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Imipenem- and Meropenem-Resistant Mutants of *Enterobacter cloacae* and *Proteus rettgeri* Lack Porins

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Carbapenems such as imipenem and meropenem are not rapidly hydrolyzed by commonly occurring β-lactamases. Nevertheless, it was possible, by mutagenesis and selection, to isolate mutant strains of *Enterobacter cloacae* and *Proteus rettgeri* that are highly resistant to meropenem and imipenem. Two alterations were noted in the *E. cloacae* mutants. First, the mutant strains appeared to be strongly derepressed in the production of β-lactamases, which reached a very high level when the strains were grown in the presence of imipenem. Second, these mutants were deficient in the production of nonspecific porins, as judged by the pattern of outer membrane proteins as well as by reconstitution assays of permeability. As with most porin-deficient mutants, their cultures were unstable, and their cultivation in the absence of carbapenems rapidly led to an overgrowth of porin-producing revertants. Analysis of the data suggests that the synergism between the lowered outer membrane permeability and the slow but significant hydrolysis of carbapenems by the overproduced enzymes can explain the resistance phenotypes quantitatively, although the possibility of alteration of the target cannot be excluded at present. With *P. rettgeri* mutants, there was no indication of further derepression of β-lactamase, but the enzyme hydrolyzed imipenem much more efficiently than the *E. cloacae* enzyme did. In addition, the major porin was absent in one mutant strain. These results suggest that a major factor for the carbapenem resistance of these enteric bacteria is the porin deficiency, and this conclusion forms a contrast to the situation in *Pseudomonas aeruginosa*, in which the most prevalent class of imipenem-resistant mutants appears to lack the specific channel protein D2 yet retains the major nonspecific porin F.

Expanded-spectrum cephalosporins such as cefotaxime and ceftazidime are active against gram-negative bacteria that produce inducible β-lactamases, such as *Enterobacter cloacae*, *Serratia marcescens*, and *Pseudomonas aeruginosa*, at least partly because they are poor inducers of this enzyme (32). The widespread use of these compounds, however, brought about the emergence of strains of these organisms that produce β-lactamase in a constitutive manner (22). Although expanded-spectrum cephalosporins appear, in conventional assays using high concentrations (0.1 to 10 mM) of substrates, stable against the chromosomally coded enzymes from these species, the agents are hydrolyzed, sometimes at rates comparable with those of the earlier cephalosporins such as cefazolin or cephaloridine under pharmacologically relevant, low concentrations (0.1 to 1 μM) (9, 29). Because of this situation, the constitutive strains show an extremely high level of resistance to expanded-spectrum cephalosporins. Imipenem and meropenem, which are carbapenems, are two of the very few β-lactam antibiotics that remain effective against these β-lactamase-overproducing bacteria, an important source of nosocomial infections.

In *P. aeruginosa*, however, the introduction of imipenem was rapidly followed by the emergence of mutants specifically resistant to imipenem (and meropenem) (3, 11, 19), which were found to lack a 43- to 45-kDa outer membrane protein (3, 19). We showed that this protein, D2, specifically facilitates the transmembrane diffusion of imipenem and meropenem and that the resistance is entirely due to this permeability defect (26, 27). In enteric bacteria, to our knowledge there is no report on high-level carbapenem-resistant mutants (but see Discussion). Because of the importance of the carbapenems as one of the "last line" drugs in infections caused by highly resistant bacteria, we wanted to see whether imipenem-resistant mutants could be isolated in these bacteria under laboratory conditions. Such mutants were indeed isolated and were shown (i) to produce β-lactamases at levels that enable them to hydrolyze carbapenems at slow but significant rates and (ii) to have a decreased outer membrane permeability apparently because of decreased levels of nonspecific porins.

MATERIALS AND METHODS

Bacterial strains. The parent strains of *E. cloacae* and *P. rettgeri* used in this study were clinical isolates, identified by the use of the API 20E system. The carbapenem-resistant mutants were derived by alternating growth in the presence of subinhibitory concentrations of the antibiotic with chemical mutagenesis. Typically the isolation required about 50 subculture steps and 3 to 4 steps of mutagenesis, which were carried out by exposing washed cells to 0.5 mg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml in 0.1 M phosphate buffer, pH 6.0, for 20 min at room temperature. The isolation of ceftazidime-resistant *E. cloacae* mutants, however, required only several passages in medium containing subinhibitory concentrations of the drug. The resistant strains were maintained as suspensions in 20% glycerol at −70°C. When they were to be maintained for extended periods as living cells, antibiotics were added at low concentrations (for example, 10 μg/ml for imipenem and meropenem) to prevent

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TABLE 1. Resistance patterns of the *E. cloacae* strains used

Strain	MIC (μg/ml)				
	Meropenem	Imipenem	Ceftazidime	Cefotaxime	Aztreonam
100	0.12	1	4	16	4
144 (selected with meropenem)	128	64	32	128	128
145 (selected with imipenem)	64	128	32	64	64
146[a] (reverted)	0.25	2	64	128	64
131 (selected with ceftazidime)	0.25	0.5	128	>128	128

[a] Strain 146 was isolated by four passages in antibiotic-free medium from an imipenem-resistant mutant, which was a sister clone of strain 145.

the overgrowth of susceptible revertants. Such revertants could be isolated easily after several passages of carbapenem-resistant strains in antibiotic-free medium.

β-Lactamase studies. For quantitation of β-lactamase activity, the hydrolysis rates of β-lactams (0.1 mM) were determined spectrophotometrically at 25°C, using sonic extracts of exponential-phase cells. One unit was defined as the activity resulting in the hydrolysis of 1 μmol of cephaloridine per min under these conditions.

For characterization of the properties of the enzyme, β-lactamases were prepared from the supernatants obtained by osmotic shock treatment of cells (10 μg of cefoxitin per ml or one-fourth the imipenem MIC was used as the inducer if the strain showed an inducible phenotype), as described earlier (18), except that the purification was carried out only up to the ammonium sulfate precipitation step. The hydrolysis of cephaloridine, cefotaxime, benzylpenicillin, and imipenem was monitored spectrophotometrically at 260, 260, 230, and 299 nm, respectively, by the use of Lambda 4 spectrophotometer (Perkin-Elmer). The K_m values for cephaloridine, determined as described earlier (18), were 0.36 and 0.55 mM, respectively, for the *E. cloacae* and *P. rettgeri* enzymes.

Isolation of outer membranes. Exponential-phase cells grown in L broth were used for the isolation of the outer membrane fraction by French press treatment, rate centrifugation, and sucrose-density centrifugation (2). In some experiments, the crude cell envelope fraction was extracted with 2% Triton X-100 for 20 min at 20°C. After centrifugation at 145,000 × *g* for 30 min, the pellet was extracted again with 2% Triton X-100 and centrifuged. This treatment solubilized most of the inner membrane material, leaving behind the outer membrane proteins as insoluble material (23).

Liposome swelling assay of permeability. The liposome swelling assay of permeability was carried out as described earlier (15). The outer membrane preparation containing 0.3 μg of total protein was added to 2.4 μmol of egg phosphatidylcholine and 0.2 μmol of dicetylphosphate (Sigma Chemical Co.) for reconstitution, and the dried mixture was resuspended in 15% (wt/vol) dextran T-40 (Pharmacia) in 5 mM Tris-HCl buffer, pH 8.0. In order to ensure that the liposomes did not contain excess porin, the assay was repeated for most strains by adding 0.03 μg of the outer membrane protein.

Permeability assay with vesicles containing β-lactamase. The permeability assay with vesicles containing β-lactamase was carried out in a manner similar to that described by Trias et al. (26), except that the *Pseudomonas saccharophilia* enzyme used in that work was substituted by *Bacillus cereus* class B β-lactamase (penicillinase type I [Sigma]). The reconstitution mixture contained 1.2 mg of enzyme, 0.4 mg of outer membrane proteins, and 44 mg of octyl-β-D-glucoside in a total volume of 0.5 ml, and this was dialyzed as previously described (26). The liposomes were separated from the β-lactamase molecules in the extravesicular space by gel filtration. Influx of the carbapenems into the vesicles, followed by their enzymatic hydrolysis, was measured by adding 0.1 mM substrate to the vesicle suspension. The results are reported as $P \times A$, i.e., the permeability coefficient multiplied by the surface area of the liposomes (26). The K_m values of the *B. cereus* enzyme were 0.2 and 0.5 mM for meropenem and imipenem, respectively.

Slab gel electrophoresis of outer membrane proteins. Slab gel electrophoresis of outer membrane proteins was carried out as described by Lugtenberg et al. (10). In order to increase the separation between the OmpA and OmpF porin, we also used gels containing 3 M urea (12). Diagonal gels (20) were also used to distinguish porins from the OmpA protein.

Other methods. Protein levels were determined by a modified Lowry method (21). MICs were determined by the agar dilution method by using Mueller-Hinton agar with an inoculum size of about 3×10^5 CFU per spot, applied with a multipoint inoculator apparatus. The results were read after 18 h of incubation at 37°C.

RESULTS

Resistance phenotypes of the *E. cloacae* mutants. As shown in Table 1, the *E. cloacae* mutants obtained by successive subculturing (and mutagenesis) in carbapenems, strains 144 and 145, have developed a high level of resistance to these compounds, as well as to other β-lactams. Although the isolation of these mutants required many steps of subculturing and mutagenesis, one of the major genetic alterations required for resistance must involve only a few changes at most, because subculturing for a short period selected revertants, such as strain 146, which had essentially regained the susceptibility of the parent strain for the two carbapenems. It should be noted that the mutant selected in the presence of ceftazidime, strain 131, was as susceptible to the carbapenems as was the parent strain, and thus its phenotype was entirely different from that of the carbapenem-resistant mutants.

Properties of the *E. cloacae* β-lactamase. The properties of the β-lactamase produced by the *E. cloacae* strains were typical of the class C, chromosomally coded enzyme of this species. Thus, the V_{max} value for cephaloridine was at least 50-fold higher than that for benzylpenicillin, and the enzyme was not strongly inhibited by clavulanic acid.

Imipenem is reported to act, for class C enzymes, as a poor substrate that nevertheless follows Michaelis-Menten kinetics (31), although it is a suicide inhibitor for the *Proteus vulgaris* enzyme, which probably belongs to class A (5, 31). Our *E. cloacae* enzyme indeed hydrolyzed imipenem extremely slowly, with a V_{max} value corresponding to 0.00005% of that of cephaloridine, and there was no burst of

TABLE 2. β-Lactamase levels in *E. cloacae* strains

Strain	Sp act[a] (U/mg of protein)	
	Uninduced	Induced[b]
100	2.6	6.0
144	5.7	56.3
145	7.0	52.4
146	6.5	35.2
131	33.1	39.5

[a] Activity was assayed by using 0.1 mM cephaloridine at 25°C.
[b] Cells were grown for more than eight generations in the presence of one-fourth the imipenem MIC.

initial hydrolysis such as that reported for *P. vulgaris* enzymes (5, 31). The V_{max} for cefotaxime was nearly an order of magnitude higher than that for imipenem. The affinity of the enzyme toward imipenem was extremely high, with a K_i of 0.03 μM (measured with cephaloridine as the substrate). Its affinity toward cefotaxime was similar (K_m, 0.06 μM), but that toward cephaloridine was very much lower (K_m, 360 μM). The enzyme isolated from the imipenem-resistant mutant 145 did not show an increased rate of hydrolysis of imipenem.

β-Lactamase levels in the *E. cloacae* mutants. We tested whether the mutants were derepressed for the production of β-lactamase. The results (Table 2) showed that there was indeed a significant derepression in the carbapenem-resistant mutants. The uninduced level in parent strain 100 was already quite high in comparison with some other strains (for example, see reference 17), but it increased more than twofold in carbapenem-resistant mutants 144 and 145, as well as in the revertant 146. More importantly, growth in the presence of one-fourth the imipenem MIC increased the level of β-lactamase to very high levels (>50 U/mg of protein) in the carbapenem-resistant mutants. This pattern, the extremely high induced level and the only moderately increased uninduced level, was quite different from the levels found in the ceftazidime-resistant strain 131, which showed a classical derepressed phenotype of high enzyme activities in both uninduced and induced cells (Table 2).

Thus, the cefotaxime and ceftazidime resistance of strain 131 can probably be explained by the overproduction of the enzyme, which hydrolyzes the expanded-spectrum cephalosporins at rates comparable with the rates of hydrolysis of cefazolin or cephaloridine at pharmacologically relevant, low concentrations (i.e., 0.1 to 1 μM) (29). However, since strain 131 remains susceptible to carbapenems (Table 1), derepression of the enzyme cannot be the sole explanation of carbapenem resistance in strains 144 and 145.

Outer membrane permeability in *E. cloacae* strains. We examined the permeability of the outer membranes in carbapenem-resistant strains. The crude outer membranes were used as the source of porins in the reconstitution of liposomes, and the permeability of the liposome membranes to small solutes, L-arabinose and glycine, was measured by the osmotic swelling of the liposomes in solutions of these compounds. The results (Table 3) show clearly that the outer membranes of resistant strains 144 and 145 have lost most of the permeability. It should be noted that in liposome swelling assays, swelling rates lower than 5 to 10% of the maximal swelling rates are difficult to determine with precision, unless liposomes made with larger amounts of porins are also used. The outer membrane of the revertant 146 appears to have regained fully the permeability of parent strain 100.

TABLE 3. Liposome swelling assay of permeability

Strain	Description	Relative permeability to:	
		Arabinose	Glycine
100	Parent	100	100
144	Meropenem resistant	<5	<5
145	Imipenem resistant	<5	ND[a]
146	Revertant	86	95

[a] ND, not determined.

Porin patterns in *E. cloacae* strains. In order to characterize the protein alterations responsible for the decreased permeability in carbapenem-resistant mutants, the outer membrane protein pattern was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In the parent strain, several protein bands were seen in the apparent molecular weight range of about 35,000, i.e., in the range where enterobacterial porins were expected to travel. The bands were identified by (i) comparison with the migration behavior of *E. cloacae* porins recently purified and characterized (8a) and (ii) regulatory response to the osmolarity of the medium, temperature, etc.

The fastest migrating band contained two proteins, a classical trimeric porin protein and OmpA, as shown by first running PAGE without heating the sample, followed by the complete heat denaturation of the sample and running the gel again in the second dimension (20). It was possible to separate these proteins by two methods. The first method, the use of gels containing 3 M urea (12), allowed us to separate the porin (lower arrowhead in the lane showing the proteins from strain 100 in Fig. 1A) from OmpA, which appeared as the most abundant band below the porin (seen in the same lane in Fig. 1A). The second method involved the extraction of the outer membrane with SDS. In *Escherichia coli*, this procedure extracts OmpA, leaving behind most of the porins (14). Similar results have been obtained in other

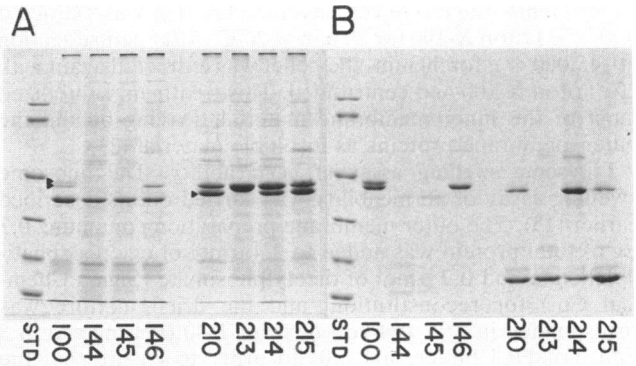

FIG. 1. Outer membrane proteins from various strains separated by SDS-PAGE. The gels contained 3 M urea so that porin F of *E. cloacae* could be resolved from the OmpA protein (see text). Samples of whole outer membranes (A) and the insoluble residues left after SDS extraction of OmpA and other proteins (B) were analyzed. The strains are listed at the bottom. STD lanes contain molecular mass standards and correspond to, from top to bottom, phosphorylase *b* (97,400 Da), bovine serum albumin (66,200 Da), ovalbumin (43,000 Da), carbonic anhydrase (31,000 Da), soybean trypsin inhibitor (21,500 Da), and lysozyme (14,400 Da). Porins of *E. cloacae* 100 are shown with arrowheads (the top arrowhead indicates porin D, while the lower one indicates porin F); the porin of *P. rettgeri* 210 is also shown with an arrowhead.

TABLE 4. Antibiotic susceptibility of *P. rettgeri* mutants

Strain	MIC (μg/ml)				
	Meropenem	Imipenem	Ceftazidime	Cefotaxime	Aztreonam
210	0.25	4	0.25	0.12	0.06
213 (selected with meropenem)	>128	32	1	1	0.5
214 (selected with imipenem)	4	32	0.12	0.25	0.06
215 (reverted)[a]	0.5	8	0.25	0.12	0.06

[a] Strain 215 was a carbapenem-susceptible revertant isolated from an old culture of strain 214.

strains of *E. cloacae* (8a). Also with strain 100, the OmpA band disappeared in the SDS-extracted sample (compare the lanes showing proteins from strain 100 in Fig. 1A and B). The faster-migrating porin resembled *E. coli* OmpF protein in its relatively large pore size and in that its synthesis became significantly repressed when 2% NaCl was added to the LB broth (8a). Because of this behavior, the porin is called "F".

The band with the intermediate mobility was also a trimeric porin, as shown by the diagonal polyacrylamide electrophoresis (results not shown). The synthesis of this porin was nearly completely repressed when the cells were grown in nutrient broth containing 0.5% glucose. Because this resembles the catabolite repressible phenotype of *Salmonella typhimurium* OmpD porin, this porin is called "D" (the upper band marked with an arrowhead in the lane for strain 100 in Fig. 1A).

In other *E. cloacae* strains, there was another porin which produced the narrowest channel and migrated slightly more slowly than the 43,000-Da marker protein (8a). This porin produces a narrow channel, resembling the OmpC porin channel of *E. coli*, and is therefore called "C". It was also somewhat similar to OmpC in that its synthesis was increased by increasing the osmolarity of the medium, for example, by adding 2% NaCl to nutrient broth (8a). In strain 100, this porin was produced at very low levels; it is not clearly visible in Fig. 1.

As seen in Fig. 1, the carbapenem-resistant mutants 144 and 145 were deficient in all three porins, whereas the susceptible revertant 146 produced the widest channel porin, F, at a high level. The ceftazidime-resistant mutant 131 also produced normal levels of porins (results not shown).

Resistant mutants of *P. rettgeri*. Carbapenem-resistant mutants were isolated from susceptible parent strain 210 by a similar procedure. As shown in Table 4, these mutants, strains 213 and 214, showed an increased resistance to carbapenems with no or only moderate levels of resistance to other β-lactams.

The β-lactamase from *P. rettgeri* was similar to the *E. cloacae* enzyme in showing higher V_{max} values for cephalosporins than for penicillins. It hydrolyzed imipenem slowly, but the relative rate of imipenem hydrolysis was much higher than with the *E. cloacae* enzyme, the V_{max} for imipenem being about 0.01% of that for cephaloridine. This relationship was not altered in imipenem-resistant mutant 214.

The uninduced cells of all strains showed a rather high level of β-lactamase activity (about 1 U/mg of protein), and induction with one-fourth the imipenem MIC increased the level to 5.2, 4.8, and 4.3 U/mg in strain 210, 213, and 214, respectively. Thus, there was no evidence of further derepression of β-lactamase in mutants 213 and 214.

When the permeability of the outer membrane was assessed by reconstituting fragments of the outer membranes into liposomes, the nonspecific permeability, as determined by the swelling rates of liposomes in L-arabinose and glycine, was clearly decreased in mutant 213, but the permeability in mutant 214 appeared to be only slightly decreased (Table 5).

The patterns of the outer membrane proteins were also altered in these mutants (Fig. 1). The diagonal gel (results not shown) and the comparison of SDS-extractable and nonextractable proteins (compare Fig. 1A and B) showed that, of the two major protein bands in the 30,000- to 40,000-Da range, the lower one (marked with arrowhead) is the porin and the upper one is equivalent to OmpA. These results confirm the study of Mitsuyama and coworkers (13), who showed that the faster migrating of the two major outer membrane proteins of *P. rettgeri* corresponds to porin. In strain 213, the major porin is apparently missing, but the porin level in strain 214 appears normal. (In another experiment [results not shown], the porin level in strain 214 was about one-third the level found in strain 210.)

If large portions of carbapenems diffuse through specific, saturable channels, then liposome swelling assays, which utilize high concentrations of test solutes (typically 20 to 40 mM), may produce misleading data (26). Because of this possibility, we reconstituted outer membrane fragments into liposomes so that they contained *Bacillus cereus* β-lactamase and then measured the meropenem influx into the liposomes by using a low concentration (0.1 mM) of the antibiotic, as described in Materials and Methods. The relative rates of penetration of meropenem were 100, 9, and 41, in vesicles reconstituted with the outer membranes of strain 210, 213, and 214, respectively. Thus, mutant 213, which lacked the major porin, was very poorly permeable to meropenem, whereas mutant 214, which still contained the major porin, showed only a moderate decrease in its permeability to this compound.

TABLE 5. Liposome swelling assay of permeability with *P. rettgeri* strains

Strain	Description	Relative permeability[a] to:	
		Arabinose	Glycine
210	Parent	100	100
213	Meropenem resistant	3, 12, 18	4, 8, 9
214	Imipenem resistant	27, 45	49, 76
215	Revertant	55	60, 80

[a] When more than one experiment was performed, values obtained in individual experiments are shown so as to indicate the degree of variability of data.

DISCUSSION

One of us has earlier shown (18) that the susceptibility of gram-negative bacteria toward β-lactam antibiotics can be

quantitatively predicted by a parameter called target access index (TAI) as follows:

$$\text{MIC} = c_{\text{inh}}(\text{TAI}^{-1} + 1) \quad (1)$$

where c_{inh} denotes the minimal drug concentration that inhibits the most sensitive of the essential penicillin-binding proteins (PBPs). TAI in turn is defined by:

$$\text{TAI} = PA/[V_{\text{max}}/(K_m + c_{\text{inh}})] \quad (2)$$

where P and A represent the permeability coefficient and the area of the outer membrane per unit weight of cells, respectively, and V_{max} and K_m are the usual kinetic constants of the periplasmic β-lactamase.

We can estimate the TAI for imipenem in the induced, parent *E. cloacae* 100. Because the *E. cloacae* outer membrane has a nonspecific permeability about equal to or only slightly lower than that of the *E. coli* outer membrane (8a) and because imipenem penetrates through *E. coli* OmpF porin only 30% faster than cephaloridine (33), we can assume that the permeability coefficient for imipenem in *E. cloacae* will not be far from the permeability coefficient for cephaloridine in *E. coli*, 8×10^{-5} cm s^{-1} (8). Induced cells of *E. cloacae* 100 contained β-lactamase with a V_{max} for cephaloridine of 28 μmol min^{-1} mg of $protein^{-1}$, or about 56 μmol min^{-1} mg of $cells^{-1}$ (Table 2). Thus the V_{max} for imipenem should be 0.00005% of this value, or 0.024 nmol min^{-1} mg of $cells^{-1}$. The c_{inh} in equation 2 can be approximated by the drug concentration that inhibits the binding of benzylpenicillin to the essential PBPs by 50% (I_{50}) (18). For imipenem, the I_{50} is reported as 0.02 μg/ml, or about 0.07 μM (24). Using the K_m of 0.03 μM and the A value of 132 cm^2 mg^{-1} (18), we get a TAI of about 2.3, which suggests that the MIC of imipenem will be only 40% higher than the I_{50}.

One mechanism used for development of β-lactam resistance is the overproduction of β-lactamase. Imipenem is indeed a good inducer of *E. cloacae* β-lactamase, and we found that in induced cells of the carbapenem-resistant mutants 144 and 145, the activity, determined by using 0.1 mM cephaloridine, reaches 55 μmol min^{-1} per mg of protein (Table 2), or about 110 μmol min^{-1} per mg of cells, an activity equivalent to the V_{max} of 506 μmol min^{-1} per mg of cells. The V_{max} for imipenem then will be 0.25 nmol min^{-1} per mg of cells, and equation 2 shows that the TAI in these β-lactamase-overproducing cells is about 0.3. Equation 1 predicts that even this huge overproduction of the β-lactamase can increase the imipenem MIC only marginally (less than threefold). In contrast, the kinetic constants of the *E. cloacae* enzyme for cefotaxime (see Results) and the outer membrane permeability for this compound (determined in *E. coli* [18]) show that the TAI decreases to 0.0008 in the overproducing strain, which explains the high resistance of strain 131 to this compound.

Clearly, therefore, further increases of the imipenem MIC requires either lowering of the outer membrane permeability, the value of P in equation 2, or increasing c_{inh} as a result of alterations of the target, the PBPs, or both. Because our mutants were strongly derepressed for the β-lactamase, the PBP assay was not possible. In any case, our mutants were shown to have lost the porins and to have a much lowered level of outer membrane permeability (see Results). We next consider whether the phenotypes of our mutants can be explained by the loss of porins superimposed on the derepression of the β-lactamase. In order to increase MIC to the level observed (i.e., 64 μg/ml [Table 1], or about 200 μM), we find from equation 1 that $200 = (\text{TAI}^{-1} + 1) \times 0.25$, which shows that the TAI value should be as low as 0.001. The only practically possible way to lower TAI further from the 0.3 value to this level is to decrease the outer membrane permeability to about 0.3% of the wild-type level. Most probably this is what happened in strains 144 and 145. We have earlier shown that porin-deficient mutants of *E. coli* producing about 0.1% of the porin molecules found in the wild-type cells (and thus with a 1,000-fold lower permeability of the outer membrane) can survive with apparently normal doubling times, at least in commonly used laboratory media (2).

These results may seem unexpected because other researchers previously reported that the MIC of imipenem either did not change (1, 4, 6, 30) or increased only slightly (25) in porin-deficient mutants of enteric bacteria. However, in contrast to our strains, all of the strains used in these earlier studies produced β-lactamase at much lower levels: the activities measured by using 0.1 mM cephaloridine (a condition identical to what we used) or nitrocefin were about 0.001 and 0.003 U/mg of protein, as reported by Hiraoka et al. (6) and Werner et al. (30), respectively. Bakken et al. (1) used *E. coli* with its extremely low and noninducible β-lactamase (although the strain produced a TEM-type enzyme, carbapenems are not readily hydrolyzed by this enzyme). Gutmann et al. (4) used *Salmonella paratyphi* A, which is generally thought not to produce significant levels of β-lactamase. Even in the study using *E. cloacae* with an inducible enzyme (25), the fully induced level was only 1 U/mg of protein, i.e., a level 50-fold lower than in our strains 144 and 145. Calculations show that in the presence of such low levels of the enzyme, even a 99.9% loss of porin will decrease TAI only to about 2, which is predicted to produce undetectable or barely detectable increase in MIC according to equation 1, which is in full agreement with the data. These considerations show once again that the synergy between the outer membrane barrier and the β-lactamase barrier is essential in the production of significant resistance (16) and that both the extremely high levels of the enzyme production and the lowering of the outer membrane permeability were needed to produce the high-level resistance found in strains 144 and 145.

This double requirement for the resistance phenotype also explains the unusual feature of the isolation protocol. Attempts to isolate high-level carbapenem-resistant mutants from wild-type *E. cloacae* in a single step have so far been unsuccessful in our hands and presumably also in other laboratories, which most likely reflects the need to overproduce the β-lactamase to an exceptionally high level, because the enzyme hydrolyzes imipenem only very slowly. The β-lactamase levels (Table 2) also explain the resistance phenotype of the revertant, strain 146. This strain has regained the OmpF-like porin yet still retained its resistance toward ceftazidime, cefotaxime, and azthreonam (Table 1). This is expected for an overproducer of a class C enzyme (18), as we see here in strain 131.

The lowered outer membrane permeability and the overproduction of the β-lactamase can thus explain the imipenem resistance of our *E. cloacae* mutants. However, we cannot prove at present that these are the only factors contributing to the resistance, because the PBP assay was not possible. Although the rapid emergence of porin-containing revertants is consistent with our interpretation, there are known cases in which the levels of porin are altered by pleiotropic mutations (for a review, see reference 16), and thus a simultaneous alteration of porin level and PBPs remains a possibility.

The β-lactamase production patterns of the *P. rettgeri*

mutants were not detectably altered. However, the *P. rettgeri* enzyme could hydrolyze imipenem much more efficiently than the *E. cloacae* enzyme could. Thus, although the enzyme activity in induced cells, measured by using cephaloridine, was about 10-fold lower than in the *E. cloacae* mutants, the specific activity of *P. rettgeri* extracts for imipenem hydrolysis was significantly higher than those of *E. cloacae* carbapenem-resistant strains 144 and 145. This leads us to expect that less-drastic decreases in outer membrane permeability may be able to produce significant carbapenem resistance in *P. rettgeri* strains.

Strain 213 was indeed deficient in a major outer membrane protein with a mobility corresponding to about 37,000, which was earlier identified as the major nonspecific porin of this organism (13). This strain showed a high-level resistance to both meropenem and imipenem (Table 4). Its outer membrane showed very low permeability in the liposome swelling assay (Table 5), in which diffusion rates less than 10% of that of the reference compound (arabinose or glycine) were difficult to determine with precision, as described above. Similar low permeability was seen when assays were carried out with liposomes containing the *B. cereus* β-lactamase (see Results). These results suggest that a major factor in the carbapenem resistance of strain 213 is the lowered outer membrane permeability, most probably due to the loss of the major porin.

The phenotype of strain 214 is more difficult to interpret. This strain appeared to produce the porin at a slightly decreased or even nearly normal level, and its outer membrane permeability toward small solutes was decreased only slightly (Table 5). A similar modest decrease in permeability was found with β-lactamase-containing liposomes (see Results). Possibly this modest decrease in permeability contributes to the low-level resistance toward carbapenems, especially toward meropenem (Table 4). However, it is also possible that other factors, such as potential alterations of the target, make an even more important contribution. We cannot rule out also the possibility that its resistance was caused by a loss of a minor protein forming a specific channel, analogous to the D2 protein of *P. aeruginosa* (26–28). However, if this were the case, we would have seen large differences in carbapenem permeability when the test was carried out with low concentrations of the drug, because the contribution of saturable, specific channels becomes larger at lower solute concentrations (26). Since such a test again showed only a small difference between parent strain 210 and mutant strain 214 (see Results), we conclude that this last explanation is unlikely.

Our results showed that enteric bacteria can develop carbapenem resistance by decreasing the content of their nonspecific, general-purpose porins. We emphasize again that the decreased permeability alone cannot produce a significant level of resistance (see above), which also requires significant rates of enzymatic hydrolysis. In other words, the extent of porin loss that is required for the generation of resistance depends on the rate of hydrolysis of carbapenems by the periplasmic β-lactamases. The enzyme produced by our *E. cloacae* strains hydrolyzed carbapenems extremely slowly, and therefore its overproduction at an exceptionally high level and a drastic decrease in outer membrane permeability were necessary for resistance, as was shown by the quantitative analysis using TAI, presented above. Such a situation is obviously unfavorable for the survival of mutant bacteria, and in the absence of carbapenems, there was a rapid overgrowth of revertants producing normal levels of porins (see Materials and Methods). This is a situation commonly seen in the cultures of mutants with severe defects in outer membrane permeability (2).

Very recently, a clinical isolate of *E. cloacae* with imipenem resistance was reported; this strain again produces high levels of β-lactamase, and its porin content is strongly decreased (7a). There are also reports in the literature of *Enterobacter* mutants with lower-level resistance to imipenem, e.g., a MIC of 5 μg/ml (7). These mutants also produce decreased levels of what appear to be porins, although the β-lactamase levels are only slightly elevated (7).

Finally, we emphasize that the loss of general-purpose porins in enteric bacteria, described in this report, forms a contrast to the common mechanism of imipenem resistance in *P. aeruginosa*, in which protein D2, forming specific channels for basic amino acids and some peptides (28), as well as imipenem and meropenem (27), is lost without any changes in the general-purpose porin, protein F. In the latter organism, the permeability of the nonspecific porin is extremely low, and many of the nutrients apparently traverse the outer membrane via specific channels. Some antibiotics showing strong activity against this organism also seem to utilize these specific pathways. In contrast, most antibiotics, including carbapenems, appear to enter enteric bacteria mostly via porin channels that show very high intrinsic permeability.

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