ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1991, p. 1093–1098
0066-4804/91/061093-06$02.00/0
Copyright © 1991, American Society for Microbiology
Vol. 35, No. 6

Association of Two Resistance Mechanisms in a Clinical Isolate of *Enterobacter cloacae* with High-Level Resistance to Imipenem

E. H. LEE,[1] M. H. NICOLAS,[2] M. D. KITZIS,[3] G. PIALOUX,[4] E. COLLATZ,[1] AND L. GUTMANN[1]*

Laboratoire de Microbiologie Médicale, Université Paris VI, 15, rue de l'Ecole de Médecine, 75270 Paris Cedex 06,[1] Laboratoire de Bactériologie, CHU Pitié-Salpétrière, Université Paris VI, 83, Boulevard de l'Hôpital, 75013 Paris,[2] Laboratoire de Microbiologie Médicale, Hôpital Saint-Joseph, 7, rue Pierre Larousse, 75674 Paris Cedex 14,[3] and Hôpital Pasteur, 211, rue de Vaugirard, 75015 Paris,[4] France

Received 17 October 1990/Accepted 21 March 1991

Carbapenem resistance was studied in a clinical isolate of *Enterobacter cloacae*, strain 201 (MIC of imipenem and meropenem, 16 μg/ml). This strain was analyzed comparatively with the carbapenem-susceptible parent strain 200, an equally susceptible revertant, 201-Rev, and in vitro-selected mutants with different levels of carbapenem resistance. All strains produced similarly high amounts of the same cephalosporinase (pI_{app} = 8.8). Strain 201 apparently lacked two major outer membrane proteins of ca. 37 and 38 kDa, while 201-Rev produced only the 37-kDa protein. The permeability coefficient, determined with cephaloridine, was reduced up to ninefold in the resistant strains which also showed a substantial reduction in the uptake of [^{14}C]meropenem. The introduction of the plasmid-borne *ampD* gene (whose product decreases the expression of *ampC*) resulted in almost complete cessation of cephalosporinase production in all strains and a substantial decrease in the MICs of the carbapenems which remained, however, 8- to 16-fold higher than those determined for the susceptible strains containing the *ampD* gene. This "residual" resistance was attributed to reduced outer membrane permeability. The contribution of cephalosporinase production was verified in a reverse experiment, in which the introduction of *ampC* into a low-level cephalosporinase producer resulted in a fourfold increase in the carbapenem MICs. From these results, we infer that reduced outer membrane permeability and high-level cephalosporinase production can operate in conjunction in clinical isolates of *E. cloacae* to confer imipenem resistance.

Acquired or uncommon resistance to imipenem has been described in only a few bacterial species. In *Bacteroides fragilis* (4), *Bacteroides distasonis* (13) *Aeromonas* spp. (14), and *Serratia marcescens* (27), resistance to imipenem is associated with an imipenem-hydrolyzing β-lactamase. In *Pseudomonas aeruginosa*, imipenem resistance is associated with a specific decrease in imipenem permeability (1, 16, 22, 24), and recently it has also been shown to be associated with a plasmid-mediated β-lactamase (26). For members of the family *Enterobacteriaceae*, non-β-lactamase-dependent resistance to imipenem has been reported only in *Enterobacter aerogenes*, in which it was linked with a decrease in the quantity of porins (12). In this study, we attempted to explain the mechanism(s) responsible for an unusually high level of carbapenem resistance observed in mutants of *E. cloacae* selected in vivo and in vitro.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Strains *E. cloacae* 200 and a putative derivative 201 were isolated, respectively, before and during moxalactam therapy of an intraabdominal sepsis. Fragment analysis of total DNA from strains 200 and 201 after digestion with three restriction endonucleases revealed no differences, and both strains contained a small, apparently cryptic plasmid. Other resistant mutants of *E. cloacae* 200, *E. cloacae* 92227, or *E. cloacae* P99 were selected in vitro on moxalactam (2 to 64 μg/ml) or cefotaxime (16 μg/ml).

All strains were grown with aeration at 37°C in Mueller-Hinton broth (Diagnostics Pasteur). The MICs of antibiotics were determined on Mueller-Hinton agar, using a multiple inoculum replicator and ca. 10^4 CFU per spot. Transformation was carried out as described previously (17).

Antibiotics and reagents. The following antibiotics were kindly provided by the indicated companies: chloramphenicol and cefotaxime, Roussel-Uclaf, (Paris, France); imipenem, Merck Sharp and Dohme-Chibret (Paris, France); meropenem (SM-7338), ICI Pharmaceuticals (Alderley Park, United Kingdom); moxalactam, Eli Lilly & Co. (Indianapolis, Ind.); nalidixic acid, Winthrop (Clichy, France); and tetracycline, Diamant (Paris, France). [^{14}C]meropenem (87.4 μCi/mg) was generously provided by ICI Pharmaceuticals.

Characterization of outer membrane proteins, lipopolysaccharide, and penicillin-binding proteins. Outer membranes were prepared by using 0.3% (wt/vol) *N*-laurylsarcosine (Sigma Chemical Co., St. Louis, Mo.) as described previously (8).

Outer membrane proteins were separated on sodium dodecyl sulfate-containing 12% polyacrylamide gels containing 20% urea. Lipopolysaccharide was extracted from cells and analyzed by polyacrylamide gel electrophoresis by the method described by Hitchcock and Brown (9).

Penicillin-binding proteins were assayed in isolated membranes, as described previously (23), with [^{14}C]meropenem.

β-Lactamase assays. Crude extracts of β-lactamase were obtained from the different strains after centrifugation (30,000 × *g*, 45 min) of sonified cells. Analytical isoelectric focusing was performed by the method of Matthew et al. (19). β-Lactamase activity was determined spectrophotometrically in sodium phosphate buffer (pH 7, 10 mM) at 30°C with a double-beam spectrophotometer (model 550S; Perkin-Elmer Corp.). One unit of β-lactamase was defined as the

* Corresponding author.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype or genotype[a]	Reference[b]
E. cloacae		
200	Cpm^s clinical isolate	
201	Cpm^r mutant of strain 200 selected in vivo	
200-MD1	Cpm^r mutant of strain 200 selected in vitro on moxalactam	
200-MD2	Cpm^r mutant of strain 200 selected in vitro on moxalactam	
201-Rev	Spontaneous revertant from strain 201	
P99	High-level cephalosporinase producer	7
P99$^-$	Mutant of strain P99 producing low levels of noninducible cephalosporinase	7
P99-M	Mutant of strain P99 selected in vitro on moxalactam	
92227	Cpm^s clinical isolate susceptible to moxalactam and cefotaxime	
92227-C	Mutant of strain 92227 selected on cefotaxime	
92227-M	Mutant of strain 92227 selected on moxalactam	
92227-CM	Mutant of strain 92227-C selected on moxalactam	
Plasmid		
pNH5	*HpaI* fragment containing *ampD* from *Escherichia coli* in pBS18	11
pEC1E	*HincII* fragment containing *ampC* from *E. cloacae* in pACYC184	10

[a] Cpm^s, carbapenem susceptible; Cpm^r, carbapenem resistant.
[b] Strains without a reference number are from this study.

amount of enzyme that hydrolyzed 1 μmol of cephaloridine per min per mg of protein at 30°C. The wavelengths (in nanometers) of maximal absorption were as follows: cephaloridine, 260; imipenem, 297; meropenem, 297; and nitrocefin, 487. K_i values were determined with cephaloridine as the substrate, and K_m and K_i values were calculated by computerized linear regression analysis of Woolf-Augustinsson-Hofstee plots (v versus v/s).

Permeability assays and uptake of [^{14}C]meropenem. Outer membrane permeability coefficients of cephaloridine for different strains were determined by the procedure described by Nikaido et al. (21). These coefficients were calculated after measuring the rate of hydrolysis of cephaloridine by intact cells (in a cuvette with an optical path length of 2 mm) as well as sonified cells from the same culture and by using the K_m and V_{max} values of the enzyme for cephaloridine. The rate of hydrolysis measured for intact cells was corrected for β-lactamase leakage by substraction of the rate of hydrolysis measured for the supernatant of the sedimented cells, which was less than 25% of the total.

Uptake of meropenem into intact cells was measured as follows. Cells grown to an optical density at 650 nm of 0.45 in Mueller-Hinton broth were centrifuged at 7,000 × g and 15°C for 10 min and resuspended in sodium phosphate buffer (pH 7) containing 5 mM $MgCl_2$. Fifty microliters of labeled meropenem (final concentration, 0.5 to 5 μM) was added to a 450-μl cell suspension and incubated at 25°C for 30 s, after which 4 ml of 7% ice-cold trichloroacetic acid was added. After 10 min on ice, samples were filtered through GF/F filters (Whatman, Clifton, N.J.), rinsed twice with 5 ml of 7% ice-cold trichloroacetic acid, and dried. The radioactivity was determined by liquid scintillation counting with Econofluor (New England Nuclear).

RESULTS

Antibiotic susceptibility and β-lactamase contents of the *E. cloacae* strains. When compared with the wild-type strain *E. cloacae* 200, the resistant strain *E. cloacae* 201, which was selected under moxalactam therapy, showed 32- to 128-fold increased MICs of the carbapenems imipenem and meropenem associated with a 64-fold increased MIC of moxalactam (Table 1). Moxalactam-resistant mutants derived from *E. cloacae* 200 were easily selected in vitro (ca. 2 × 10^{-8}) on moxalactam (64 μg/ml). Mutants 200-MD1 and 200-MD2 were chosen because they showed, respectively, an intermediate level of resistance and a level of resistance similar to that of strain 201 (Table 2). The resistant mutants selected either in vivo or in vitro showed no changes in the MICs of chloramphenicol, nalidixic acid, or trimethoprim, while only a twofold increase in the MIC of ticarcillin was observed (data not shown). A spontaneous revertant from *E. clocacae* 201, *E. clocacae* 201-Rev, was selected in vitro and displayed MICs of β-lactams very similar to those for *E. cloacae* 200 (Table 1).

Examination of the β-lactamase content of these strains revealed only one β-lactamase with a pI of ca. 8.8, which is in agreement with the pI of the cephalosporinase produced in this species (2). This β-lactamase was produced in high and very similar amounts by all strains derived from *E. cloacae* 200 (Table 1).

In an attempt to obtain similar resistance phenotypes with other strains of *E. cloacae*, mutants from either *E. cloacae* 92227, a clinical isolate producing low amounts of cephalosporinase, or *E. cloacae* P99 (7), which produces high amounts of cephalosporinase were selected in vitro on moxalactam. Resistant mutants could be selected from both strains (strains 92227-M and P99-M in Table 2), and they had specific β-lactamase activities similar to those of the parental strains. They showed high levels of resistance to moxalactam and 4- to 32-fold increased MICs of carbapenems. In contrast, when resistant mutants of *E. cloacae* 92227 were selected on cefotaxime, only high-level cephalosporinase producers were selected. For these strains the MICs of cefotaxime were high (512 μg/ml) and the MICs of moxalactam were moderate, but there was no increase in the MICs of carbapenems (strain 92227-C in Table 2). Plating of 92227-C on moxalactam (64 μg/ml) yielded mutants, such as 92227-CM, that were highly resistant to moxalactam and that had resistances to the carbapenems similar to those of 92227-M and P99-M (Table 1).

Role of the β-lactamases. Since *E. cloacae* 200 and its derivatives produced high amounts of the same cephalosporinase, we assessed the possible role of the β-lactamase in the resistance to imipenem. We first examined whether qualitative changes occurred in the enzymes of the resistant

TABLE 2. MICs of β-lactam antibiotics for different strains of *E. cloacae* before or after introduction of the plasmid-borne *ampD* gene

Strain	MIC (μg/ml) in the absence of *ampD*				β-Lactamase activity (mU/mg)[a]	MIC (μg/ml) in the presence of *ampD*				β-Lactamase activity (mU/mg)
	Imipenem	Meropenem	Moxalactam	Cefotaxime		Imipenem	Meropenem	Moxalactam	Cefotaxime	
200	0.5	0.125	8	512	9,462	0.25	0.03	1	2	19
201	16	16	512	>512	4,635	4	0.5	8	4	19
200-MD1	2	2	512	>512	7,150	1	0.125	4	2	18
200-MD2	16	16	512	>512	9,038	4	0.5	8	4	27
201-Rev	0.5	0.125	4	512	5,324	0.25	0.03	1	2	23
92227	0.5	0.125	0.125	0.5	31	0.125	0.03	0.06	0.125	19
92227-C	0.5	0.25	16	512	21,932	0.125	0.03	0.25	1	16
92227-M	2	4	256	16	28	0.25	0.125	4	0.5	10
92227-CM	2	8	512	>512	36,243	0.25	0.25	4	2	31
P99	0.5	0.125	16	256	10,839	0.12	0.03	1	1	18
P99-M	2	2	512	512	12,010	0.5	0.25	16	4	27
P99$^-$	0.06	0.015	0.25	0.25	6	ND[b]	ND	ND	ND	
P99$^-$ (*ampC*)[c]	0.25	0.06	4	64	3,780	ND	ND	ND	ND	

[a] Values are means from two or three determinations. Variations were within ±10%.
[b] ND, not determined.
[c] Plasmid pEC1E containing the *ampC* gene was introduced into *E. cloacae* P99$^-$.

mutants which could explain the decrease in their susceptibilities to imipenem. The rates of hydrolysis of carbapenems by the enzymes of the different strains were similar and very low compared with that of nitrocefin (Table 3). Similarly, the K_m for nitrocefin as well as the K_i for the carbapenems were not significantly different. These results, together with the lack of changes in pI, indicate the absence of qualitative differences between the enzymes in these strains.

The direct contribution of the enzyme was analyzed next by introduction of the plasmid-borne *ampD* gene into *E. cloacae* 200 and its derivatives. The *ampD* product decreases *ampC* expression, thereby preventing the production of the cephalosporinase (11). As shown in Table 2, when *ampD* was present, the production of the cephalosporinase was decreased more than 450-fold in the wild-type strain *E. cloacae* 200, as well as in derivatives 201, 200-MD1, and 200-MD2 selected on moxalactam. This decrease was associated with a decrease in the MICs of carbapenems (2- to 32-fold), moxalactam (8- to 32-fold), and cefotaxime (≥256-fold). The decrease in the MICs of the carbapenems was more pronounced in the resistant derivatives selected on moxalactam, and this was particularly obvious for meropenem. It should be noted that in the presence of up to 1× the MIC of imipenem, cephalosporinase production was induced maximally 1.8-fold in the high-level producers, whether or not they contained *ampD*. Similar results were obtained when the *ampD* gene was introduced into 92227-M, 92227-CM, or P99-M (Table 2).

Further evidence of the influence of the cephalosporinase on the MICs of carbapenems was obtained by introducing, into strain P99$^-$, the plasmid pEC1E (10) which carries the cephalosporinase-encoding gene *ampC*. Since P99$^-$ is a noninducible, low-level producer of cephalosporinase, the effect of the cephalosporinase produced by the *ampC* gene can be directly deduced from the MICs. As shown in Table 2, a 100-fold increase in the production of cephalosporinase was associated with a 4-fold increase in the MICs of carbapenems.

Characterization of the outer membrane proteins. Although, in the resistant strains, the reduced cephalosporinase production in the presence of plasmid-borne *ampD* was associated with a significant decrease in the MICs of the carbapenems and moxalactam, the MICs of these antibiotics were still 2- to 16-fold higher than those observed for the wild-type strain. Thus, we investigated whether a change in the outer membrane structure could account for this "residual" resistance in the different resistant mutants derived from *E. cloacae* 200 and *E. cloacae* 92227. No change in the lipopolysaccharide or penicillin-binding proteins (labeled with [^{14}C]meropenem) was observed (data not shown). Examination of the outer membrane proteins, however, showed that in the resistant derivatives of *E. cloacae* 200, two outer membrane proteins of ca. 37 and 38 kDa had apparently disappeared (Fig. 1). An obvious correlation between the intensities of other proteins with higher or lower molecular weights and imipenem susceptibility was not observed (data not shown). Two-dimensional polyacrylamide gel electrophoresis showed that these proteins were among the most acidic (data not shown) and, thus, were likely to be porins (20). *E. cloacae* 200-MD1, which showed

TABLE 3. Kinetics of hydrolysis of β-lactam antibiotics by the cephalosporinases of *E. cloacae* 200 and its derivatives[a]

Strain	Rate of hydrolysis[b]			K_m (μM), nitrocefin	K_i (μM)	
	Imipenem[c]	Meropenem[c]	Nitrocefin		Imipenem	Meropenem
200	1	0.9	11.578	98.6	0.33	0.62
201	0.6	0.5	6.968	67.5	0.89	0.34
200-MD1	1.2	1.3	14.156	48.7	0.66	0.58
200-MD2	1.3	1.3	12.990	83.8	0.78	0.52

[a] See Table 1.
[b] Rate of hydrolysis at 100 μM expressed in nanomoles per minute per milligram of protein. Values are means from two or three determinations. Variations were within ±18%.
[c] Only the initial rate of hydrolysis (<2 min) was considered.

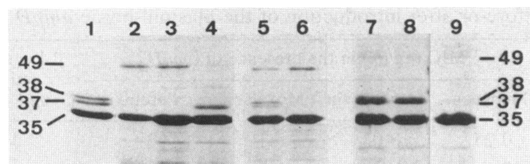

FIG. 1. Outer membrane proteins of *E. cloacae* 200, *E. cloacae* 92227, and their derivatives. Lanes: 1, *E. cloacae* 200; 2, *E. cloacae* 201; 3, *E. cloacae* 201 (containing *ampD*); 4, *E. cloacae* 201-Rev; 5, *E. cloacae* 200-MD1; 6, *E. cloacae* 200-MD2; 7, *E. cloacae* 92227; 8, *E. cloacae* 92227-C; 9, *E. cloacae* 92227-M. Outer membrane proteins were separated on 12 and 10% acrylamide gels (lanes 1 through 6 and lanes 7 through 9, respectively) containing 20% urea. Molecular weights are given in kilodaltons.

a lower level of resistance to carbapenems than *E. cloacae* 201 and 200-MD2 did, had only a ca. fourfold decrease in the amount of the two porins (as determined by scanning densitometry; data not shown). In the imipenem-susceptible revertant from strain 201, only the 37-kDa protein reappeared, although in slightly increased amounts (Fig. 1). Similar results were obtained with the carbapenem-resistant derivative 92227-M (Fig. 1). The carbapenem-susceptible mutant 92227-C, which was selected on cefotaxime, showed only a very discrete reduction in the amounts of the 37- and 38-kDa porins. Introduction of *ampD* into the carbapenem-resistant strain 201, which caused an increased susceptibility to carbapenems, was not associated with obvious changes in the outer membrane protein profile (Fig. 1).

Permeability of the outer membrane of *E. cloacae* to cephaloridine. Washed exponential-phase cells of *E. cloacae* 200 and its derivatives 201, 200-MD1, and 200-MD2 were incubated, as intact cells or after sonication, in the presence of 200 μM cephaloridine, and the rate of hydrolysis was determined spectrophotometrically with a cuvette with an optical path length of 2 mm. By using the K_m (221 μM) of the cephalosporinase for cephaloridine and the area of the cell surface and by following the calculation of Nikaido et al. (21), the permeability coefficients for cephaloridine were determined. The permeability coefficients determined for the resistant strains *E. cloacae* 201 (17 nm/s) and 200-MD2 (21 nm/s) were seven- to eightfold lower than that for the wild-type strain 200 (142 nm/s). The permeability coefficient for *E. cloacae* 200-MD1 (44 nm/s), which showed lower levels of resistance to imipenem and meropenem and a lesser decrease in the quantity of porins than strains 201 and MD2 did, decreased only 3.5-fold.

Uptake of [^{14}C]meropenem. Since imipenem and meropenem, like cephaloridine, are zwitterionic compounds, one could expect that there were also decreases in the rates of diffusion of the two carbapenems into the resistant derivatives of *E. cloacae* 200. However, there is no proof that, in *E. cloacae*, these compounds diffuse entirely through the same channels. Since the cephalosporinase of *E. cloacae* did not hydrolyze carbapenems at a sufficient rate to allow assessment of their permeability coefficients, we assayed the uptake of [^{14}C]meropenem. As shown in Fig. 2, after incubation for 30 s with different concentrations of [^{14}C]meropenem, substantially less uptake of [^{14}C]meropenem was observed for *E. cloacae* 201, 200-MD2, and 200-MD1 compared with that observed for *E. cloacae* 200. The revertant strain *E. cloacae* 201-Rev showed an uptake intermediate between those observed for *E. cloacae* 200 and the resistant mutants.

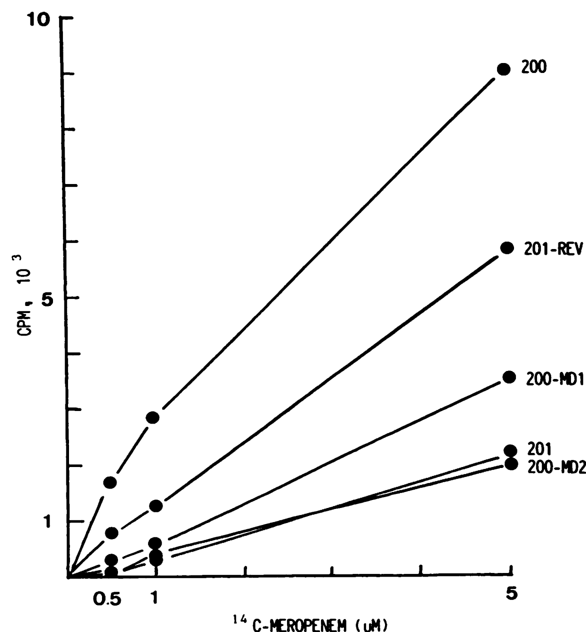

FIG. 2. [^{14}C]meropenem uptake by *E. cloacae* 200 and its derivatives. 200, *E. cloacae* 200; 201, *E. cloacae* 201; 201-REV, *E. cloacae* 201-Rev; 200-MD1, *E. cloacae* 200-MD1; 200-MD2, *E. cloacae* 200-MD2.

DISCUSSION

Resistance to imipenem has been well studied in *P. aeruginosa* (1, 16, 22). It was demonstrated that the decrease in the quantity of the outer membrane protein D2, which functions as a specific channel for imipenem, was responsible for this resistance (24, 25). A contribution of the chromosomal cephalosporinase to the level of imipenem resistance was described by Livermore and Yang (15), who reported that different mutants of *P. aeruginosa* that produce low levels of noninducible cephalosporinase show 16-fold reduced MICs of imipenem.

In this study, using mutants selected in vivo and in vitro, we explored the mechanisms involved in the resistance to imipenem in several strains of *E. clocae*. Subsequent to the selection of a carbapenem-resistant mutant under moxalactam therapy, we selected other mutants in vitro; these mutants were either from the susceptible clinical isolate or from other susceptible strains of *E. cloacae*.

We demonstrated that in the resistant derivatives of *E. cloacae* 200 and *E. cloacae* 92227, which were selected on moxalactam, there was a net decrease in the amounts of two outer membrane proteins, the putative porins. For the resistant derivatives of *E. cloacae* 200, we showed that a reduced permeability coefficient for cephaloridine was correlated with a reduced uptake of [^{14}C]meropenem. This suggests a decrease in the permeabilities for carbapenems in the resistant strains. Two additional observations reinforce this hypothesis. (i) *E. cloacae* 200-MD1, which had an intermediate level of resistance to imipenem and meropenem, showed a relatively smaller decrease in the quantities of the two porins and also a smaller decrease in the permeability coefficient for cephaloridine than the more resistant strains *E. cloacae* 201 and 200-MD2 did. (ii) In the revertant strain *E. cloacae* 201-Rev, susceptibilities to carbapenem and moxalactam were restored simultaneously with the reappearance, although in slightly increased quantities, of only the 37-kDa

porin and an increase in the uptake of [^{14}C]meropenem. This uptake, however, was not quite as high as that observed for *E. cloacae* 200. This supports a correlation between the amounts of, at least, the 37-kDa porin and susceptibility to carbapenem. However, the precise role of the porins in carbapenem resistance must ultimately be assessed in vitro by using, for instance, proteoliposome swelling assays with total outer membrane proteins and isolated porins. A diffusion pathway for carbapenems that would be provided by membrane components other than the classical porins (but that would not necessarily be functional in the mutants we described here) may also exist, since we recently selected a carbapenem-susceptible mutant in vitro which lacked both the 37- and the 38-kDa proteins (data not shown).

The presence of the cephalosporinase undoubtedly contributes to the susceptibilities of the different strains to carbapenem. However, high-level production per se was not responsible for the high level of resistance observed in the resistant mutants. This was well demonstrated by the very close quantitative and qualitative similarities (pI, K_m, K_i, rate of hydrolysis) of the enzymes present in *E. cloacae* 200 and its resistant derivatives.

Three arguments, however, substantiate the contribution of the caphalosporinase to the different resistance phenotypes. (i) The very low amount of the noninducible cephalosporinase in *E. cloacae* P99$^-$ was accompanied by eightfold reduced MICs of carbapenems compared with those of the isogenic high-level producer P99; (ii) the introduction of *ampC* into P99$^-$ increased cephalosporinase production ca. 80-fold and led to a 4-fold increase in the MICs of the carbapenems; and (iii) the introduction of *ampD* into *E. cloacae* 200, *E. cloacae* 92227, and their derivatives selected on moxalactam, which decreased cephalosporinase production more than 500-fold, led to a 2- to 32-fold decrease in the MICs of the carbapenems.

In the context of these observations, it may appear surprising that mutant 92227-C, which was selected on cefotaxime and produced high levels of cephalosporinase spontaneously, showed no increase in the MICs of carbapenems with respect to those of wild-type strain 92227. However, the identities of the MICs observed for the mutant 92227-C and the parental strain 92227, which is a low cephalosporinase producer, were explained by induction of the cephalosporinase with imipenem in strain 92227 (7,835 mU/mg in the presence of 0.2 μg of imipenem). This is similar to what was observed previously for *P. aeruginosa* (15) and is also supported by the decreased MICs of imipenem for the two strains after introduction of the *ampD* gene.

Two studies (3, 18) tested the imipenem susceptibilities of *E. cloacae* strains with decreased outer membrane permeabilities. Marchou et al. (18) selected a mutant (218 R2) on ceftriaxone that produced high levels of cephalosporinase and less of the 37- and 38-kDa outer membrane proteins in conjunction with reduced permeability. The MIC of moxalactam, however, was only 10 μg/ml, and the mutant was not resistant to imipenem. Frère et al. (5), using a mathematical model, calculated a fivefold decrease in the permeability of this mutant to various β-lactams, including moxalactam, but could not explain the absence of resistance to imipenem. Bush et al. (3) selected a permeability mutant (Mx^r) on moxalactam that was highly resistant to this compound (>2,000 μg/ml) and that was also resistant to imipenem (32 μg/ml). A low-level cephalosporinase producer like 92227-M, mutant Mx^r, resembled the former somewhat in its moxalactam and imipenem resistance patterns, but the outer membrane protein profile of the Mx^r mutant is not known. Our conclusion that the permeability for imipenem may be modified in this type of mutant is in agreement with that of Frère et al. (6), who analyzed the data of Bush et al. (3).

If we consider the reduced outer membrane permeabilities of the mutants described here to be primarily responsible for the resistance to imipenem and meropenem, the production of the cephalosporinase in high amounts (spontaneously or after induction) should increase the level of resistance. Thus, the slow penetration of the carbapenems into the resistant strains should enhance the effect of the slow hydrolysis by the cephalosporinase and thereby decrease the quantity of antibiotic that is able to reach its target.

It should be noted, finally, that, from our data and those of others (3), moxalactam may be a very effective selector of such permeability mutants.

ACKNOWLEDGMENTS

This work was supported by a grant from the Institut National de la Santé et de la Recherche Médicale (CJF 89-01) and grants in aid from ICI Pharmaceuticals and Merck Sharp and Dohme-Chibret.

We thank C. Harcour for secretarial assistance.

REFERENCES

1. **Büscher, 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.
2. **Bush, K.** 1989. Classification of β-lactamases: groups 1, 2a, 2b, and 2b′. Antimicrob. Agents Chemother. **33:**264–270.
3. **Bush, K., S. K. Tanaka, D. P. Bonner, and R. B. Sykes.** 1985. Resistance caused by decreased penetration of β-lactam antibiotics into *Enterobacter cloacae*. Antimicrob. Agents Chemother. **27:**555–560.
4. **Cuchural, G. J., Jr., M. H. Malamy, and F. P. Tally.** 1986. β-Lactamase-mediated imipenem resistance in *Bacteroides fragilis*. Antimicrob. Agents Chemother. **30:**645–648.
5. **Frère, J. M.** 1989. Quantitative relationship between sensitivity to β-lactam antibiotics and β-lactamase production in gram-negative bacteria. I. Steady-state treatment. Biochem. Pharmacol. **38:**1415–1426.
6. **Frère, J. M., B. Joris, M. Crine, and H. H. Martins.** 1989. Quantitative relationship between sensitivity to β-lactam antibiotics and β-lactamase production in gram-negative bacteria. II. Non-steady-state treatment and progress curves. Biochem. Pharmacol. **38:**1427–1433.
7. **Goldner, M., D. G. Glass, and P. C. Fleming.** 1969. Spontaneous mutant with loss of β-lactamase in *Aerobacter cloacae*. J. Bacteriol. **97:**961.
8. **Gutmann, L., R. Williamson, N. Moreau, M. D. Kitzis, E. Collatz, J. F. Acar, and F. W. Goldstein.** 1985. Cross-resistance to nalidixic acid, trimethoprim, and chloramphenicol associated with alterations in outer membrane proteins of *Klebsiella*, *Enterobacter* and *Serratia*. J. Infect. Dis. **151:**501–507.
9. **Hitchcock, P., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154:**269–277.
10. **Honoré, N., M. H. Nicolas, and S. T. Cole.** 1986. Inducible cephalosporinase production in clinical isolates of *Enterobacter cloacae* is controlled by a regulatory gene that has been deleted from *Escherichia coli*. EMBO J. **5:**3709–3714.
11. **Honoré, N., M. H. Nicolas, and S. T. Cole.** 1989. Regulation of enterobacterial cephalosporinase production: the role of membrane bound sensory transducer. Mol. Microbiol. **3:**1121–1130.
12. **Hopkins, J. M., and K. J. Towner.** 1990. Enhanced resistance to cefotaxime and imipenem associated with outer membrane protein alterations in *Enterobacter aerogenes*. J. Antimicrob. Chemother. **25:**49–55.
13. **Hurlbut, S., G. J. Cuchural, and F. P. Tally.** 1990. Imipenem resistance in *Bacteroides distasonis* mediated by a novel β-lactamase. Antimicrob. Agents Chemother. **34:**117–120.

14. **Iaconis, J. P., and C. C. Sanders.** 1990. Purification and characterization of inducible β-lactamases in *Aeromonas* spp. Antimicrob. Agents Chemother. **34**:44–51.
15. **Livermore, D. M., and Y. J. Yang.** 1987. β-Lactamase lability and inducer power of newer β-lactam antibiotics in relation to their activity against β-lactamase-inducibility mutants of *Pseudomonas aeruginosa*. J. Infect. Dis. **155**:775–782.
16. **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.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Marchou, B., F. Bellido, R. Charnas, C. Lucain, and J. C. Pechère.** 1987. Contribution of β-lactamase hydrolysis and outer membrane permeability to ceftriaxone resistance in *Enterobacter cloacae*. Antimicrob. Agents Chemother. **31**:1589–1595.
19. **Matthew, M., A. M. Harris, M. J. Marshall, and G. W. Ross.** 1975. The use of analytical isoelectric focusing for detection and identification of β-lactamases. J. Gen. Microbiol. **88**:169–178.
20. **Nikaido, H., and Y. Nakae.** 1979. The outer membrane of gram-negative bacteria. Adv. Microb. Physiol. **20**:163–250.
21. **Nikaido, H., E. Y. Rosenberg, and J. Foulds.** 1983. Porin channels in *Escherichia coli*: studies with β-lactams in intact cells. J. Bacteriol. **153**:232–240.
22. **Quinn, J. P., 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. **Spratt, B. G.** 1975. Distinct penicillin-binding proteins involved in the division, elongation and shape of *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **72**:2999–3003.
24. **Trias, J., J. Dufresne, R. C. Levesque, and H. Nikaido.** 1989. Decreased outer membrane permeability in imipenem-resistant mutants of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **33**:1201–1206.
25. **Trias, J., and H. Nikaido.** 1990. Outer membrane protein D2 catalyzes facilitated diffusion of carbapenems and penems through the outer membrane of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **34**:52–57.
26. **Watanabe, M., S. Iyobe, M. Inoue, and S. Mitsuhashi.** 1991. Transferable imipenem resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **35**:147–151.
27. **Yang, Y., P. Wu, and D. M. Livermore.** 1990. Biochemical characterization of a β-lactamase that hydrolyzes penems and carbapenems from two *Serratia marcescens* isolates. Antimicrob. Agents Chemother. **34**:755–758.