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Imipenem Resistance in *Pseudomonas aeruginosa* Resulting from Diminished Expression of an Outer Membrane Protein

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The mechanism of *Pseudomonas aeruginosa* resistance to imipenem in five imipenem-susceptible clinical isolates and in their resistant counterparts was investigated. The frequency for selecting imipenem-resistant variants ranged from 2.7×10^{-5} to 2.1×10^{-8} and was comparable to those for other β-lactams. Cross-resistance between imipenem and other β-lactam compounds was not observed. In all imipenem-resistant variants, induction of chromosomal β-lactamase by imipenem was markedly diminished compared with that in the susceptible parent strain. This was not the case for other inducers such as ampicillin or cefoxitin, suggesting an impaired uptake of imipenem as an explanation for resistance. Analysis of the outer membrane proteins revealed a marked decrease of either a 46- or a 45-kilodalton protein. The lipopolysaccharide of the outer membrane in the imipenem-resistant variants was not altered.

Within the last 2 years, several reports have emphasized the development of resistance to imipenem in clinical *Pseudomonas aeruginosa* isolates (1, 8, 10, 19, 22, 24). Emergence of resistance was observed especially in patients suffering from either cystic fibrosis or nosocomial lower respiratory tract infections (1, 19, 22). Consequently, development of cross-resistance between imipenem and other β-lactam compounds must be evaluated.

Enzymatic inactivation of imipenem has been reported thus far only for *Pseudomonas maltophilia* (21). Since resistance to newer cephalosporins and broad-spectrum penicillins is often chromosomally mediated, we studied five imipenem-susceptible clinical isolates of *P. aeruginosa* and their derived imipenem-resistant counterparts for the production of imipenem-inactivating enzymes and for outer membrane alterations. As it was not clear whether mutation or selection of a resistant subpopulation occurred, the term "variants" will be used for the resistant counterparts.

MATERIALS AND METHODS

Strains. The *P. aeruginosa* strains used were recent clinical isolates from our laboratory and were identified by standard procedures (12). Strain FRA was kindly supplied by G. Seibert, Frankfurt, Federal Republic of Germany. None of the strains exhibited resistance to antipseudomonal β-lactam compounds.

MICs. MICs were determined in Mueller-Hinton broth (E. Merck AG, Darmstadt, Federal Republic of Germany) by twofold serial dilutions representing a final inoculum of 5×10^5 CFU/ml (microdilution procedure).

Selection of resistant variants. Resistant variants were selected by plating 0.05 ml of an overnight culture in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) onto Mueller-Hinton agar plates (Merck) containing the antibiotic in twofold serial dilution steps above the MIC. The frequency of resistant variants was expressed as the ratio of CFU grown in the presence of the antibiotic to the CFU of the control (grown without an antibiotic). All assays were carried out in triplicate.

Kinetics of β-lactamase production. β-Lactamase production was studied during exponential growth. Overnight cultures were diluted 1:100 and grown at 37°C in Iso-Sensitest broth (Oxoid Ltd., Basingstoke, England) in a shaking incubator (150 rpm). Enzyme production was evaluated either in the absence of an inducing agent or in the presence of 50 μg of ampicillin or cefoxitin per ml, 5 μg of ceftriaxone per ml, or 0.25 μg of imipenem per ml as inducers. Aliquots were removed after 2, 4, 6, 8, 12, and 24 h and analyzed for their β-lactamase production. Bacteria were harvested by centrifugation, washed with 0.155 M sodium chloride, centrifuged again, and then subjected to ultrasonic disruption. Details of the procedure were described previously (5). Total protein was determined by the method of Markwell et al. (18), and β-lactamase activity was quantified by using the chromogenic cephalosporin compound PADAC (Calbiochem, Frankfurt, Federal Republic of Germany) as described by Schindler and Huber (23). Final substrate concentrations were 3×10^{-5} M in all assays. One enzyme unit (1 U) was defined as the amount of enzyme hydrolyzing 1 μmol of substrate per min at 37°C; enzyme activity was then expressed in units per milligram of protein in the cell-free supernatant. Isoelectric focusing was carried out with commercially available gel sheets (pH range, 3.5 to 9.5; LKB Produkter, Bromma, Sweden), as described more fully elsewhere (5). Cell-free supernatants were screened for aminopeptidase and dipeptidase activity. Aminopeptidase activity was measured at 405 nm with the chromogenic substrate glycyl-L-proline-4-nitroanilide (Calbiochem) by the method of Fujita et al. (11), and dipeptidase activity was measured with the substrate glycyl-DL-phenylalanine (Sigma Chemical Co., Munich, Federal Republic of Germany) at 255 nm as outlined by Campbell (3). All assays were performed in a 0.05 M triethanolamine buffer adjusted to pH 7.4. For measurements in the visible range, the Gemsaec Fast Analyzer (Electro-Nucleonics, Breda, The Netherlands) was used, and for measurements in the UV range, a double-beam spectrophotometer (model UV-Vis 555; The Perkin-Elmer Corp., Überlingen, Federal Republic of Germany) was used. Moreover, all supernatants were monitored for 10 min at 299 nm with imipenem as the substrate at a final concentration of 8×10^{-5} M to detect possible breakdown of imipenem. For

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TABLE 1. Frequency of resistant variants from clinical *P. aeruginosa* isolates at twice the MIC of the selecting agent[a]

Strain	Frequency of strains resistant to:				
	Piperacillin	Ceftazidime	Aztreonam	Imipenem	Cefotaxime
FRA[b]	2.4×10^{-6}	3.2×10^{-6}	5.0×10^{-7}	2.7×10^{-5}	2.0×10^{-5}
Pip^r				4.9×10^{-6}	
Cfz^r				7.5×10^{-6}	
Azt^r				6.5×10^{-5}	
Imi^r	4.2×10^{-6}	4.1×10^{-6}	3.2×10^{-7}		
526[b]	8.0×10^{-7}	1.3×10^{-6}	1.2×10^{-6}	9.0×10^{-7}	
Pip^r				7.5×10^{-7}	
Cfz^r				1.9×10^{-6}	
Azt^r				1.8×10^{-6}	
Imi^r	6.5×10^{-7}	1.8×10^{-6}	8.0×10^{-7}		
912[b]	3.0×10^{-6}	3.5×10^{-6}	1.3×10^{-7}	3.4×10^{-7}	
Pip^r				1.2×10^{-6}	
Cfz^r				6.1×10^{-7}	
Azt^r				4.3×10^{-7}	
Imi^r	1.7×10^{-6}	3.0×10^{-6}	1.0×10^{-7}		
2206[b]	1.2×10^{-6}	3.5×10^{-6}	7.0×10^{-7}	2.1×10^{-8}	
Pip^r				2.5×10^{-8}	
Cfz^r				1.6×10^{-8}	
Azt^r				2.0×10^{-8}	
Imi^r	7.2×10^{-7}	1.4×10^{-6}	7.7×10^{-7}		
4178[b]	3.8×10^{-7}	1.5×10^{-7}	6.1×10^{-7}	4.4×10^{-7}	
Pip^r				1.0×10^{-6}	
Cfz^r				5.4×10^{-7}	
Azt^r				8.6×10^{-7}	
Imi^r	1.6×10^{-7}	2.1×10^{-7}	2.8×10^{-7}		

[a] Resistant variants were selected in the presence of piperacillin (Pip^r), ceftazidime (Cfz^r), aztreonam (Azt^r), and imipenem (Imi^r).
[b] Wild-type strains.

this purpose, the COBAS FARA analyzer (La Roche, Basel, Switzerland) was used as described by Saino et al. (21). Experiments were carried out at least twice independently of each other, and the mean values are given.

Hydrolysis of β-lactam compounds. The periplasmic β-lactamase produced by the FRA strain was partially purified by extraction of periplasmic proteins with 1% (vol/vol) phenethanol and 5 mM EDTA (Sigma) followed by column chromatography on Sephadex G 100s (Pharmacia, Freiburg, Federal Republic of Germany) (3). The enzyme was incubated with appropriate amounts of β-lactams, resulting in approximately 90% saturation of the enzyme. Aliquots were assayed at different time intervals for residual enzyme activity with PADAC as described previously (6).

Outer membrane proteins. Outer membrane proteins were prepared from overnight cultures in Iso-Sensitest broth by differential solubilization of the cytoplasmic membrane with sodium lauryl sarcosinate after ultrasonic treatment (9). Membranes were harvested by centrifugation at 40,000 × *g* for 60 min at 4°C. Prior to electrophoresis, samples were boiled for 5 min at 100°C in 62 mM Tris hydrochloride buffer, adjusted to pH 6.8, containing 20% (vol/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol, 2% sodium dodecyl sulfate, and 0.01% (vol/vol) bromphenol blue. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels were prepared by the method of Lugtenberg et al. (17) with 11.4% acrylamide in the separating gel. A 60-μg portion of protein from each sample was applied to the gel (11 by 14 cm, 1.5 mm thick) and subjected to electrophoresis at 27 mA until the dye front reached the bottom of the gel. The gels were stained with Coomassie brilliant blue R250; Bio-Rad equipment and reagents (Bio-Rad Laboratories, Munich, Federal Republic of Germany) were used. A set of marker proteins was obtained from Pharmacia.

Lipopolysaccharide analysis. Lipopolysaccharide was isolated by using phenol-water as described by Westphal and Jann (25) and purified further by treatment with Cetavlon (cetyltrimethylammonium bromide; Roth, Karlsruhe, Federal Republic of Germany) and by ethanol precipitation (25). A 20-μg portion of lipopolysaccharide of the respective strain was applied to the gel; conditions corresponded to those maintained for the outer membrane analysis. Gels were developed by the silver-staining procedure (16).

Antibiotics and reagents. Antibiotics were kindly supplied by the following sources: ampicillin and cefotaxime, Hoechst AG, Frankfurt, Federal Republic of Germany; ceftazidime, Cascan-Glaxo, Wiesbaden, Federal Republic of Germany; piperacillin, Cyanamid Lederle, Munich, Federal Republic of Germany; ceftriaxone, La Roche; and cefoxitin and imipenem, MSD Sharp & Dohme, Munich, Federal Republic of Germany. All other reagents, if not specified otherwise, were obtained from Merck and were of the highest purity available.

RESULTS

Selection of resistant variants and their resistance patterns. Resistant variants were selected from five clinical isolates that were susceptible to β-lactam compounds active against *P. aeruginosa*. There were no striking differences in the frequencies of variants obtained with the different agents (Table 1). Variants exhibiting combined resistance to imipenem and other β-lactams such as piperacillin or ceftazidime required two selection procedures, the first in the

TABLE 2. MICs for the wild-type strains and their resistant variants

Strain and variants	MIC (μg/ml) (geometric mean and range)			
	Piperacillin	Ceftazidime	Aztreonam	Imipenem
Wild type	4.6 (2–8)	2.0 (1–4)	5.3 (2–16)	1.1 (1–2)
Imi^r variants	5.6 (2–16)	2.3 (2–4)	5.6 (2–16)	12.1 (8–16)
Pip^r variants	256 (128–512)	48.5 (16–64)	42 (16–64)	1.5 (1–2)
Pip^r Imi^r variants	304 (128–512)	45.3 (16–64)	36.9 (8–64)	13.4 (8–16)
Cfz^r variants	107 (64–256)	45.3 (32–64)	32 (32)	1.5 (1–2)
Cfz^r Imi^r variants	90.5 (64–256)	54.3 (16–64)	32 (32)	11.3 (8–16)
Azt^r variants	55.7 (16–512)	18.4 (8–128)	55.7 (32–128)	1.7 (1–2)
Azt^r Imi^r variants	53.8 (16–512)	22.6 (8–128)	45.3 (32–64)	13.4 (8–16)

presence of imipenem and the second in the presence of a penam, a cephem, or aztreonam. In general, resistant variants could be isolated with a frequency of approximately 10^{-7} at twice the MIC of the selecting agent independent of the agent considered (Table 1). In most cases, resistant variants could be selected at up to eight times the MIC (data not shown). The frequency of variants obtained by selecting with different agents at eight times the MIC decreased two- to fourfold. Variants selected in the presence of ceftazidime (Cfz^r), piperacillin (Pip^r), or aztreonam (Azt^r) exhibited cross-resistance with each other but remained susceptible to imipenem (Table 2). Variants selected in the presence of imipenem (Imi^r) exhibited resistance exclusively to imipenem (Table 2).

To ensure that imipenem resistance was independent of resistance acquired to β-lactams other than imipenem, Imi^r variants were subjected to a second selection procedure in the presence of piperacillin, ceftazidime, or aztreonam, and all Pip^r, Cfz^r, and Azt^r variants were exposed to imipenem. Frequencies of variants in the second selection step resembled those observed in the first step. Resistance acquired in the first step remained unaffected by the second selection procedure (Table 2).

Role of antibiotic-inactivating enzymes. It is well established that *P. aeruginosa* produces type Id β-lactamase, which can be induced in the presence of β-lactam derivatives. Consequently, β-lactamase production in the wild-type strains as well as in the Imi^r variants was studied in the presence of various β-lactams as inducers (ampicillin, cefoxitin, ceftriaxone, and imipenem). The induction potency of ceftriaxone proved to be marginal, whereas ampicillin, cefoxitin, and, above all, imipenem were more efficacious inducers. With ampicillin or cefoxitin as the inducer, no differences were observed in the amount of β-lactamase produced in a wild-type strain and its Imi^r variant (Fig. 1). However, with imipenem as the inducer, appropriate concentrations led to much stronger induction of the type Id enzyme in all wild-type strains than in their Imi^r variants (Fig. 2).

As imipenem is known to be inactivated by enzymes other than β-lactamases such as dipeptidases, the cell-free supernatants of the wild-type strains and their Imi^r variants were monitored for dipeptidase activity with the substrate glycyl-L-proline-4-nitroanilide. In none of the supernatants could dipeptidase or aminopeptidase activity be detected (data not shown). Moreover, hydrolysis of imipenem could not be detected in any of the cell-free supernatants.

In a further experiment, the possible inactivation of imipenem by large amounts of β-lactamase could be ruled out. The partially purified β-lactamase of the FRA strain was incubated with various β-lactams. After every 15 min, ali-

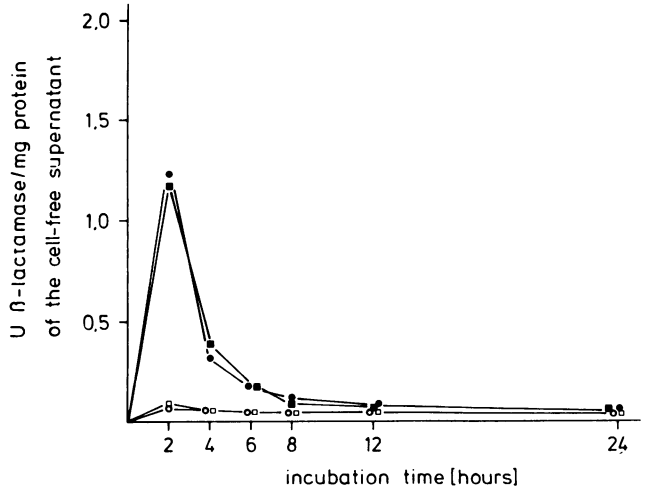

FIG. 1. Spontaneous ampicillin-inducible β-lactamase production of the FRA wild-type strain and the Imi^r variant. Symbols: ○, wild-type strain without inducer; ●, wild-type strain with 50 mg of ampicillin per liter as inducer; □, Imi^r variant without inducer; ■, Imi^r variant with 50 mg of ampicillin per liter as inducer.

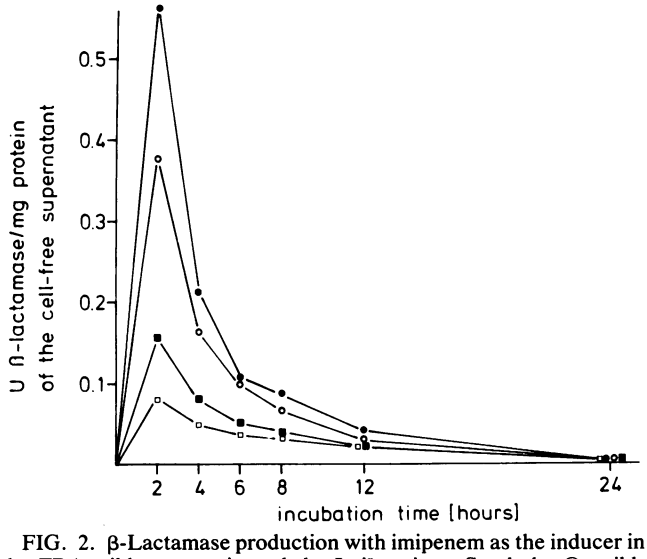

FIG. 2. β-Lactamase production with imipenem as the inducer in the FRA wild-type strain and the Imi^r variant. Symbols: ○, wild-type strain with imipenem at 0.125 mg/liter as inducer; ●, wild-type strain with imipenem at 0.25 mg/liter as inducer; □, Imi^r variant with imipenem at 0.125 mg/liter as inducer; ■, Imi^r variant with imipenem at 0.25 mg/liter as inducer.

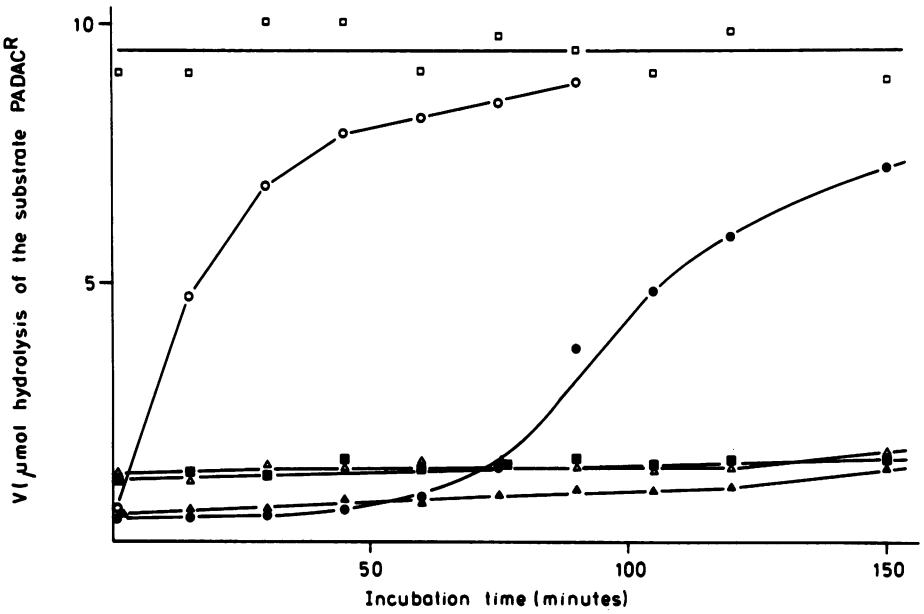

FIG. 3. Hydrolysis of various β-lactam compounds by the partially purified type Id enzyme from the FRA wild-type strain. Residual enzyme activity was quantified with the chromogenic compound PADAC. Symbols: □, control assay; ○, 250 mg of piperacillin per liter; ●, 25 mg of cefotaxime per liter; ▲, 250 mg of ceftazidime per liter; △, 5 mg of aztreonam per liter; ■, 5 mg of imipenem per liter.

quots were taken and analyzed for residual enzyme activity (Fig. 3). Hydrolysis of PADAC is known to be competitively inhibited by penams and cephems (2): piperacillin was the most rapidly broken down, followed by cefotaxime. Hydrolysis of ceftazidime was marginal, whereas hydrolysis of imipenem or aztreonam was not detectable within 2.5 h. Even at a 10-fold-lower concentration (0.5 μg/ml), imipenem proved to be stable against enzymatic breakdown. It should be mentioned that concentrations of the β-lactams investigated differed widely according to their affinity to the enzyme.

The outer membrane. The lack of enzymatically mediated breakdown and the impaired induction potency of imipenem in Imi^r variants suggest that resistance of *P. aeruginosa* to imipenem might be attributed to an impaired penetration of imipenem through the outer membrane proteins of the wild-type strain, of a variant selected in the presence of cefotaxime, and of an Imi^r variant. The outer membrane of the Ctx^r variant resembled the outer membrane of the wild-type strain, whereas a marked decrease in an outer membrane protein was observed in the Imi^r variant (Fig. 4). As can be calculated from the protein standards, the molecular mass of this protein was 46,000 daltons. Since lipopolysaccharide is

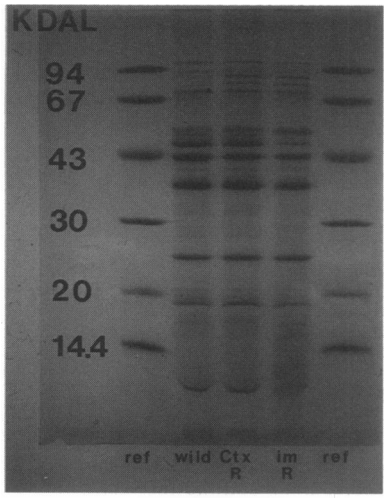

FIG. 4. Outer membrane proteins of the FRA wild-type strain, the Ctx^r variant, and the Imi^r variant. In the Imi^r variant, a 46,000-dalton protein is markedly diminished, as revealed by the standard proteins.

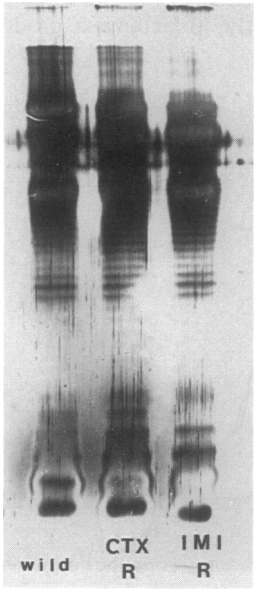

FIG. 5. Electrophoretic analysis of the lipopolysaccharide of the outer membranes of the wild-type strain FRA and the Ctx^r and Imi^r variants. No discrepancies between the wild-type strains and the variants were revealed in the core or in the O-antigen region.

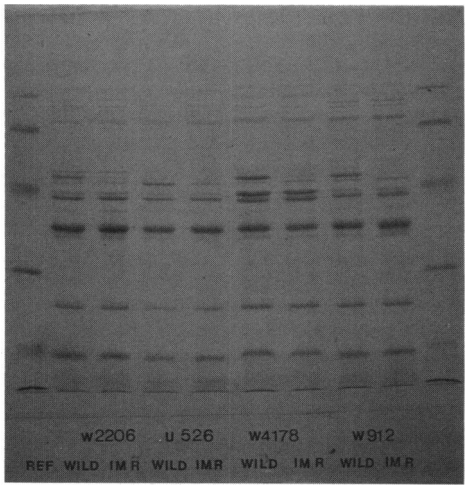

FIG. 6. Comparison of the outer membrane proteins of four imipenem-susceptible isolates with their resistant counterparts. A marked decrease of a 45,000- or 46,000-dalton protein is revealed in the Imi^r variants.

known to contribute to resistance to β-lactams in *P. aeruginosa* (13), lipopolysaccharide was studied by electrophoretic analysis. Neither the Ctx^r nor the Imi^r variant showed differences from the wild-type strain (Fig. 5). To ensure that the loss of the 46,000-dalton protein was not a unique finding for the FRA strain, outer membrane proteins of four other strains and their corresponding Imi^r variants were investigated; in each case, one of two closely related bands became barely detectable in the Imi^r variant (Fig. 6). In one strain (strain 912), only one band appeared (in the wild-type strain only), which became faintly visible.

DISCUSSION

The alterations of the outer membrane proteins, the markedly reduced induction potency of imipenem in the Imi^r variants, and the lack of enzymatic inactivation suggest that impaired penetration of imipenem through the outer membrane of *P. aeruginosa* is responsible for imipenem resistance. This assumption is supported further by the findings reported by Quinn et al., who observed no differences among strains in the penicillin-binding proteins to account for the observed development of resistance to imipenem (20). On the other hand, they observed that imipenem-resistant isolates lacked an outer membrane protein that was present in the susceptible counterpart (20). Moreover, our findings can rule out an alteration of the lipopolysaccharide in the outer membrane as a possible explanation for resistance. It should be noted that the influence of lipopolysaccharide on the susceptibility of the organism to β-lactam antibiotics is not specific to a single component, as is the case for imipenem, but differentiates either on the basis of relative hydrophobicity or on the basis of electrical charge (13). Therefore, it appears unlikely that lipopolysaccharide alterations contribute substantially to imipenem resistance in *P. aeruginosa*.

The marked decrease of either a 45- or a 46-kilodalton protein in the outer membrane of the Imi^r variants suggests that these proteins participate in the penetration of imipenem. The relative mobility of both proteins on sodium dodecyl sulfate-polyacrylamide gel electrophoresis was dependent on the temperature (data not shown). Furthermore, the expression of the 46-kilodalton protein in wild-type strains could be reduced by addition of Casamino Acids (Difco Laboratories, Detroit, Mich.) to the culture medium (data not shown). These findings led us to suggest that the 45- and 46-kilodalton proteins correspond to proteins D1 and D2 described by Hancock and Carey (14). Porin function was reported for the glucose-inducible protein D1 (14, 15); analogous findings have not been reported so far for protein D2, which is phenotypically expressed to some extent even in the absence of glucose (14, 15). At present, it is not yet clear whether proteins D1 and D2 interact with each other to form protein D. Nevertheless, it is likely that the porin function of these two proteins is much less pronounced in the Imi^r variants than in their susceptible counterparts, as revealed by the reduced induction potency of imipenem in the Imi^r variants. As there is not a complete lack of proteins D1 or D2, the approximately 20-fold decrease of susceptibility can be explained. Alteration of protein D1 or D2 will not substantially affect the growth of *P. aeruginosa*, as two distinct glucose uptake systems are known (15). Comparison of the uptake of [^{14}C]imipenem by the wild-type strains and their resistant counterparts to assess the penetration rate is a problem experimentally mainly owing to the low specific activity of the labeled compound available. Nevertheless, it seems that the uptake of imipenem is reduced in the resistant variants (unpublished observations).

The lack of cross resistance between imipenem and β-lactams other than imipenem is worthy of mention. Thus, therapeutic use of imipenem in severe nosocomial infections will not result in the selection of *P. aeruginosa* populations completely resistant to all β-lactams. On the other hand, recently performed clinical studies indicate clearly that a 20-fold increase in the MIC of imipenem may cause therapeutic failure in the treatment of severe *P. aeruginosa* infections (10, 19, 20, 22). Regarding the selection frequencies for Imi^r variants, it can be easily understood that resistant variants emerge above all in diseases characterized by high concentrations of bacteria, e.g., cystic fibrosis. It should be kept in mind that frequencies of resistant variants do not differ widely among imipenem, other β-lactams, and quinolone compounds (7).

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