JOURNAL OF BACTERIOLOGY, Dec. 1993, p. 7793–7800
0021-9193/93/247793-08$02.00/0

Vol. 175, No. 24

Genetic Definition of the Substrate Selectivity of Outer Membrane Porin Protein OprD of *Pseudomonas aeruginosa*

HONGJIN HUANG AND ROBERT E. W. HANCOCK*

Department of Microbiology, University of British Columbia, Vancouver, British Columbia, V6T 1Z3 Canada

Received 29 June 1993/Accepted 12 October 1993

Earlier studies proved that *Pseudomonas aeruginosa* OprD is a specific porin for basic amino acids and imipenem. It was also considered to function as a nonspecific porin that allowed the size-dependent uptake of monosaccharides and facilitation of the uptake of quinolone and other antibiotics. In the present study, we utilized *P. aeruginosa* strains with genetically defined levels of OprD to characterize the in vivo substrate selectivity of this porin. An *oprD*::Ω interposon mutant was constructed by gene replacement utilizing an in vitro mutagenized cloned *oprD* gene. In addition, OprD was overexpressed from the *lac* promoter by cloning the *oprD* gene into the broad-host-range plasmid pUCP19. To test the substrate selectivity, strains were grown in minimal medium with limiting concentrations of the carbon sources glucose, gluconate, or pyruvate. In minimal medium with 0.5 mM gluconate, the growth rates of the parent strain H103 and its *oprD*::Ω mutant H729 were only 60 and 20%, respectively, of that of the OprD-overexpressing strain H103(pXH2). In contrast, no significant differences were observed in the growth rates of these three strains on glucose or pyruvate, indicating that OprD selectively facilitated the transport of gluconate. To determine the role of OprD in antibiotic uptake, nine strains representing different levels of OprD and OprF were used to determine the MICs of different antibiotics. The results clearly demonstrated that OprD could be utilized by imipenem and meropenem but that, even when substantially overexpressed, it could not be significantly utilized by other β-lactams, quinolones, or aminoglycosides. In addition, competition experiments confirmed that imipenem had common binding sites with basic amino acids in the OprD channel, but not with gluconate or glucose.

A gram-negative bacterial cell is surrounded by an outer membrane, which plays an important role as a permeability barrier for many solutes (20). Certain proteins in the outer membrane, known as porins, form aqueous channels which allow the nonspecific diffusion of small hydrophilic molecules (19, 21). The outer membrane of *Pseudomonas aeruginosa* is notorious for its low permeability relative to that of most other gram-negative bacteria, including *Escherichia coli* (1, 40). To compensate for this low permeability and to permit the uptake of essential nutrients available at low concentrations in the environment, certain substrate-specific transport systems are present in the *P. aeruginosa* outer membrane. For example, OprP allows the facilitated permeation of phosphate (4), OprB (formerly called protein D1) has a binding site for glucose (13, 34), iron-repressible outer membrane proteins (IROMP) permit uptake of Fe^{3+}-siderophore complexes when produced under the conditions of iron deprivation (35), and OprD (formerly called protein D2) (12, 14) facilitates the diffusion of basic amino acids and small peptides containing these amino acids (32). These channels may be utilized by compounds which are structural analogs of the natural substrate. One example involves OprD, which can also serve as a specific channel for imipenem, which has a structure resembling that of basic amino acids (32).

Imipenem is a low-molecular-weight broad-spectrum carbapenem antibiotic with high potency against *P. aeruginosa* (36). However, during clinical therapy of *P. aeruginosa*, imipenem-resistant isolates arise at a significant rate (22). The major cause of resistance is loss of a specific outer membrane protein, OprD (5–7, 15). Interestingly, OprD mutants are not cross resistant to other classes of β-lactam antibiotics. Instead, resistance is limited to the zwitterionic carbapenem antibiotics such as meropenem and panipenem (12, 31, 33). Nevertheless, it was suggested that OprD had significant nonspecific permeability to monosaccharides (and disaccharides) and was generally involved as a porin for antibiotic (β-lactam) uptake (24, 33, 38, 39).

Fluoroquinolones have recently emerged as one of the most effective classes of antibiotics. In *P. aeruginosa*, the outer membrane has been proposed to act as a significant permeability barrier to the antimicrobial activity of fluoroquinolone antibiotics (2, 8). Some, but not all, fluoroquinolone-resistant mutants are cross resistant to imipenem and lack OprD. For example, Michea-Hamzehpour et al. demonstrated that decreased fluoroquinolone susceptibility was associated with a decrease or loss of OprD and proposed that OprD can catalyze the facilitated diffusion of fluoroquinolone as it does for imipenem (16, 17).

To further investigate the substrate selectivity of the OprD channel, a series of isogenic mutants expressing different levels of OprD were constructed in this study. This has permitted the demonstration that OprD can be significantly utilized by imipenem, meropenem, and gluconate, but not by other antibiotics, including fluoroquinolones, or by glucose or pyruvate. In addition, competition experiments confirmed that imipenem shared common binding sites with basic amino acids in the OprD channel, but not with gluconate or glucose.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *P. aeruginosa* PAO1 H103 was used as the OprD-containing wild-type strain (37). *P. aeruginosa* H636 was an *oprF*::Ω derivative of strain H103 created by gene replacement with an Ω fragment-mutated *oprF* gene (37). *P. aeruginosa* H729 was an OprD-defective (*oprD*::Ω) interposon mutant created in this study. *E. coli* DH5α F′ [F′ *endA1 hsdR17* (r_K^- m_K^+)

* Corresponding author.

supE44 thi-1 recA1 gyrA96 relA1 λ⁻ ϕ80d*lacZ*ΔM15 Δ(*lacZYA argF*) *U169*] was used for primary cloning (37). *E. coli* S17-1 (30), which contains a mutated RP1 plasmid integrated into the chromosome, was utilized for biparental mating of plasmids from *E. coli* to *P. aeruginosa*. *E. coli* CE1248 (F⁻ *recA-56 phoE proAB phoR-69 ompB-471 thr leu thi pyrF thy ilvA his lacY argG tonA rspL cod dra utr glpR*) (29) was a strain with mutations preventing the production of porins OmpF, OmpC, and PhoE. *E. coli* CE1248(pBK19R) contained the *oprD* gene which was cloned as the 2.1-kb *Bam*HI-*Kpn*I fragment into pTZ19R in the same orientation as the *lac* promoter (14).

Plasmid pUCP19 (27) was used as the vector for the overexpression of OprD in *P. aeruginosa*. It is a broad-host-range plasmid which can be maintained in both *E. coli* and *P. aeruginosa*. For making the OprD-defective mutant, plasmids pNOT19, pMOB3, and pUC4KAPA were used (28). pNOT19 is derived from pUC19 with the unique *Nde*I site changed to a *Not*I site. pMOB3 contains the MOB3 cassette as a 5.8-kb *Not*I fragment which is composed of a portable *oriT*, the *sacB* gene from *Bacillus subtilis* as a counterselectable marker, and a chloramphenicol resistance gene allowing positive selection of both the *oriT* and the *sacB* genes. The plasmid pUC4KAPA, obtained from Pharmacia, contained a 1.3-kb fragment which was derived from Tn*901*, which encoded the enzyme aminoglycoside phosphotransferase (conferring kanamycin resistance), and was flanked by symmetrical restriction enzyme recognition sites.

Strains were routinely grown on Luria broth (LB) medium (1.0% Bacto Tryptone, 0.5% yeast extract, 0.5% NaCl) or LB agar containing, in addition, 2% Bacto agar. For experiments involving growth on specific carbon sources, *P. aeruginosa* strains were grown on BM2 minimal medium (18). VBMM medium (28) is VB medium containing 0.3% trisodium citrate as a carbon source and is selective for *P. aeruginosa*, since *E. coli* cannot utilize citrate. Antibiotics were used in selective media at the following concentrations: ampicillin, 75 μg/ml (for *E. coli*; replaced by 500 μg of carbenicillin per ml for *P. aeruginosa*); chloramphenicol, 25 μg/ml; kanamycin, 35 μg/ml (for *E. coli*) and 300 μg/ml (for *P. aeruginosa*); streptomycin, 500 μg/ml (for *P. aeruginosa*).

Construction of pXH1 for making an OprD-defective mutant in *P. aeruginosa*. A kanamycin resistance (Km^r)-conferring Ω fragment from pUC4KAPA was isolated as the 1.3-kb *Sal*I fragment and cloned into the *Xho*I site on the *oprD* gene, yielding pBK19R::Ω, which left 0.6 and 0.7 kb, respectively, of chromosomal DNA sequence on either side of the Km^r cassette. Then, the 3.6-kb *Bam*HI-*Kpn*I fragment containing the *oprD*::Ω insert was cloned into the similarly cleaved pNOT19. Subsequently, the MOB3 cassette was isolated as a 5.8-kb *Not*I fragment from pMOB3 and inserted into the unique *Not*I site on pNOT19 with *oprD*::ΩKm^r to generate plasmid pXH1 (~12 kb) (Fig. 1A).

Overexpression of the *oprD* gene in *P. aeruginosa*. To overexpress OprD, the 2.1-kb *Bam*HI-*Kpn*I fragment from pBK19R containing the *oprD* gene was cloned into pUCP19 to form the plasmid pXH2, so that the direction of expression of the *oprD* gene was in the same orientation as the *lac* promoter (Fig. 1B). It was then transformed into *E. coli* S17-1 and mobilized back into *P. aeruginosa* H103, H636, and H729.

DNA procedure. All DNA techniques followed the protocols outlined by Sambrook et al. (23). Plasmids were transferred from *E. coli* to *P. aeruginosa* by biparental mating. Successful transfers were monitored by evaluating antibiotic resistance and with a quick-lysis plasmid preparation.

Growth experiments. For growth experiments, each strain was grown to mid-exponential phase on minimal medium with the specific carbon source glucose, gluconate, or pyruvate. Then, the strains were subcultured 1:50 into prewarmed fresh media containing the indicated levels of saccharides and were grown with shaking at 37°C. Samples (1 ml each) were taken at regular intervals for measurements of the optical density at 600 nm. Growth rates (μ [expressed in 1/hours]) were calculated by the equation $\mu = \ln 2/g$, where g was the apparent doubling (generation) time which was determined from semilogarithmic plotting of the growth curve. Each experiment was repeated three times independently.

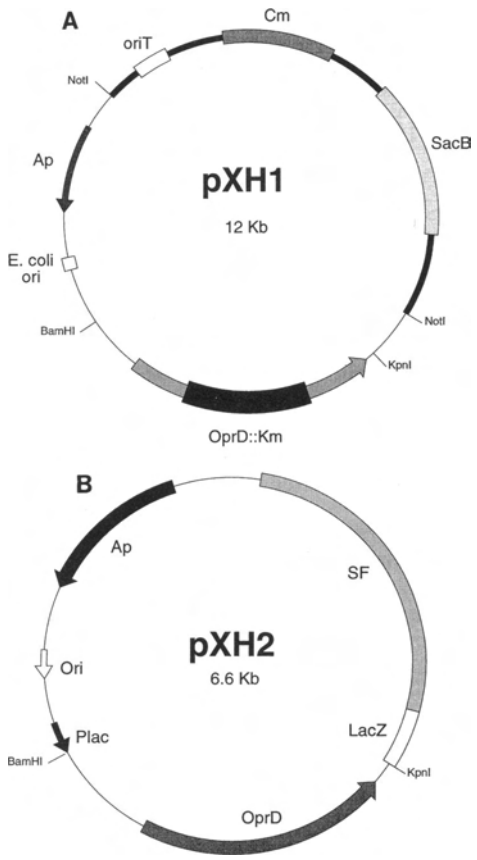

FIG. 1. (A) Diagram of plasmid pXH1 utilized for allele replacement mutagenesis. The more lightly shaded arrow between the *Bam*HI and *Kpn*I restriction sites represents the *P. aeruginosa oprD* gene coding region, whereas the black thick bar in the middle represents the 1.3-kb kanamycin resistance Ω interposon that was used to interrupt the *oprD* gene. The fragment between the two *Not*I sites is the 5.8-kb MOB3 cassette. The orientations of the *oprD* gene and ampicillin resistance marker are indicated. Abbreviations: Ap, ampicillin resistance gene; Km, kanamycin resistance Ω interposon; Cm, chloramphenicol resistance gene; *E. coli* ori, *E. coli*-specific origin of replication; oriT, origin of transfer. (B) Diagram of plasmid pXH2 utilized for the overexpression of the *oprD* gene. The dark shaded arrow between the *Bam*HI and *Kpn*I restriction sites represents the *P. aeruginosa oprD* gene coding region. The orientations of the ampicillin resistance gene, the *oprD* gene, the origin of replication, and the *lac* promoter are indicated. The 1.8-kb stabilizing fragment is for the maintenance of the plasmid in *P. aeruginosa*. Abbreviations: Ap, ampicillin resistance gene; Ori, origin of replication for *E. coli*; Plac, *lac* promoter; SF, stabilizing fragment.

MIC determinations. To determine MICs, each strain was grown overnight in Mueller-Hinton medium. Mueller-Hinton or other medium agar plates containing serial twofold dilutions of appropriate antimicrobial agents were inoculated with 10^3

cells in a 10-μl volume. MICs were determined at least three times and were assessed after 18 h of incubation at 37°C. The MICs were recorded as the lowest antibiotic concentrations at which cell growth was inhibited. The influence of basic amino acids and gluconate on imipenem susceptibilities of *P. aeruginosa* strains was determined by using BM2 minimal medium (18) containing a 20 mM carbon source (glucose or succinate) supplemented with a basic amino acid or glucose or gluconate.

RESULTS

Construction of a defined OprD-defective mutant in *P. aeruginosa*. Our previous data indicated that two gene regions were involved in the expression of the *oprD* gene. One turned out to be the *oprD* structural gene, and the other region (the *opdE* gene) might encode a protein influencing the expression of OprD (14). In keeping with this hypothesis, the cloned *oprD* gene in *E. coli* was expressed poorly from its own promoter (14) and the level of OprD observed in the outer membrane of *P. aeruginosa* was influenced by both the growth medium and the carbon source (13). All of the OprD-defective mutants of *P. aeruginosa* studied to date were genetically undefined, and many were from clinical sources. Therefore, to permit study of the substrate selectivity of OprD, it was necessary to make a defined OprD-lacking mutant so that we could exclude the potential artifacts of leaky or secondary mutations and strain backgrounds.

The improved method described by Schweizer for allele replacement (28) was utilized to replace the wild-type *oprD* gene in strain H103 with an *oprD*::Ω interposon-mutated gene. Southern hybridization of chromosomal DNA with a ^{32}P-labelled *oprD* gene probe confirmed that in strain H729, the *oprD* chromosomal gene was interrupted by a 1.3-kb *Sal*I fragment containing the Km-Ω interposon (Fig. 2). In the Southern blot, a 4.5-kb band was present in both the mutant and parent strains (Fig. 2), which indicated that there might be another gene with high homology to the *oprD* gene in *P. aeruginosa*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the outer membrane proteins of H729 confirmed the lack of OprD (Fig. 3, lanes 8 and 9).

Overexpression of the *oprD* gene in *P. aeruginosa*. To overexpress the *oprD* gene in *P. aeruginosa*, the *oprD* gene was subcloned into plasmid pUCP19 to form plasmid pXH2, so that the direction of expression of the *oprD* gene was in the same orientation as the *lac* promoter. pXH2 was then mobilized back into *P. aeruginosa* wild-type strain H103 and the *OprF*::Ω mutant strain H636. Since *P. aeruginosa* does not have the *lac* repressor gene, isopropyl-β-D-thiogalactopyranoside (IPTG) was not added to the medium. SDS-PAGE of the outer membrane proteins demonstrated that in strain H103(pXH2), OprD was expressed to a level almost equivalent to that of *P. aeruginosa* major outer membrane protein OprF (Fig. 3, lane 4), and in H636(pXH2) OprD appeared to be the prominent outer membrane protein (Fig. 3, lane 7).

Plasmid pXH2 was also transferred into the OprD-defective strain H729. The results indicated that the loss of OprD could be complemented and that the *oprD* gene was also overexpressed in H729(pXH2) (Fig. 3, lane 10).

Function of OprD in sugar transport. Specific porins, with the exception of the sucrose porin of *E. coli* (26), are generally poorly permeable to nonspecific substrates. OprD was considered to be a nonspecific pore which allowed the size-dependent uptake of small hydrophilic molecules such as monosaccharides (33, 38, 39). To investigate the role of OprD in sugar transport, the above-described set of isogenic strains expressing different levels of OprD were used. Control experiments

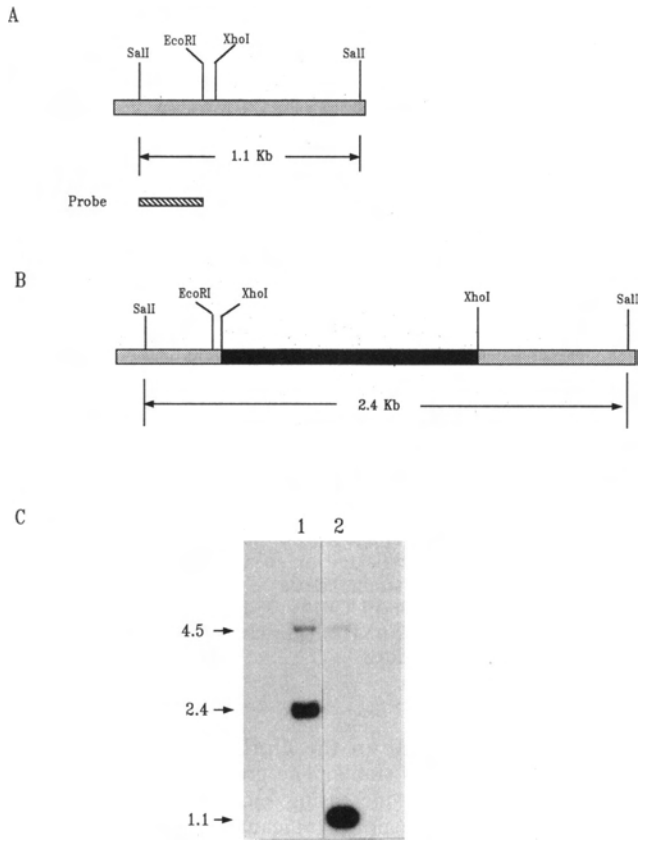

FIG. 2. Genomic Southern hybridization illustrating the interruption of the *oprD* gene (shaded bar) with a kanamycin resistance Ω interposon (black bar). The physical maps of the wild-type (A) and the mutant (B) *oprD* gene region are shown. Genomic DNAs were digested to completion (C) with *Sal*I and were hybridized to the ^{32}P-labelled 0.3-kb *Sal*I-*Eco*RI fragment diagrammed in panel A. Lanes: 1, H729; 2, H103. The molecular sizes of the fragments (in kilobases) are indicated on the left.

demonstrated very similar rates of growth for all three strains on LB (growth rate, 1.26 doublings per h for all three strains) and Mueller-Hinton broth (growth rate, 1.10 to 1.23 doublings per h). Therefore, strains were grown in the BM2 minimal medium with either glucose, gluconate, or pyruvate as carbon sources, at concentrations varying from 0.5 to 10 mM. Growth curves were constructed for each sugar at each concentration and were utilized to calculate growth rates. We reasoned that if OprD could facilitate the transport of a certain sugar at the growth rate-limiting concentrations, different growth rates depending on the *oprD* gene expression level would be observed.

For gluconate, at a concentration of 0.5 mM, the growth rates of wild-type strain H103 and OprD-defective strain H729 were only 60 and 20%, respectively, of that of OprD-overexpressing strain H103(pXH1), and these differences were statistically significant ($P < 0.05$ by Student's t test) (Fig. 4A). These data were thus consistent with outer membrane permeation being the rate-limiting step for growth on gluconate and further suggested OprD was the major porin involved in gluconate passage across the outer membrane under growth-limiting conditions.

As the initial concentration of gluconate in the medium was increased, the growth rates for H103 and H729 increased,

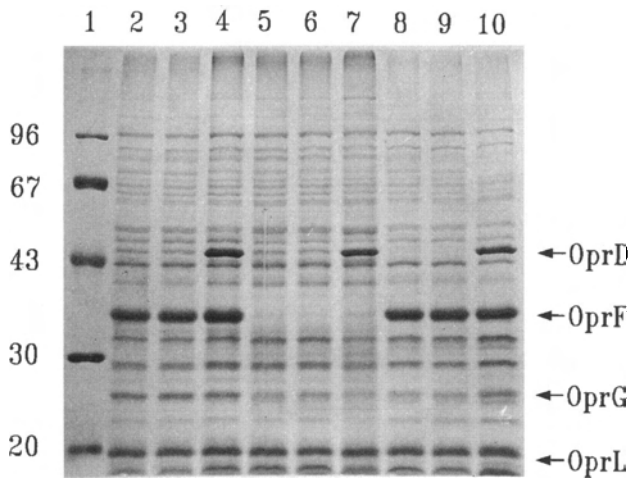

FIG. 3. SDS-polyacrylamide gel electrophoretograms demonstrating overexpression and mutagenesis of the *oprD* gene. The banding position of OprD is indicated by an arrow on the right. Lanes: 1, molecular marker; 2, strain H103; 3, H103(pUCP19); 4, H103 (pXH2); 5, H636; 6, H636(pUCP19); 7, H636(pXH2); 8, H729; 9, H729(pUCP19); 10, H729(pXH2). For each lane, 20 μg of outer membrane proteins was added.

whereas the growth rate of the OprD-overexpressing strain H103(pXH2) remained stable. The growth rates of the three strains converged with increasing saccharide concentrations and eventually became not significantly different at 10 mM gluconate. This result indicated that outer membrane permeation through OprD ceased to become rate limiting at high saccharide concentrations, at which other porins could substitute for OprD (Fig. 4A).

To further exclude the possibility that the low growth rate of the OprD-defective strain H729 resulted from metabolic disturbances due to the mutation, another carbon source, glucose, was used as a control. At a glucose concentration of 0.5 mM, the growth rates for H103 and H729 were 110 and 90% that of H103(pXH2), which were not significantly different ($P > 0.5$). As the glucose concentration was increased, the growth rates for all three strains increased but remained very close for the three strains (Fig. 4B). These results were reasonable since, as previously demonstrated, in the presence of glucose *P. aeruginosa* strains induce a specific porin, OprB. The OprB levels in all three strains were found to be the same, approximately fivefold lower than the level of OprD in strain H103(pXH2). These results indicated that loss or overexpression of OprD did not affect the normal growth of the cell.

Since growth in pyruvate leads to higher expression levels of OprD in *P. aeruginosa* (13), it was questioned whether OprD could also allow the specific passage of pyruvate. The results showed no significant difference in the growth rates of *P. aeruginosa* H103(pXH2), H103, and H729 at pyruvate concentrations of from 1 to 10 mM (Fig. 4C), indicating that OprD was not able to function as the predominant channel for the transport of pyruvate.

Antibiotic susceptibility. To reexamine the role of OprD in quinolone uptake and also to confirm, in genetically defined strains, that it is a specific pore for imipenem, MICs were determined by the agar dilution procedure in Mueller-Hinton medium (Table 1). Nine strains which represented different expression levels of the *oprD* and *oprF* gene were utilized. The rationale behind this experiment was that, if OprD could act as channel for certain antibiotics, the overexpression of this porin would increase susceptibility, whereas the lack of the porin would decrease susceptibility to these antibiotics; otherwise, the amount of OprD would not influence antibiotic susceptibility.

Two carbapenems were used (imipenem and meropenem); the MICs for wild-type strain H103 were 4.0 and 0.5 μg/ml, respectively. The OprD-overexpressing strain H103(pXH2) showed the lowest MICs, 0.5 to 1.0 μg/ml (imipenem) and 0.06 to 0.12 μg/ml (meropenem), which were four- to eightfold lower than the MICs for H103. In contrast, the OprD-defective

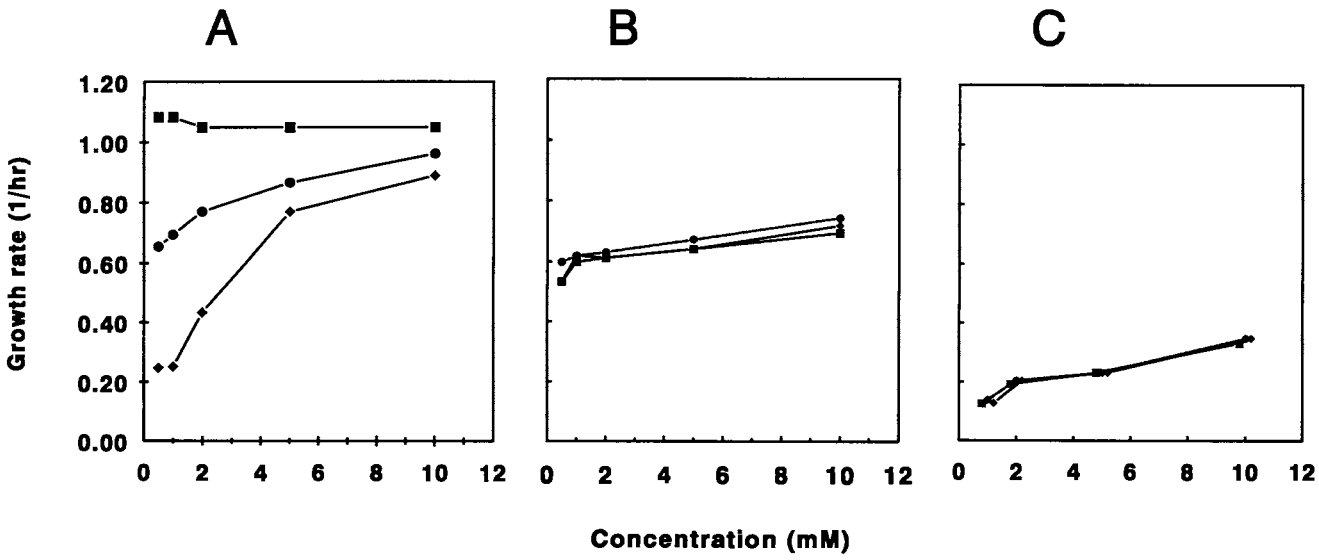

FIG. 4. Influence of substrate concentrations on the growth rates of H103 (circles), its OprD-overexpressing strain H103(pXH2) (squares), and the OprD-defective strain H729 (diamonds). (A) Gluconate; (B) glucose; (C) pyruvate. Datum points are average results from three experiments, with variation of ≤10% between experiments.

TABLE 1. Influence of OprD expression level on antibiotic susceptibilities of *P. aeruginosa* strains

Strain	Level of[a]		MIC (μg/ml)[b]						
	OprD	OprF	IMIP	MERO	CTX	CFP	CIP	FLER	GM
H103	+	+++	4.0	0.50	4.0	1.0	0.12	0.50	2.0
H103(pUCP19)	+	+++	4.0	0.50	8.0	4.0	0.12	0.50	2.0
H103(pXH2)	+++	+++	1.0	0.06	4.0	4.0	0.12	0.50	2.0
H729	−	+++	16.0	2.0	8.0	2.0	0.12	0.50	4.0
H729(pUCP19)	−	+++	16.0	2.0	4.0	4.0	0.12	0.50	2.0
H729(pXH2)	+++	+++	0.5	0.06	8.0	4.0	0.12	0.50	4.0
H636	+	−	4.0	0.50	8.0	2.0	0.06	0.50	2.0
H636(pUCP19)	+	−	4.0	0.50	8.0	4.0	0.06	0.50	2.0
H636(pXH2)	+++	−	1.0	0.12	8.0	4.0	0.06	0.50	2.0

[a] Levels of different outer membrane proteins as observed in control experiments such as that shown in Fig. 3.
[b] Abbreviations: IMIP, imipenem; MERO, meropenem; CTX, cefotaxime; CFP, cefpirome; CIP, ciprofloxacin; FLER, fleroxacin; GM, gentamicin. All MICs were determined three times on Mueller-Hinton medium.

strain H729 showed the highest MICs, 16 and 2.0 μg/ml for imipenem and meropenem, respectively, which were fourfold higher than the MICs for H103 (Table 1). The overexpression of OprD from plasmid pXH2 in the OprD-defective strain H729 restored susceptibility to levels equivalent to those observed in H103(pXH2). In contrast, the loss by mutation of OprF did not influence the MICs of carbapenems, indicating that OprF did not function as a major uptake route for carbapenems (Table 1).

Other β-lactams (cefotaxime and cefpirome), quinolones (ciprofloxacin and fleroxacin), and an aminoglycoside (gentamicin) did not show significant differences in MICs, regardless of the expression levels of OprD (Table 1). (note that the two- to fourfold increase in susceptibility to cefotaxime and cefpirome of strains containing plasmid pUCP19 or pXH2 was due to the plasmid-encoded β-lactamase). The additional absence of OprF had little effect on antibiotic resistance. These results indicated that OprD did not significantly facilitate the passage of these antibiotics across the outer membrane.

We also examined the influence of OprD expression on the antibiotic susceptibility of *E. coli*. The *oprD* gene was overexpressed to high levels in *E. coli* CE1248(pBK19R), a strain with mutations preventing the production of porins OmpF, OmpC, and PhoE. However, even in this background, the MICs of the carbapenems were much lower than those for *P. aeruginosa* strains, and overexpression of OprD had no effect, presumably because of the higher intrinsic outer membrane permeability of *E. coli*.

Competition experiments. Since OprD can facilitate the permeation of basic amino acids (23, 30) and the results described above suggested a role in uptake of gluconate and zwitterionic carbapenems, competition experiments were performed as described by Fukuoka et al. (11) to determine whether common sites were shared by these substrates. The susceptibilities of the isogenic variants to imipenem were determined by using BM2 medium supplemented with basic amino acids or gluconate and were compared with the results obtained with unsupplemented BM2 glucose or succinate, respectively (Table 2). The MICs for H103 and H103(pXH2) were increased 8- to 16-fold and 4- to 8-fold, respectively, by the addition of 50 mM basic amino acids. However, only a twofold effect was observed for the OprD-deficient mutant H729. Such a change in MIC is usually considered not significant. In addition, the effect of L-lysine concentrations in BM2 glucose medium on the susceptibilities of the isogenic variants to imipenem was determined. Figure 5 showed that the MICs for H103 and H103(pXH2) increased as the concentration of L-lysine increased, until they reached the same level as the OprD-deficient mutant H729. In contrast, the susceptibility of the OprD-defective strain H729 was not significantly influenced by the addition of basic amino acids. These results suggested that imipenem and basic amino acids shared the common binding sites in the OprD channel.

The same competition experiments were also performed with gluconate or glucose at concentrations of 20, 50, 100, and 150 mM. In contrast to the results for the basic amino acids, the susceptibilities of H103 and H103(pXH2) were not affected by the presence of gluconate or glucose (the twofold increase in the presence of gluconate was also observed for the negative control H729), indicating that no common OprD-binding sites were involved for gluconate and imipenem.

DISCUSSION

In this paper, we report the construction of a set of genetically defined strains which were utilized to define the in vivo substrate profile of porin OprD. Previous studies with intact cells and model membrane systems have indicated a role

TABLE 2. Effects of basic amino acids and gluconate on imipenem susceptibilities of *P. aeruginosa* strains expressing different levels of OprD

Strain	OprD level	MIC (μg/ml) in the following media[a]:						
		BM2[b]	BM2[b] + 50 mM L-lysine	BM2[b] + 50 mM L-arginine	BM2[b] + 50 mM L-histidine	BM2[c]	BM2[c] + 20–150 mM gluconate	BM2[c] + 20–150 mM glucose
H729	−	4.0	8.0	8.0	8.0	4.0	8.0	4.0
H103	+	0.5	4.0	8.0	8.0	1.0	2.0	1.0
H103(pXH2)	+++	0.25	1.0	4.0	4.0	0.5	1.0	0.5

[a] All MICs were measured three times.
[b] Gluconate was used as the carbon source in BM2.
[c] Glucose was used as the carbon source in BM2.

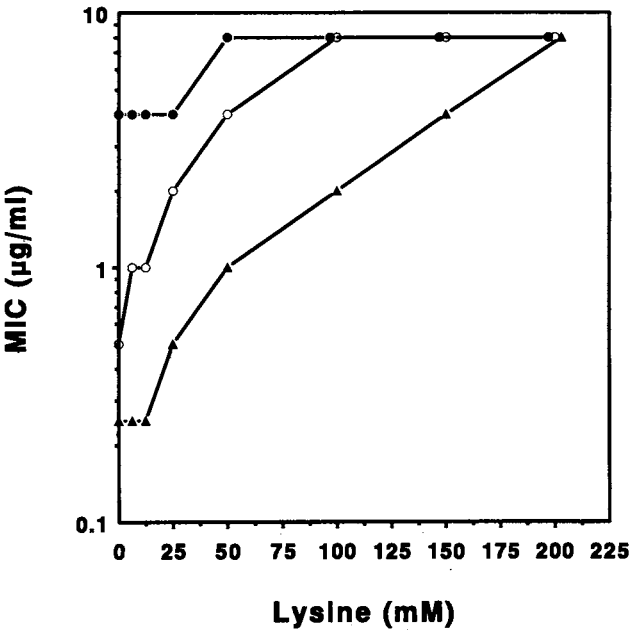

FIG. 5. Effects of L-lysine concentration in BM2 glucose medium on the susceptibilities of H103 (open circles), its OprD-overexpressing strain H103(pXH2) (triangles), and the OprD-defective strain H729 (filled circles) to imipenem.

for uptake of basic amino acids (32) and the zwitteronic carbapenems, including imipenem and meropenem (33). Consistent with these analyses, competition experiments (Table 2) confirmed (11) that common binding sites were involved. However, the utilization of defined OprD-deficient and OprD-overexpressing strains in the present study permitted reexamination of the role of OprD in the uptake of carbon sources and other antibiotics.

Previous work demonstrated that OprF was the major porin for uptake of di-, tri-, and tetrasaccharides (3). However, OprF levels did not influence growth on gluconate. Consistent with this, it was demonstrated here that OprD was the major porin for gluconate in that its absence (in strain H729 *oprD*::Ω) led to a nearly threefold decrease in growth rate on 0.5 to 1.0 mM gluconate (compared with the parent strain H103), whereas its overexpression resulted in a 70% increase in growth rate (Fig. 4A). As expected for an outer membrane diffusion-limited process, these differences disappeared at high concentrations, at which concentrations other porins including OprF might be expected to function adequately. In addition, no significant differences in the rates of growth on glucose, pyruvate, or rich media were observed regardless of the concentration or OprD expression levels (Fig. 4), indicating that the results for gluconate were not due to metabolic disturbances caused by the loss or overexpression of OprD. The result obtained for glucose does not indicate that OprD is unable to permit the passage of glucose, since these studies were performed in strains capable of being induced for the glucose porin OprB. However, given the approximately fivefold-higher level of OprD than OprB in strain H103(pXH2) grown on glucose, it seems likely that OprD has at best a minor role in glucose uptake, as confirmed in part by the data in Table 2. To see whether gluconate might be an analog of basic amino acids and imipenem, we compared their three-dimensional structures by using the computer program HyperChem. Except for the common carboxyl group, gluconate was not structurally related to the basic amino acids or imipenem. This was also confirmed by competition experiments, which suggested that common binding sites were not involved in imipenem and gluconate passage through OprD. Therefore, to explain how the OprD channel might facilitate the transport of gluconate, we suggest three possibilities. Firstly, since both basic amino acids and imipenem contain carboxyl groups and since the only difference between gluconate and glucose is that the former has a carboxyl group, the carboxyl group might function in directing the molecules to the channel and in binding to sites within the channel. Secondly, there might be two functional domains in the OprD channel, one for the binding of basic amino acids and the other one for gluconate. Thirdly, given the low outer membrane permeability of *P. aeruginosa*, gluconate may pass through the channel in a nonspecific fashion. This last suggestion would make the OprD channel analogous to the sucrose porin which has the properties of both substrate-specific and general porins (9, 25, 26). However, the exact mechanism of gluconate uptake through the OprD channel will be determined only by further studies.

OprD is only moderately expressed in wild-type strains like H103 in most growth media (Fig. 3). Nevertheless, this level of expression is sufficient to enhance uptake of imipenem and meropenem, since the OprD-deficient mutant H729 was fourfold more resistant to these antibiotics than the parent strain H103. However, the level of OprD in strain H103 was insufficient to permit maximal uptake, and the outer membrane permeability of imipenem and meropenem was still rate limiting, since overexpression of OprD led to enhanced susceptibility to both antibiotics. The MICs of imipenem for different clinical isolates have been reported in the literature to vary from 0.5 to 4.0 μg/ml for apparently susceptible isolates to between 8.0 and 32 μg/ml for posttherapy-resistant isolates. It is not possible to directly compare these literature studies with the investigation reported here, since these clinical strains had different genetic backgrounds and since different techniques and media were used. Also, it is known that there are at least one regulatory locus (*opdE*) (14) and one poorly understood multiple-antibiotic resistance locus (*nfxC*) (10) that can influence *oprD* expression and imipenem susceptibility. Furthermore, the potential for obtaining double mutants when utilizing clinical strains or when selecting directly with antibiotics has been described previously (41). This indicates the importance of utilizing truly isogenic strains obtained without direct antibiotic selection. Other authors have also suggested a role for OprD in uptake of quinolones (16, 17) or other antibiotics (24) in *P. aeruginosa*. These conclusions are based in part on poorly defined clinical or experimental animal isolates or on in vitro model liposome swelling experiments that have been criticized on other grounds (3, 31, 33). We consider in vivo experiments utilizing genetically defined isogenic variants to be more definitive. If OprD were to be involved in the uptake of quinolones and other antibiotics, one would expect that the substantial overexpression of OprD in H103(pXH2) and H729(pXH2) would increase the normal low outer membrane permeability of *P. aeruginosa* and thus decrease the MICs of these antibiotics. In contrast, no significant alteration in susceptibility was observed. Therefore, we conclude that OprD is not capable of being utilized by antibiotics other than the zwitterionic carbapenems. The strains reported here will provide the basis for studies of structure function relationships in OprD.

ACKNOWLEDGMENTS

This work was supported by grants from the Canadian Cystic Fibrosis Foundation and the Medical Research Council of Canada. H. Huang was the recipient of a Canadian Fibrosis Foundation studentship.

We thank Richard Siehnel for his helpful advice.

REFERENCES

1. **Angus, B. L., A. M. Carey, D. A. Caron, A. M. B. Kropinski, and R. E. W. Hancock.** 1982. Outer membrane permeability in *Pseudomonas aeruginosa*: comparison of a wild-type with an antibiotic-supersusceptible mutant. Antimicrob. Agents Chemother. **21**:299–309.
2. **Bedard, J., S. Chamberland, S. Wong, T. Schollaaardt, and L. E. Bryan.** 1989. Contribution of permeability and sensitivity to inhibition of DNA synthesis in determining susceptibilities of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Alcaligenes faecalis* to ciprofloxacin. Antimicrob. Agents Chemother. **33**:1457–1464.
3. **Bellido, F., N. L. Martin, R. Siehnel, and R. E. W. Hancock.** 1992. Reevaluation, using intact cells, of the exclusion limit and role of porin OprF in *Pseudomonas aeruginosa* outer membrane permeability. J. Bacteriol. **174**:5196–5203.
4. **Benz, R., and R. E. W. Hancock.** 1987. Mechanism of ion transport through the anion-selective channel of the *Pseudomonas aeruginosa* outer membrane. J. Gen. Physiol. **89**:275–295.
5. **Buscher, K. H., W. Cullmann, W. Dick, and W. Opferkuch.** 1987. Imipenem resistance in *Pseudomonas aeruginosa* resulting from diminished expression of an outer membrane protein. Antimicrob. Agents Chemother. **31**:703–708.
6. **Buscher, K. H., W. Cullmann, W. Dick, S. Wendt, and W. Opferkuch.** 1987. Imipenem resistance in Pseudomonas aeruginosa is due to diminished expression of outer membrane proteins. J. Infect. Dis. **156**:681–684.
7. **Buscher, K. H., W. Cullmann, and W. Opferkuch.** 1987. Resistance of *Pseudomonas aeruginosa* is independent of β-lactamase production. J. Infect. Dis. **156**:700–701.
8. **Diakos, D. L., V. T. Lolands, and G. G. Jackson.** 1988. Alterations in outer membrane proteins of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **32**:785–787.
9. **Forst, D., K. Schulein, T. Wacker, K. Diederichs, W. Kreutz, R. Benz, and W. Welte.** 1993. Crystallization and preliminary X-ray diffraction analysis of ScrY, a specific bacterial outer membrane porin. J. Mol. Biol. **229**:258–262.
10. **Fukuda, H., M. Hosaka, K. Hirai, and S. Iyobe.** 1990. New norfloxacin resistance gene in *Pseudomonas aeruginosa* PAO. Antimicrob. Agents Chemother. **34**:1757–1761.
11. **Fukuoka, T., N. Masuda, T. Takenouchi, N. Sekine, M. Iijima, and S. Ohya.** 1991. Increase in susceptibility of *Pseudomonas aeruginosa* to carbapenem antibiotics in low-amino-acid media. Antimicrob. Agents Chemother. **35**:529–532.
12. **Fukuoka, T., S. Ohya, T. Narita, M. Katsuta, M. Iijima, M. Masuda, N. Yasuda, H. Trias, and H. Nikaido.** 1993. Activity of the carbapenem panipenem and role of the OprD (D2) protein in its diffusion through the *Pseudomonas aeruginosa* outer membrane. Antimicrob. Agents Chemother. **37**:322–327.
13. **Hancock, R. E. W., and A. M. Carey.** 1980. Protein D1—a glucose inducible, pore-forming protein from the outer membrane of *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **8**:105–109.
14. **Huang, H., R. Siehnel, F. Bellido, E. Rawling, and R. E. W. Hancock.** 1992. Analysis of two gene regions involved in the expression of the imipenem-specific, outer membrane porin protein OprD of *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **97**:267–274.
15. **Lynch, M. J., G. L. Drusano, and H. L. T. Mobley.** 1987. Emergence of resistance to imipenem in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **31**:1892–1896.
16. **Michea-Hamzehpour, M., Y. X. Furet, and J.-C. Pechere.** 1991. Role of protein D2 and lipopolysaccharide in diffusion of quinolones through the outer membrane of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **35**:2091–2097.
17. **Michea-Hamzehpour, M., C. Lucain, and J.-C. Pechere.** 1991. Resistance to pefloxacin in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **35**:512–518.
18. **Nicas, T. I., and R. E. W. Hancock.** 1980. Outer membrane-protein H1 of *Pseudomonas aeruginosa*: involvement in adaptive and mutational resistance to ethylenediaminetetraacetate, polymyxin B, and gentamicin. J. Bacteriol. **143**:872–878.
19. **Nikaido, H.** 1989. Outer membrane barrier as mechanism of antimicrobial resistance. Antimicrob. Agents Chemother. **33**:1831–1836.
20. **Nikaido, H., and M. Vaara.** 1987. Outer membrane, p. 7–22. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
21. **Nikaido, H., and M. Vaara.** 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49**:1–32.
22. **Quinn, J., E. J. Dudek, C. A. diVincenzo, D. A. Lucks, and S. A. Lerner.** 1986. Emergence of resistance to imipenem during therapy for *Pseudomonas aeruginosa* infections. J. Infect. Dis. **154**:289–294.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Satake, S., E. Yoshihara, and T. Nakae.** 1990. Diffusion of β-lactam antibiotics through liposome membranes reconstituted from purified porins of the outer membrane of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **34**:685–690.
25. **Schmid, K., R. Ebner, K. Jaheris, J. W. Lengeler, and F. Titgemeyer.** 1991. A sugar-specific porin, ScrY, is involved in sucrose uptake in enteric bacteria. Mol. Microbiol. **5**:941–950.
26. **Schulein, K., K. Schmid, and R. Benz.** 1991. The sugar-specific outer membrane channel ScrY contains functional characteristics of general diffusion pores and substrate-specific porins. Mol. Microbiol. **5**:2233–2241.
27. **Schweizer, H. P.** 1991. Cloning vector for *Escherichia-Pseudomonas* cloning derived from pUC18/19. Gene **97**:109–112.
28. **Schweizer, H. P.** 1992. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable *oriT* and the counter-selectable *Bacillus subtilis sacB* marker. Mol. Microbiol. **6**:1195–1204.
29. **Siehnel, R., C. Egli, R. Benz, and R. E. W. Hancock.** 1992. Overexpression in *Escherichia coli* and functional analysis of a novel PPi-selective porin, OprO, from *Pseudomonas aeruginosa*. J. Bacteriol. **174**:471–476.
30. **Simon, R., U. Preifer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology **1**:784–790.
31. **Trias, J., J. Duferesne, R. C. Levesque, and H. Nikaido.** 1989. Decreased outer membrane permeability in imipenem-resistant mutants of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **33**:1201–1206.
32. **Trias, J., and H. Nikaido.** 1990. Protein D2 channel of the *Pseudomonas aeruginosa* outer membrane has a binding site for basic amino acids and peptides. J. Biol. Chem. **265**:15680–15684.
33. **Trias, J., and H. Nikaido.** 1990. Outer membrane protein D2 catalyzes facilitated diffusion of carbapenem and penem through the outer membrane of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **34**:52–57.
34. **Trias, J., E. Y. Rosenberg, and H. Nikaido.** 1988. Specificity of the glucose channel formed by protein D1 of *Pseudomonas aeruginosa*. Biochim. Biophys. Acta **938**:493–496.
35. **Watanabe, N.-A., T. Nagasu, K. Katsu, and K. Kitoh.** 1987. E-0702, a new cephalosporin, is incorporated into *Escherichia coli* cells via the *tonB*-dependent iron transport system. Antimicrob. Agents Chemother. **31**:497–504.
36. **William, J. R.** 1985. Activity of imipenem against *Pseudomonas aeruginosa* and *Bacteroides species*. Rev. Infect. Dis. **7**:S411–S416.
37. **Woodruff, W. A., and R. E. W. Hancock.** 1988. Construction and characterization of *Pseudomonas aeruginosa* protein F-deficient mutants after in vivo and in vitro insertion mutagenesis of the cloned gene. J. Bacteriol. **170**:2592–2598.

38. **Yoshihara, E., and T. Nakae.** 1989. Identification of porins in the outer membrane of *Pseudomonas aeruginosa* that form small diffusion pores. J. Biol. Chem. **264:**6297–6301.
39. **Yoshihara, E., H. Yoneyama, and T. Nakae.** 1991. *In vitro* assembly of the functional porin trimer from dissociated monomers in *Pseudomonas aeruginosa*. J. Biol. Chem. **266:**952–957.
40. **Yoshimura, F., and H. Nikaido.** 1982. Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes. J. Bacteriol. **152:**636–642.
41. **Zhou, X. Y., M. Kitzis, and L. Gutmann.** 1993. Role of cephalosporinase in carbapenem resistance of clinical isolates of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **37:**1387–1389.