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Outer Membrane Protein D2 Catalyzes Facilitated Diffusion of Carbapenems and Penems through the Outer Membrane of *Pseudomonas aeruginosa*

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The outer membrane of imipenem-resistant mutants of *Pseudomonas aeruginosa* with decreased permeability to imipenem was shown by Western (immuno-) blotting to contain protein D1 and to lack protein D2. Protein D2 was purified and was shown to allow the permeation of imipenem at a rate higher than expected from its molecular weight. Spontaneous imipenem-resistant mutants of *P. aeruginosa* PAO1 appeared at a frequency of 10^{-8} in the laboratory and did not synthesize protein D2. Experiments performed with intact cells carrying plasmid pHN4 containing the gene for L-1 β-lactamase from *Pseudomonas maltophilia* showed that this channel could also be used by SM-7338, Sch 33755, and Sch 33440 but apparently not by Sch 34343 or Sch 29482.

Nutrients and waste products have to go through the outer membrane of gram-negative bacteria to reach the cytoplasm and the outer medium, respectively. This membrane is semipermeable and acts as a molecular sieve allowing the passage of small hydrophilic molecules (21). The overall permeability of the outer membrane usually depends on the number and properties of the pore-forming proteins that are generically called porins (21). Although most of the small hydrophilic molecules utilize this nonspecific pathway, there are some specific channels that allow the passage of compounds that have an insufficient or negligible rate of permeation through the porin channels and of compounds that are found in the external medium at low concentrations (21). Some of these specific channels are synthesized at higher levels when the substrate is present in the medium of growth: for example, the well-known lambda phage receptor of *Escherichia coli*, protein LamB, which allows the passage of maltose and maltodextrins (16), and the D1 protein of *Pseudomonas aeruginosa*, which allows the permeation of glucose (26), are induced by maltose and glucose, respectively. Other specific permeation systems, such as the Tsx protein that shows a high specificity for nucleosides in *E. coli* (18) and the TonB-related iron and cobalamine uptake systems of the outer membrane, function in the uptake of building blocks for the cell rather than of substrates for energy generation. They are derepressed when the substrate is missing from the medium or present at very low concentrations. The TonB-dependent transport systems in *E. coli* are energy dependent and include the following outer membrane receptors: BtuB, which is involved in the transport of vitamin B_{12}; and Cir, FutA, FhuE, FhuA, FecA, FepA, and Fiu, which are siderophore receptors (21).

The main targets of β-lactams in gram-negative bacteria are the penicillin-binding proteins, which are located in the inner membrane (24). Thus, to reach their target, β-lactams have to go first through the outer membrane. The diffusion of hydrophilic β-lactams into the gram-negative bacterial cell takes place mainly through the nonspecific pore-forming proteins (20, 21, 29), although some β-lactams can go into the periplasm more rapidly by utilizing specific channels (8, 19, 25). Compounds that mimic the siderophores, such as catechol-cephalosporins, are able to go through the Fiu and Cir channels in *E. coli*, and when these outer membrane proteins are overexpressed, there is a significant decrease of the MIC (8). It has been suggested that other antibiotics such as imipenem, a carbapenem, use a specific channel of the outer membrane of *P. aeruginosa* (25). Some imipenem-resistant strains do not show cross-resistance to other antibiotics, cannot hydrolyze or modify imipenem, and do not show any alteration in the affinity or copy number of penicillin-binding proteins. On the other hand, they lack a protein in the outer membrane (4–6, 17, 22, 23). We demonstrated earlier that these imipenem-resistant strains had a lower outer membrane permeability to imipenem but not to cephaloridine, another dipolar ionic β-lactam, and that imipenem could use a specific saturable channel to go through the outer membrane of *P. aeruginosa* (25).

The outer membrane protein missing in the imipenem-resistant strains is a major protein, which exhibits a heat-modifiable behavior and has a molecular weight in the range of 45,000 to 49,000, depending on the strain (4–6, 17, 22, 23). These are characteristics of proteins of the D group in the *P. aeruginosa* outer membrane (10, 11), and it has been suggested that imipenem uses protein(s) belonging to this group for influx into the periplasm (4–6). Two major proteins belong to the D group, D1 and D2. They are difficult to separate through standard gel electrophoresis, since they migrate with similar apparent molecular weights both before heating (36,000 and 35,500, respectively) and after heating (45,000 and 44,500, respectively) (10, 11). Protein D1 is a glucose-inducible pore that facilitates specifically the diffusion of glucose and xylose while allowing the slower, nonspecific passage of other low-molecular-weight compounds, including imipenem (26). Protein D2 has been described as being able to form a small pore through the outer membrane (27). We identify in this paper outer membrane protein D2 as being responsible for the specific transport of imipenem and describe some of the properties of the channel. This channel can also be used by other penems and carbapenems.

MATERIALS AND METHODS

Chemicals. Imipenem was a gift of Merck Sharp & Dohme Research Laboratories, Rahway, N.J.; SM-7338 was a gift of Pharmaceutical Group ICI Americas, Inc., Wilmington,

* Corresponding author.

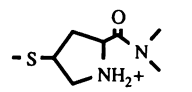
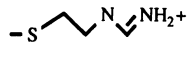
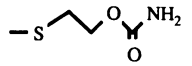
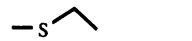
	X	Z	R
SM-7338	C	$-CH_3$	
Imipenem	C	-H	
Sch 34343	S	-H	
Sch 33755	S	-H	
Sch 33440	S	-H	
Sch 29482	S	-H	

FIG. 1. Structures of penems and carbapenems used in this study.

Del.; Sch 34343, Sch 33755, Sch 33440, and Sch 29482 were a gift of Schering Corp., Bloomfield, N.J.; tetracycline was purchased from Sigma Chemical Co., St. Louis, Mo. The structures of the penems and carbapenems used are shown in Fig. 1.

Bacterial strains, plasmid, and growth conditions. The *P. aeruginosa* strains used were a standard laboratory strain, PAO1, and clinical isolates including imipenem-susceptible strains 1-pre, 2-pre, 3-A, and 3-C and imipenem-resistant strains 1-post, 2-post, and 3-B (22). All strains were grown in Luria broth (LB) or in medium 63 (M63) (7) with glucose or sodium succinate as the carbon source. Plasmid pHN4 contained the gene coding for L-1 β-lactamase from *Pseudomonas maltophilia*, an enzyme capable of hydrolyzing imipenem (9, 25), and was introduced into *P. aeruginosa* strains as described previously (25).

Susceptibility of penems and carbapenems to L-1 β-lactamase. L-1 β-lactamase was obtained from an imipenem-induced culture of strain PAO1(pHN4) grown in LB. The enzyme used was purified as previously described by anion-exchange chromatography from a sample obtained by ammonium sulfate fractionation of the supernatant of a cell osmotic shock (25). The hydrolysis of β-lactams was monitored spectrophotometrically in 10 mM sodium phosphate buffer (pH 6) containing 5 mM $MgCl_2$ at the wavelengths indicated: imipenem, 300 nm; SM-7338, 296 nm; Sch 34343, 321 nm; Sch 33755, 320 nm; Sch 33440, 315 nm; and Sch 29482, 323 nm. The K_m for each β-lactam was determined from a Lineweaver-Burk plot.

Permeability assays with intact cells. Intact-cell experiments were performed with strains 3-B and 3-C carrying pHN4. Cells were grown in 5 ml of LB overnight. The medium contained 100 μg of tetracycline per ml to ensure that the plasmid would not be lost and 30 μg of imipenem per ml to induce the synthesis of L-1 β-lactamase. Flasks containing 40 ml of LB with 30 μg of imipenem per ml were inoculated with 0.4 ml of the overnight culture, and cells were harvested by centrifugation at room temperature at the late exponential phase of growth. Bacteria were washed twice with 10 mM sodium phosphate buffer (pH 6) supplemented with 5 mM $MgCl_2$ and were resuspended in the same buffer for the experiment. The rates of hydrolysis of 50 μM antibiotic by the intact cells and the sonic extract of the cells were obtained spectrophotometrically. The measurement was made nearly simultaneously at two wavelengths, i.e., the wavelength at which β-lactam hydrolysis produced maximal changes of absorbance (given above) and at 384 nm, at which none of the β-lactams absorbed, by using the wavelength programming mode of a Perkin-Elmer 4B spectrophotometer connected to an IBM PC/XT computer. The optical density at 384 nm was used to correct for changes in light scattering caused by slow settling of cells. To measure the extent of leakage of enzyme into the medium, we measured the rate of β-lactam hydrolysis with the supernatants obtained by the centrifugation of the intact-cell preparations (25).

The apparent permeability coefficients (P) were calculated by the method of Nikaido et al. (20) with the modifications of Yoshimura and Nikaido (28). We should emphasize, however, that one parameter, P, does not completely describe the diffusion through specific channels, although it is sufficient to describe diffusion processes through nonspecific channels. Thus, the flux of solutes (v) through nonspecific channels is described by Fick's first law of diffusion,

$$v = PA(C_o - C_i) \quad (1)$$

where A, C_o, and C_i denote the area of the membrane, the solute concentration outside, and the solute concentration inside, respectively. In contrast, if the diffusion occurs through a symmetrical channel with one ligand binding site inside the channel, modification of equation 3 of Benz et al. (1) leads to the following formula (K. B. Gehring, Ph.D. thesis, University of California, Berkeley, 1988):

$$v = V_{max}A(C_o - C_i)/(C_o + C_i + K_m) \quad (2)$$

where it is clear that we need at least two coefficients, V_{max} and K_m, to describe the diffusion process. Since under the conditions of our assay, C_i is much smaller than K_m, the equation simplifies to

$$v = [V_{max}/(K_m + C_o)]A(C_o - C_i) \quad (3)$$

If C_o is also much smaller than K_m, equation 3 further simplifies to

$$v = (V_{max}/K_m)A(C_o - C_i) \quad (4)$$

which has the form of equation 1, with V_{max}/K_m, a constant, replacing P.

We assumed that equation 4 will hold and calculated P. However, since the K_m of the outer membrane channel for imipenem is around 0.1 mM (25) and the C_o used was 50 μM, equation 3 more correctly describes the situation, and V_{max}/K_m would have been overestimated by a factor of up to 1.5. We feel that this is not a serious problem in view of a fairly large error inherent in the determination of permeability coefficients.

Purification of the outer membrane. Cells were grown in LB and M63 with glucose or sodium succinate until the late exponential phase of growth. They were harvested by centrifugation and washed with 20 mM Tris hydrochloride (pH 8) containing 5 mM $MgCl_2$ and were broken with a French press at 10,000 lb/in^2. Cells were removed by centrifugation at 3,000 × g for 20 min, and membranes were pelleted by centrifugation of the supernatant at 100,000 × g for 1 h. Outer membranes were purified by sucrose gradient centrifugation by the method of Hancock and Carey (10). The outer membrane fraction recovered was pelleted by centrifugation at 100,000 × g for 1 h and washed twice with 20 mM Tris

hydrochloride buffer (pH 8) containing 5 mM $MgCl_2$ and 1% Triton X-100. The pellet was finally resuspended in the same buffer.

Purification of outer membrane proteins D1 and D2. Protein D1 was purified from *P. aeruginosa* PAO1 by a modification of the method of Hancock and Carey (11). Cells were grown in M63 with glucose, and the outer membrane was isolated as described above. Protein D1 was extracted from the outer membrane with a solution containing 20 mM Tris hydrochloride (pH 8), 10 mM EDTA, and 1% Triton X-100. The supernatant obtained by centrifugation at 100,000 × *g* for 1 h was loaded onto a DEAE-Sephacel column preequilibrated with a solution containing 20 mM Tris hydrochloride (pH 8), 10 mM EDTA, and 0.1% Triton X-100. The column was extensively washed with the same buffer containing 0.1 M NaCl, and the protein was eluted in a 0.1 to 0.3 M NaCl gradient in the same buffer. Fractions shown to contain protein D1 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were pooled, concentrated by ethanol precipitation, and run in preparative SDS-11% PAGE. Guide strips of the gel were cut and stained with Coomassie brilliant blue. The appropriate area of the gel was cut out, and the protein was electroeluted and ethanol precipitated.

Protein D2 was purified from strain PAO1 grown in M63 with sodium succinate so that the production of D1 was repressed. The outer membrane was isolated as described above. It was washed twice with 20 mM Tris hydrochloride buffer (pH 8) containing 5 mM $MgCl_2$ and 1% Triton X-100 by resuspension and centrifugation and extracted with a small volume of a solution containing 20 mM Tris hydrochloride (pH 8), 10 mM EDTA, and 1% Triton X-100. The supernatant after centrifugation at 100,000 × *g* for 1 h was loaded onto a DEAE-Sephacel column and washed extensively with a buffer solution containing 20 mM Tris hydrochloride (pH 8), 10 mM EDTA, and 0.1% Triton X-100. Protein D2 was eluted in a 0 to 0.1 M NaCl gradient in the same buffer. The fractions shown to contain protein D2 were pooled, concentrated by ethanol precipitation, and run in preparative SDS-11% PAGE. The band containing protein D2 was electroeluted from the gel and ethanol precipitated as described above.

Antiserum production and immunochemical detection. Antisera were obtained with proteins D1 and D2 purified from strain PAO1 as described above. Two adult female rabbits were immunized every 2 weeks by intramuscular injection of 100 μg of each protein and bled after the third immunization. Western (immuno-) blotting was performed as described by Burnette (3) with modifications described by Bers and Garfin (2). The proteins were separated by SDS-11% PAGE and were transferred electrophoretically to a nitrocellulose membrane (0.45-μm pore size; Sartorius Filters, Hayward, Calif.). The membranes were probed by anti-D1 and anti-D2 sera, and the bound antibodies were detected with goat anti-rabbit alkaline phosphatase conjugate using the bromochloroindolyl phosphate-nitro blue tetrazolium reagent (14).

Electrophoresis. SDS-PAGE was performed essentially as described by Hancock and Carey (10) with the following modifications. Samples containing 20 μg of protein were electrophoresed by using a 11 or 15% polyacrylamide (acrylamide-bisacrylamide, 44:0.8) resolving gel and a 4% stacking gel. Proteins were detected with Coomassie blue staining or silver staining by the method of Heukeshoven and Dernick (12).

Liposome swelling assay of permeability. Liposomes were made by suspending a dried film containing 6 μmol of egg phosphatidylcholine, 0.3 μmol of dicetylphosphate, and 10 μg of outer membrane protein D2 in 5 mM Tris hydrochloride buffer (pH 7.5) containing dextran T-40 (Pharmacia Diagnostics, Piscataway, N.J.), as described earlier (29). The liposomes were then diluted in isoosmotic solutions of the test solute, and the decrease of the optical density was recorded. The concentration of dextran T-40 used was 5% because if liposomes were made in higher concentration of dextran, isoosmotic solutions of imipenem could not be produced owing to the low solubility of the compound. Protein D2 used for the assay came from the elution of the DEAE column.

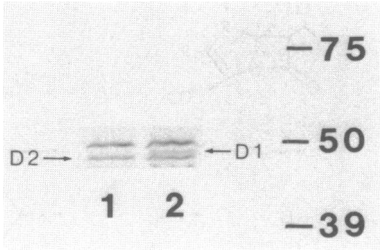


FIG. 2. SDS-PAGE of silver-stained *P. aeruginosa* outer membranes. PAO1 grown in M63 with succinate (lane 1) and M63 with glucose (lane 2). The positions of migration of standard proteins are shown on the right, with their sizes in kilodaltons.

Isolation of imipenem-resistant mutants from PAO1. LB plates containing 10 μg of imipenem per ml were inoculated with an overnight LB culture of strain PAO1. The plates were incubated for 48 h at 37°C. The colonies that grew were inoculated in LB. Overnight cultures were centrifuged, washed twice with 20 mM Tris (pH 8) containing 5 mM $MgCl_2$, and disrupted by ultrasonic treatment using a Soniprep 150 Ultrasonic Disintegrator (MSE, Crawley, England) with a microprobe (3 cycles of 30 s each). The crude sonic extracts were screened for protein D2 content by SDS-PAGE followed by Western blotting.

Other methods. Protein concentration was determined by the method of Lowry et al. (15) using bovine serum albumin (Sigma) as the standard.

RESULTS

Purification of outer membrane proteins D1 and D2 from strain PAO1. When *P. aeruginosa* PAO1 was grown in M63 with succinate as the carbon source, protein D1 was undetectable even with silver staining, but it was fully induced in M63-glucose; in contrast, protein D2 was synthesized under both growth conditions (Fig. 2). Since the synthesis of protein D1 was repressed when the cells were grown with succinate, we purified protein D2 from PAO1 grown under these conditions to avoid any cross contamination between proteins D1 and D2. Protein D1 was purified from cells grown in M63 with glucose. Both proteins were extracted with Triton X-100–EDTA buffer and eluted from an anion-exchange column with a gradient of NaCl. Protein D2 eluted first at a lower ionic strength (0.1 M NaCl), and we were able to remove it completely from the column before the elution of protein D1. The proteins were further purified by preparative SDS-PAGE, and after electroelution the proteins were obtained at a high level of purity (Fig. 3), which allowed us to raise specific antibodies against each protein.

Immunological identification of proteins D1 and D2 in susceptible and resistant strains of *P. aeruginosa*. Since the

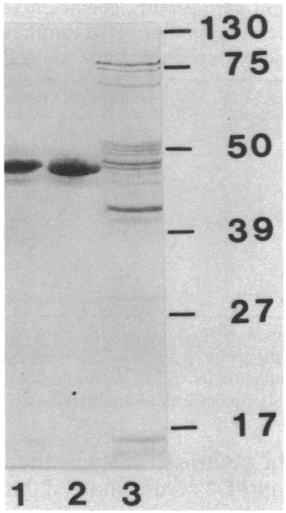


FIG. 3. SDS-PAGE of protein D1 (lane 1), protein D2 (lane 2), and outer membrane of *P. aeruginosa* grown in M63 with glucose (lane 3). Molecular size markers in kilodaltons are shown to the right.

different clinical isolates showed some variability in the apparent molecular weight(s) of the missing protein(s) as shown by gel electrophoresis (4–6, 17, 22, 23), we could not identify the missing protein(s) solely on the basis of its molecular weight. We therefore used anti-protein D1 and anti-protein D2 sera to probe the outer membranes of the strains of clinical origin.

The outer membrane proteins of *P. aeruginosa* clinical isolates and PAO1 were separated in two different gels and transferred to a nitrocellulose membrane. Western blotting with antibodies against D1 and D2 (Fig. 4) showed that protein D1 was present in all the strains tested. There was no significant difference in the amount of protein D1 between the imipenem-susceptible and imipenem-resistant strains. In contrast, protein D2 was present only in the susceptible strains and PAO1; it was absent in the resistant strains.

Spontaneous imipenem-resistant mutants from PAO1 were obtained at a frequency of approximately 10^{-8}. When we examined by Western blotting the production of protein D2 in 23 imipenem-resistant strains isolated in two separate experiments, we could not detect the presence of this protein in any of the strains.

Permeability properties of D2 channel. The permeability properties of outer membrane protein D2 were assayed by reconstituting proteoliposomes with egg phosphatidylcholine, dicetylphosphate, and purified protein D2. The results of the osmotic swelling assay using these liposomes are shown in Fig. 5. Diffusion rates of the compounds tested were normalized to the permeation rate of glycine. Clearly, protein D2 allowed the diffusion of small hydrophilic molecules such as amino acids and sugars, and the diffusion rates for these compounds showed a monotonous dependence on their molecular weights. We were not able to detect the diffusion of cephaloridine with this method. In contrast, protein D2 showed a clear specificity for imipenem, because the rate of liposome swelling in imipenem was much higher than the one expected from its molecular weight.

Hydrolysis of penems and carbapenems by L-1 β-lactamase. As preliminaries for the measurement of outer membrane permeability in intact cells, we tested whether various β-lactams were susceptible to hydrolysis by L-1 β-lactamase. All the penems and carbapenems tested were hydrolyzed by

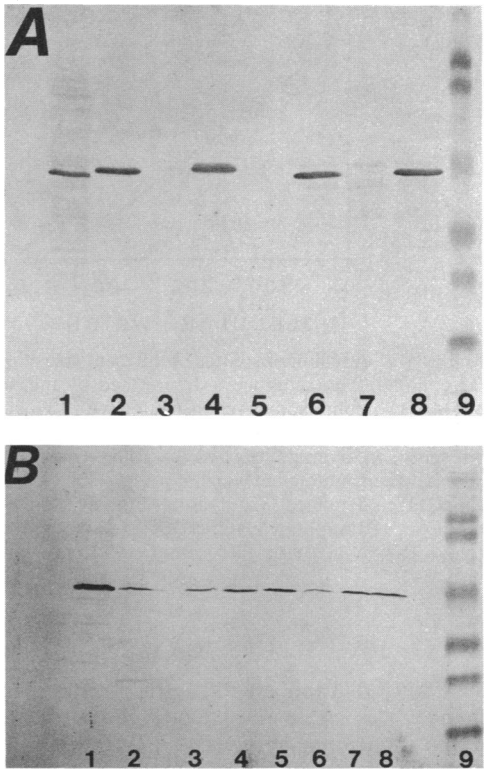


FIG. 4. Western blotting of outer membranes of *P. aeruginosa* strains. Lanes: 1, PAO1; 2, 1-pre; 3, 1-post; 4, 2-pre; 5, 2-post; 6, 3-A; 7, 3-B; 8, 3-C; 9, molecular weight standards. (A) Gel probed with anti-D2 sera. (B) Gel probed with anti-D1 sera. Prestained SDS-PAGE standards (Bio-Rad Laboratories, Richmond, Calif.) with the indicated M_r values were used: phosphorylase *b*, 130,000; bovine serum albumin, 75,000; ovalbumin, 50,000; carbonic anhydrase, 39,000; soybean trypsin inhibitor, 27,000; and lysozyme, 17,000.

this enzyme. The K_m values calculated from the Lineweaver-Burk plots and the relative V_{max} values are given in Table 1.

Permeability of the outer membrane measured with intact-cell method. Since all the penems and carbapenems tested were rapidly hydrolyzed by L-1 β-lactamase, we were able to measure the transport through the outer membrane of *P. aeruginosa* of these compounds by using the Zimmermann-Rosselet method (20). The results of the intact-cell experiment, expressed as apparent permeability coefficients (see Materials and Methods), are shown in Table 2. The outer membrane permeabilities of strain 3-C, which synthesizes protein D2, were much higher for imipenem, SM-7338, Sch 33755, and Sch 33440 than were those of strain 3-B, which does not synthesize protein D2. In contrast, there were no significant differences between the two strains for the transport of Sch 34343 and Sch 29482.

Among the compounds that penetrated the outer membrane via the D2 channel, imipenem showed the highest apparent permeability coefficient, followed by the other carbapenem, SM-7338, and then by Sch 33755. However, it should be emphasized that the computation of apparent permeability coefficients is based on assumptions that are not totally warranted, and we cannot claim that the relative rates of permeation among these compounds will remain similar under all possible conditions (see Materials and Methods).

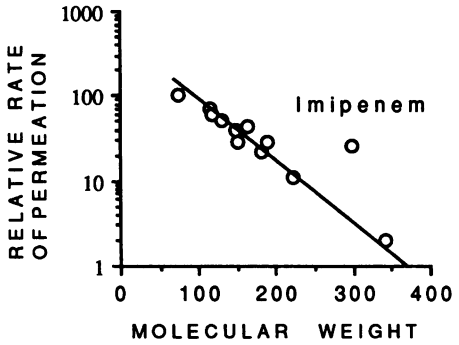


FIG. 5. Rates of diffusion of solutes of different sizes through the protein D2 channel. The rates were determined by measuring the swelling rates of proteoliposomes reconstituted with 10 μg of protein D2. The rates were normalized to that of glycine, taken as 100. Each point represents an average of two to four experiments. The compounds and their molecular weights are glycine, 75; L-proline, 115; L-valine, 117; L-leucine, 131; glycylglycine, 132; L-methionine, 149; D-arabinose, 150; L-phenylalanine, 164; D-glucose, 180; glycylglycylglycine, 189; *N*-acetyl-D-glucosamine, 221; imipenem, 299; and maltose, 342.

DISCUSSION

The possibility that imipenem uses a specific outer membrane channel was suggested when Quinn et al. (22) first reported that some imipenem-resistant *P. aeruginosa* strains lacked an outer membrane protein with an apparent molecular weight of 45,000 to 49,000. Several laboratories reported similar findings (4–6, 17), and this protein was thought to belong to the D group in the classification of Hancock and Carey (10). This group contains two major proteins, D1 and D2. It was difficult to identify the protein responsible for imipenem diffusion, because these two proteins often had very similar migration rates on gel electrophoresis and proteins from clinical isolates frequently showed differences in relative migration rates. Because of this difficulty, we used immunological approaches for identification with antisera obtained by immunization with D1 and D2 purified from a standard laboratory strain, *P. aeruginosa* PAO1, in which the behavior of these proteins has been clearly established (10). With anti-D1 antiserum, we found that similar levels of protein D1 were present in all the strains probed, regardless of their imipenem susceptibility or resistance. Thus, it is unlikely that D1 is responsible for the facilitated diffusion of imipenem. This conclusion is consistent with the data showing that this protein produces a channel that facilitates the diffusion of glucose and its structural analogs (26), substrates that have no obvious structural resemblance to imipenem. We also showed earlier that purified D1 allowed only the nonspecific and therefore relatively slow penetration of imipenem (26). In contrast, probing the outer membrane protein gels with anti-D2 serum showed that this protein was absent in all the imipenem-resistant strains but present in all the susceptible strains (Fig. 4). In addition, all of the imipenem-resistant laboratory isolates newly obtained by us did not produce D2. Thus, the protein forming the "imipenem channel" was clearly identified as D2.

This conclusion is also consistent with the permeability properties of the purified D2 protein, examined by reconstitution into liposomes. Thus, the osmotic swelling behavior of such proteoliposomes showed first that the D2 protein allowed diffusion of various sugars and amino acids of diverse structure. Here the channel behaves as a nonspecific pore, since the diffusion rates were smoothly and inversely related to the molecular weights of the solutes (Fig. 5; 27). It is known that specific outer membrane channels such as LamB (16) and protein D1 (26) behave as nonspecific channels for solutes that do not bind to the substrate-binding sites, allowing the slow, nonspecific diffusion of various small solute molecules. In addition, however, the D2 channel allowed the diffusion of imipenem at a rate much higher than expected from its molecular weight (Fig. 5). This is the behavior expected for a specific ligand whose diffusion is facilitated by the channel, as was previously shown for glucose and xylose in the D1 channel (26) and for maltose and maltodextrins in the LamB channel (16). We emphasize also that the difference between the diffusion rate of a specific ligand, imipenem, and those of nonspecific solutes is greatly underestimated in this assay because of the following reason. The diffusion rate of a specific ligand reaches a maximum at a fairly low ligand concentration: in the case of imipenem, the half saturation occurs at about 0.1 mM (25). In contrast, the nonspecific diffusion rates for structurally unrelated molecules keep increasing in proportion to the solute concentration, as seen with diffusion through the porin channel (1). Because very high concentrations of solutes (about 10 mM or higher) must be used for the liposome swelling assay, in this assay the diffusion rates of unrelated molecules are much increased in relation to that of the specific ligand. We expect, therefore, that the D2 channel will behave essentially as a truly specific channel at low concentrations of solutes.

What structural features of imipenem make it bind to the presumptive binding site within the D2 channel? To answer this question, we studied the diffusion of several penems and carbapenems through the D2 channel. We found that L-1 β-lactamase of *P. maltophilia* was able to hydrolyze, in addition to imipenem, such carbapenems and penems as SM-7338, Sch 34343, Sch 33755, Sch 33440, and Sch 29482;

TABLE 1. Hydrolysis of penems and carbapenems by L-1 β-lactamase

Antibiotic	K_m (μM)	Relative V_{max}[a]
Imipenem	240	20
SM-7338	280	19
Sch 34343	239	13
Sch 33755	181	4.5
Sch 33440	131	1.8
Sch 29482	249	16

[a] Relative V_{max} values are expressed as the percentage of the V_{max} for penicillin G.

TABLE 2. Apparent permeability coefficients (see Materials and Methods) of outer membranes of D2-synthesizing strain 3-C and nonsynthesizing strain 3-B[a]

Substrate	Permeability coefficient (nm/s) for strain:	
	3-B	3-C
Imipenem	6	736
SM-7338	5.5	73
Sch 34343	18.5	22.7
Sch 33755	9	40
Sch 33440	7.5	24
Sch 29482	24.5	23.3

[a] The hydrolysis rates by leaked-out enzymes (measured with supernatants obtained by centrifugation of cell suspension) were between 10 and 60% of rates measured with intact cells. Rates with intact cells were corrected for these rates of hydrolysis by the leaked-out enzymes.

and this finding enabled us to measure the diffusion rates of these compounds into the periplasm by the determination of their hydrolysis rates in intact cells. Such experiments done with plasmid HN4-containing strains 3-C and 3-B, which did and did not produce D2, respectively, showed that imipenem, Sch 33755, and Sch 33440 penetrated faster through the D2-containing outer membrane (Fig. 4 and Table 2). In contrast, the diffusion rates of Sch 34343 and Sch 29482 across the outer membrane were not affected by the presence or absence of D2. Comparison of the structures of these two groups of compounds indicates that those utilizing the D2 channel for penetration all contain positively charged groups in the *R* moiety (Fig. 1). This is consistent with our recent observation (J. Trias and H. Nikaido, manuscript in preparation) that protein D2 facilitates the diffusion of basic amino acids, lysine, arginine, and histidine, and small peptides containing these amino acids. It seems likely that the structure involving the positively charged substituent *R* and the carboxyl group in C-2 mimics the structure of basic amino acids.

The fact that SM-7338, Sch 33755, and Sch 33440, together with imipenem, all utilize the D2 channel predicts some cross-resistance between these compounds. Indeed, in a multicenter study of susceptibility to SM-7338, it was found that *P. aeruginosa* strains that showed intermediate resistance to imipenem but remained susceptible to other conventional β-lactams were also resistant to SM-7338 (13).

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