGAAATCGGCGCT CT, TTCATCACCGGCAGGTT, CCTGATCGCTGACGA AT, AGGTCGCTTCTCAAGTCGCTT, and TCGCGCTGT ATTCATTTGGC. The nucleotide sequence was determined by the dideoxy chain termination method (22) by using BcaBEST (Takara) and Sequenase Version 2.0 (U.S. Biochemicals).

Western blot (immunoblot) analysis. Anti-OprD2 rabbit antibody was precipitated with 33% ammonium sulfate and was dissolved in an equal amount of phosphate-buffered saline (PBS). One milliliter of the antibody solution was mixed with 5.5 ml of the outer membrane (37.4 mg of protein) prepared from the OprD2-deficient *P. aeruginosa* strain (strain TNP002) in the presence of 0.1% sodium azide, and the mixture was incubated at 4°C overnight by gentle shaking. The mixture was centrifuged at 134,000 × *g* for 1 h at 15°C, and the supernatant was used as anti-OprD2 immunoglobulin G (IgG). Imipenem-resistant mutants were grown in L broth containing 200 μg of carbenicillin per ml (plasmids carry *amp*) at 37°C overnight, harvested, and washed once with PBS, and then the mutants were suspended in an appropriate amount of PBS. A portion of the cell suspension was mixed with an equal amount of a sample buffer described by Laemmli (12) and was heated at 95°C for 5 min. Whole-cell lysate was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel (10%) electrophoresis as described previously (12). Protein bands were blotted onto a polyvinylidene difluoride membrane (Millipore) at 300 mA for 20 min and were visualized with anti-OprD2 IgG as described previously (30).

Northern blot analysis. RNA was isolated by the procedure described by Aiba et al. (1). Cells were grown in L broth containing 100 μg of carbenicillin per ml at 37°C overnight, diluted 100-fold with L broth containing 5 mM $MgCl_2$ and 100 μg of carbenicillin per ml, and then incubated until the cells reached the exponential phase of growth. Cells were harvested by centrifugation and were mixed with 500 μl of a solution containing 0.5% SDS, 20 mM sodium acetate, and 10 mM EDTA (pH 5.5) (solution A). The cell suspension was mixed with an equal amount of phenol saturated with 20 mM sodium acetate and 10 mM EDTA (pH 5.5), and then the mixture was incubated at 60°C for 5 min with gentle shaking. The mixture was centrifuged at 13,000 × *g* for 3 min at room temperature. The water phase (400 μl) was mixed with 1 ml of ethanol and was centrifuged at 13,000 × *g* for 5 min. RNA was washed with 70% ethanol and was dissolved in 400 μl of solution A. This washing procedure was repeated twice. RNA was dissolved in solution A and was quantified at 260 nm. An appropriate amount of RNA was precipitated and dissolved in 10 μl of sample buffer containing 20 mM 3-(*N*-morpholino)propane sulfonic acid (pH 7.0), 5 mM sodium acetate, 50% formamide, 2.2 M formaldehyde, and 10 mM EDTA, and then the mixture was heated at 65°C for 15 min. The sample was subjected to agarose gel (0.8%) electrophoresis in the presence of formaldehyde as described earlier (21) and was transferred to a nylon membrane (Hybond-N$^+$; Amersham) by capillary blotting. RNA was hybridized with a ^{32}P-labelled *oprD* probe (*Bam*HI through *Kpn*I fragment) and was visualized by exposure to X-ray film.

Other techniques. The MICs of the antibiotics were determined by the twofold agar dilution method with Mueller-Hinton medium (Difco). Protein was quantified by the method of Lowry et al. (13).

RESULTS

Isolation of the imipenem-resistant mutants carrying defective *oprD* on the plasmid. To isolate defective *oprD* encoded by the cloned gene, we streaked TNP038 cells with a defective chromosomal *oprD* gene (OprD2$^-$) and harboring plasmid pTN003 (*oprD*$^+$) on medium containing 6.25 μg of imipenem per ml and 300 μg of carbenicillin per ml (for selection of the plasmid). Twenty-three imipenem-resistant mutants were obtained. To see whether the imipenem resistance in these mutants was due to the absence of OprD2, whole-cell lysates of all the mutants were analyzed by the Western blot method with anti-OprD2 rabbit IgG. All the mutants tested lacked OprD2 (Fig. 1). We isolated plasmids from the imipenem-resistant mutants, and the plasmid DNA was subjected to *Bam*HI digestion. Analysis of 23 plasmid DNAs showed essentially two restriction profiles. Figure 2A shows the *Bam*HI digestion profile of the representative plasmid DNAs. One type, which included 11 plasmids, had

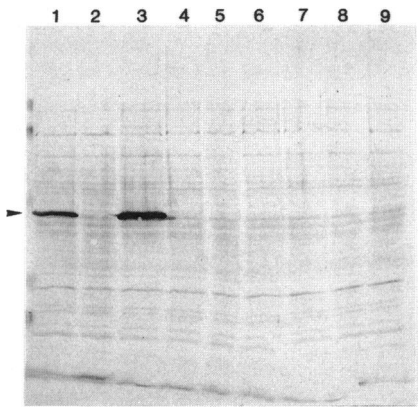

FIG. 1. Western blot analysis of whole-cell lysate of the imipenem-resistant mutant. Samples (20 μg of protein) were subjected to SDS-polyacrylamide gel (10%) electrophoresis. Protein was blotted to a polyvinylidene difluoride membrane (Millipore) at 300 mA for 20 min and visualized with anti-OprD2 IgG. Lanes: 1, PAO2003; 2, TNP031; 3, TNP038; 4, TNP042; 5, TNP043; 6, TNP044; 7, TNP045; 8, TNP046; 9, TNP047. The arrowhead indicates the location of OprD2.

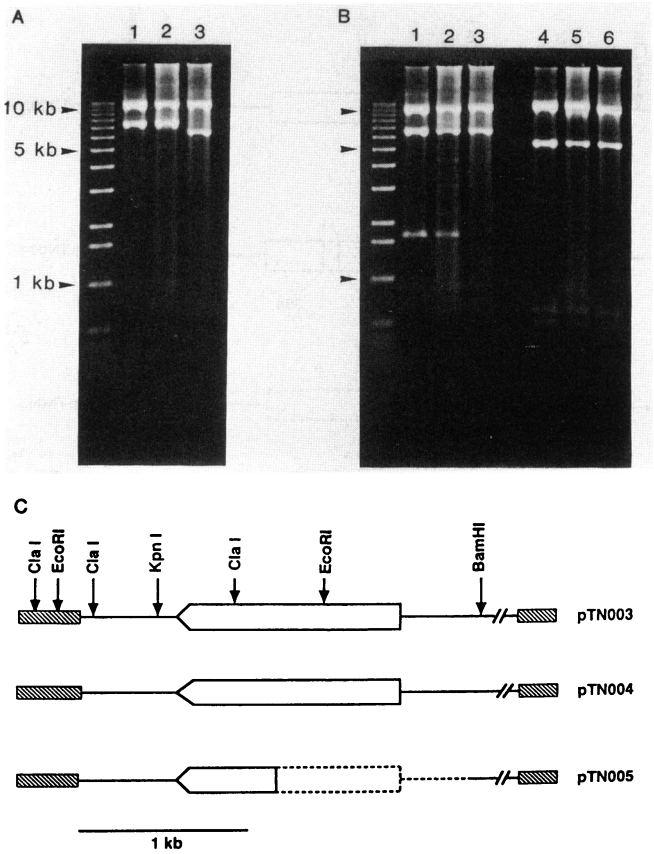

FIG. 2. Restriction profile and map of mutant *oprD*. (A) Agarose gel profile of the plasmid DNA digested with *Bam*HI. Lanes 1, pTN003; 2, pTN004; 3, pTN005. Molecular size markers are shown on the left. (B) Agarose gel profile of the plasmid DNA digested with *Eco*RI and *Cla*I. Approximately 500 ng of plasmid DNA was digested with the respective restriction enzyme. Lanes: 1 to 3, *Eco*RI treatment; 4 to 6, *Cla*I treatment; 1 and 4, pTN003; 2 and 5, pTN004; 3 and 6, pTN005. Molecular size markers are shown on the left. (C) Restriction map of *oprD* and its flanking region. The boxes represent the location of the OprD2 structural gene. The hatched boxes represent vector DNA. Arrows indicate the restriction sites and the relevant cloning sites. The physical distances are arbitrary.

nearly the same restriction profile and size as pTN003 DNA. Another type, which included 12 plasmids, showed a reduced size of DNA, suggesting the presence of a deletion.

We next digested DNA isolated from a representative plasmid of the former type of mutation, pTN004, with *Eco*RI and *Cla*I. We found no observable difference in the restriction map of pTN004 compared with that of pTN003 (Fig. 2B and C). Therefore, we hypothesized that the plasmid pTN004 has a point mutation(s) or a small deletion(s). To test this hypothesis, we investigated plasmid pTN005 DNA, a representative of the latter type of mutation, and found that it possessed no *Eco*RI cleavage site but retained the *Cla*I site (Fig. 2B). Therefore, it is likely that pTN005 has a large deletion (about 1.2 kb) extending from the upstream region of the initiation codon (ATG) to the OprD2 coding region somewhere between the *Eco*RI and the *Cla*I sites (Fig. 2C).

Nucleotide sequence analysis of the mutant *oprD* on the plasmid. To determine the precise location and nature of the *oprD* mutation(s) on the mutant plasmid, we subcloned *Bam*HI-*Hin*dIII fragments from both plasmids into pBluescript II SK(−) and SK(+) (Stratagene) for sequencing of the coding and the anticoding strands, respectively. For analysis of the anticoding strand, pBluescript II SK(+) harboring the *Bam*HI-*Hin*dIII fragment was digested with *Kpn*I or *Cla*I and was ligated with T4 DNA ligase. Thus, the transformant carrying the *Bam*HI-*Kpn*I or the *Bam*HI-*Cla*I fragment was obtained. We determined the nucleotide sequences of both pTN004 and pTN005 for both strands using oligonucleotides complementary to *oprD* as primers. We found that pTN004 had an 11-bp deletion from nucleotides 395 to 405 from the initiation codon (ATG). This small deletion caused a frameshift downstream (Fig. 3), introducing a new termination codon at nucleotides 654 to 656. On the other hand, we found that pTN005 had a 1,204-bp deletion from nucleotides −519 to 685 from the initiation codon. This large deletion covered the initiation codon and putative Shine-Dalgarno (GGAG; nucleotides −12 to −9), −10 (TAAGTT; nucleotides −84 to −79), and −35 (TCGCCA; nucleotides −107 to −102) sequences. It is most likely, therefore, that *oprD* of pTN005 would not be transcribed.

Because pTN003 is a derivative of RSF1010, the number of the plasmid copies should be 15 to 20 per cell (3, 4). Accordingly, one may argue that the imipenem-resistant derivatives may harbor multiple plasmids with heterogeneous mutations. However, this seems unlikely, since (i) the restriction maps of the plasmid DNA seem to be homogeneous (Fig. 2) and (ii) the possible occurrence of a double mutation in a single host is less likely. This assumption was supported by the fact that the mutation rate was 10^{-7} to 10^{-8}.

Northern blot analysis of imipenem-resistant mutants harboring the defective *oprD* gene. The nucleotide sequence of *oprD* on pTN004 suggests that TNP042 transcribes the mutant *oprD* gene into defective mRNA and synthesizes the polypeptide out of sense, resulting in premature termination. In contrast, the mutant *oprD* gene on pTN005 (TNP043) is unlikely to be transcribed because of a large deletion including a putative promoter region. In order to test this hypothesis, we investigated the transcription of mutant *oprD* by Northern blot analysis. PAO2003 (wild type for *oprD*) and

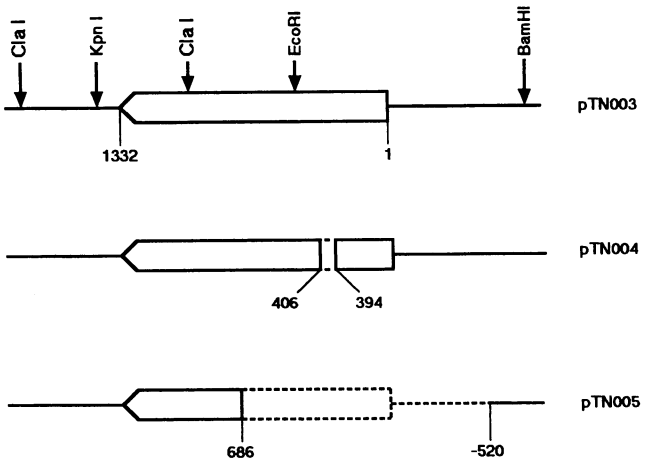

FIG. 3. Schematic representation of mutant *oprD*. Numbers represent the position of nucleotides from the adenine of the initiation codon. The boxes represent the location of the OprD2 structural gene. Arrows indicate the restriction sites. The physical distances are arbitrary.

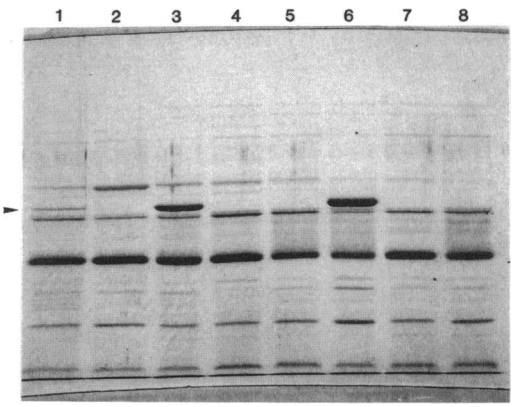

FIG. 5. Electrophoretic analysis of the outer membrane proteins. Purified outer membrane (15 μg of protein) was subjected to SDS-polyacrylamide gel (10%) electrophoresis. Lanes: 1, PAO2003; 2, TNP031; 3, TNP038(pTN003); 4, TNP042(pTN004); 5, TNP043 (pTN005); 6, TNP048(pTN006); 7, TNP049(pTN007); 8, TNP050 (pTN008). The arrowhead indicates the location of OprD2.

TNP038 (*oprD*$^+$/*oprD*) carrying cloned *oprD*$^+$ produced a single RNA band of about 1.5 kb. The size of *oprD* mRNA appeared to be reasonable, as deduced from the nucleotide sequence of *oprD* (Fig. 4, lanes 1 and 3) (28). In contrast, we were unable to detect mRNA complementary to *oprD* DNA in TNP031 carrying defective chromosomal *oprD* (Fig. 4, lane 2). This result showed that the OprD2 defect in TNP031 was due to an inability to transcribe *oprD*. We detected the plasmid-borne *oprD* mRNA in strain TNP042 (*oprD*/*oprD* mutant) (Fig. 4, lane 4). The size of the mRNA appeared to be almost the same as that derived from wild-type *oprD* (Fig. 4, lanes 1 and 4). The amount of mRNA expressed in TNP042 was somewhat less than that expressed in TNP038. On the other hand, mRNA complementary to *oprD* DNA in TNP043 (*oprD*/*oprD* mutant) was undetectable (Fig. 4, lane 5).

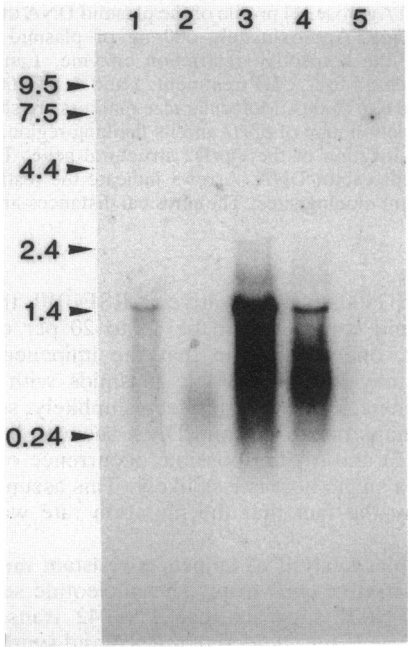

FIG. 4. Northern blot analysis of RNAs from the imipenem-resistant mutant. Lanes 1, PAO2003; 2, TNP031; 3, TNP038; 4, TNP042; 5, TNP043. The amounts of RNA applied for agarose gels (0.8%) electrophoresis were 50 μg for PAO2003 and TNP031 and 5 μg for TNP038, TNP042, and TNP043. Bands were visualized by hybridization with the *Bam*HI-*Kpn*I fragment (2.3 kb) of *oprD*. Size markers (in kilobases) are shown on the left.

The frameshift mutation in *oprD* of TNP042 introduces an additional nonsense codon and, therefore, may produce a truncated OprD2 with an M_r of about 21 kDa. It is possible that the truncated protein is located in the outer membrane but does not function as the imipenem-permeable channel. To test this possibility, we analyzed OprD2 peptide in purified outer membrane by the Western blot method. Results showed that neither intact OprD2 nor truncated protein was detectable in the TNP042 outer membrane (data not shown). This was also confirmed by Coomassie blue staining (Fig. 5, lane 4). OprD2 was undetectable in the TNP043 outer membrane, as predicted (Fig. 5, lane 5).

MIC determination. OprD2-defective *P. aeruginosa* is resistant to imipenem, but its susceptibility to other antibiotics is unchanged (27). To test whether the strain carrying the *oprD*/*oprD* mutant genotype also showed the same antibiotic susceptibility to the cell with a chromosomal *oprD* mutation, we determined the MICs of several antibiotics. MICs of imipenem for both mutants appeared to be 12.5 μg/ml, indicating that the mutants gained imipenem resistance (Table 2). Both mutants showed the same susceptibilities as their parent strain TNP038 to other antibiotics (Table 2).

It is possible, although less likely, that the upstream region of the *Bam*HI site and the downstream region of *Kpn*I site play some role in *oprD* expression. Therefore, we subcloned *Bam*HI through *Kpn*I fragments from pTN003 into pBluescript II SK(−) and constructed a fusion plasmid with pKT240 (pTN006). This fusion plasmid carrying only the *Bam*HI-*Kpn*I fragment containing *oprD* and short flanking regions fully expressed OprD2, indicating that this 2-kb fragment, including *oprD*, was sufficient to express OprD2 normally (Fig. 5, lane 6). On the other hand, transformants possessing pTN007 or pTN008, carrying *Bam*HI through

TABLE 2. MICs of antibiotics for *P. aeruginosa* derivatives[a]

Strain	Plasmid	MIC (μg/ml)						
		IPM	CEZ	CAZ	GM	OFLX	CP	TC
PAO2003		0.78	>800	1.56	1.56	0.2	50	12.5
TNP031		12.5	>800	1.56	3.13	0.39	50	12.5
TNP038	pTN003	0.78	>800	1.56	1.56	0.39	50	12.5
TNP042	pTN004	12.5	>800	1.56	1.56	0.39	50	12.5
TNP043	pTN005	12.5	>800	1.56	1.56	0.39	50	12.5
TNP048	pTN006	0.78	>800	1.56	1.56	0.39	25	12.5
TNP049	pTN007	12.5	>800	1.56	1.56	0.39	50	12.5
TNP050	pTN008	12.5	>800	1.56	1.56	0.39	50	12.5

[a] About 5×10^3 CFU of cells per 5 μl was inoculated onto Mueller-Hinton medium containing antibiotic, and growth of the cells was scored after 18 to 20 h of incubation at 37°C. The medium used for the preculture of strains harboring plasmid contained 200 μg of carbenicillin per ml, since these plasmids have the Amp^r marker. Abbreviations: IPM, imipenem; CEZ, cefazolin; CAZ, ceftazidime; GM, gentamicin; OFLX, ofloxacin; CP, chloramphenicol; TC, tetracycline.

*Kpn*I fragment from pTN004 or pTN005, respectively, failed to express OprD2 in their outer membranes (Fig. 5, lanes 7 and 8); consequently, they were imipenem resistant (Table 2). On the basis of these results, we concluded that imipenem resistance in TNP042 is caused by a deletion of 11 nucleotides, leading to the generation of a frameshift mutation and a premature termination codon. In TNP043, the cause is a deletion of about 1.2 kb nucleotides encompassing the sequence upstream of the promoter region through the coding region that abolished transcription.

DISCUSSION

P. aeruginosa has intrinsic resistance to many structurally diverse antibiotics (8). One of the reasons for this antibiotic resistance is the presence of a tight penetration barrier at the outer membrane (7). Imipenem is a potent β-lactam antibiotic against *P. aeruginosa*. Imipenem-resistant *P. aeruginosa* often lacks OprD2, suggesting that OprD2 functions as a route for imipenem penetration (24, 25). We previously provided direct evidence (27), by the transfer of cloned *oprD* into an OprD2$^-$ imipenem-resistant host, that imipenem crosses the outer membrane through the OprD2 channel. The level of OprD2 expression in most imipenem-resistant mutants appeared to be undetectably low by the immunoblotting method (23, 25).

We addressed the question of why the imipenem-resistant mutants clearly eliminated OprD2. Our strategy was to isolate mutant plasmids carrying defective *oprD* by selecting the imipenem-resistant host (defective in chromosomal *oprD*) harboring cloned *oprD*. Twenty-three independent mutant plasmids were isolated. These mutants could be divided into two major DNA types on the basis of the restriction map. One type of mutant, including 11 plasmids, showed a restriction map indistinguishable from that of wild-type *oprD*, and the other type, including 12 plasmids, showed small DNA fragments. Analysis of one of each representative plasmid-borne mutant *oprD* gene revealed the following interesting results. The former type of mutant had an 11-bp deletion, causing a frameshift mutation and generating a new termination codon. Since Western blotting analysis did not detect any trace of OprD2, it is likely that the other 10 mutants had similar mutations. To confirm the result of DNA sequencing, we used the Northern blot method to analyze mRNA transcribed from the plasmid *oprD* gene (pTN004). The amount of mRNA purified from the cell carrying this mutant *oprD* gene appeared to be slightly less than that from the cell harboring the plasmid containing the healthy *oprD*, but the size of the mRNA was comparable. We searched for OprD2 peptide translated from this mutant mRNA, but the immunoblotting technique could not detect any trace of the OprD2 peptide. Two interpretations can explain these findings. One is that the mutant mRNA had a shorter life, because a smaller amount of mRNA was observed (20). The other is that because of the frameshift mutation and the generation of a new termination codon, the OprD2 peptide translated from the mutant mRNA was abnormal. Such abnormal protein would be a target of intracellular scavenger proteases, resulting in a rapid degradation (11). The latter possibility was tested by introducing pTN004 into an *E. coli* host carrying the *lon* mutation. However, we were unable to detect any trace of OprD2 peptide in an assay by the immunoblotting technique using anti-OprD2 IgG. Since this result does not rule out a possible proteolytic cleavage of defective OprD2, which of the above possibilities is the more likely remains speculative. Whatever the precise mechanism, the imipenem resistance was due to the deletion in *oprD*, resulting in a frameshift mutation and the generation of a premature termination codon. Although we have not analyzed the rest of the mutants of this group, it is likely that a somewhat similar mechanism exists for these other mutants.

The representative strain from another group showed a reduced size of *oprD* DNA. Nucleotide sequencing of this gene showed a large deletion encompassing a region from upstream to downstream across the promoter region. This mutant gene can never be transcribed into mRNA, since it lacks promoter, Shine-Dalgarno, −10, and −35 sequences. In fact, analysis of mRNA showed undetectable RNA complementary to *oprD* DNA. Since we found 12 independent mutants with a restriction map similar to that of pTN005, it is highly likely that these mutant *oprD* genes had a large deletion as described above. We assume this type of deletion mutation is another type of mechanism of imipenem resistance.

Imipenem resistance in *P. aeruginosa* is largely attributable to the elimination of OprD2 from the outer membrane, making the outer membrane less permeable to imipenem. This mechanism of imipenem resistance is probably due to the affinity of OprD2 to imipenem. It was reported that the imipenem and basic amino acids diffuse across the *P. aeruginosa* outer membrane via the OprD2 channel in a specific manner (25, 26). This was supported by the observation that the MIC of imipenem for *P. aeruginosa* became high in the presence of about 10 to 50 mM basic amino acids (10). One would expect, therefore, to isolate a mutant *oprD* that develops a low affinity to imipenem resulting in intermediate imipenem resistance. However, we have never encountered such a mutant expressing OprD2 with a lower functional level. We await the results of ongoing studies to find consistent and cohesive answers to these questions.

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