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Characterization of IMI-1 β-Lactamase, a Class A Carbapenem-Hydrolyzing Enzyme from *Enterobacter cloacae*

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In 1984, a year prior to the U.S. approval of imipenem for clinical use, a wound isolate and a bile isolate of *Enterobacter cloacae* were obtained from two patients in a California hospital. These isolates were resistant to imipenem, penicillins, and inhibitor combinations; early cephalosporins such as cephalothin, cefamandole, and cefoxitin; and cefoperazone. However, they were susceptible (MICs, <4 μg/ml) to cefotaxime, ceftriaxone, ceftazidime, and moxalactam. Both strains produced an apparent TEM-1 β-lactamase; an inducible NmcA-type imipenem-hydrolyzing β-lactamase, IMI-1, with a pI of 7.05; and an inducible β-lactamase with a pI of 8.1, typical of an *E. cloacae* AmpC β-lactamase. Purified IMI-1 hydrolyzed imipenem and benzylpenicillin at modest rates, but more slowly than cephaloridine. The enzyme was inhibited by clavulanic acid and tazobactam. EDTA did not inhibit the cephaloridine-hydrolyzing activity. The β-lactamase gene encoding IMI-1, *imiA1*, was cloned from *E. cloacae* 1413B. Sequence analysis identified the *imiA1* gene as encoding a class A serine β-lactamase. Both the *imiA1* DNA and encoded amino acid sequences shared greater than 95% identity with the NmcA gene and its encoded protein. DNA sequence analysis also identified a gene upstream of *imiA1* that shares >95% identity with *nmcR* and that may encode a regulatory protein. In conclusion, IMI-1, a carbapenem-hydrolyzing β-lactamase inhibited by clavulanic acid, was identified as a group 2f, class A, carbapenem-hydrolyzing cephalosporinase.

Most clinical isolates of *Enterobacter cloacae* resistant to expanded-spectrum cephalosporins are susceptible to imipenem (19, 27). These isolates usually hyperproduce a chromosomal β-lactamase that hydrolyzes cephaloridine rapidly, cefotaxime and ceftazidime slowly, and imipenem at very low rates (7, 26). In addition, carbapenems often have an added advantage against these isolates, in that they exhibit higher rates of permeation than the cephalosporins (19). As a result, imipenem-resistant clinical isolates of *E. cloacae* are unusual and have been described primarily in strains with porin alterations combined with hyperproduction of cephalosporinase activity (9).

In 1984, before the approval of any carbapenem in the United States for clinical use, two strains of imipenem-resistant, cefotaxime-susceptible *E. cloacae* were identified in a single hospital in California. Initial characterization of these strains showed that a novel, inducible cephalosporinase was produced (12). However, this enzyme did not have the characteristics expected for a metallo-β-lactamase, which at that time was the only class of β-lactamase known to be capable of hydrolysis of the carbapenem skeleton (23). Recently, another imipenem-resistant, cefotaxime-susceptible *E. cloacae* clinical isolate has been described from France (17). The NmcA β-lactamase responsible for the carbapenem resistance in this strain is not a class B metallo-β-lactamase but, instead, a class A serine β-lactamase (15). A similar class A β-lactamase that is responsible for imipenem resistance has been cloned and sequenced from a British strain, *Serratia marcescens* S6 (16).

In the studies described herein, the 1984 North American strain, *E. cloacae* 1413B, is shown to produce an enzyme similar to the European class A carbapenem-hydrolyzing β-lactamases. This strain and its β-lactamase production have been characterized on the basis of microbiological, biochemical, and genetic evidence.

(The information in this report was presented at the 26th [12] and 34th [22] Interscience Conferences on Antimicrobial Agents and Chemotherapy.)

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains tested in the study are listed in Table 1. The cloning experiments were performed with Luria broth or Luria broth agar (Miller) supplemented, when required, with kanamycin (25 μg/ml) and ampicillin (100 μg/ml).

Clinical isolates. *E. cloacae* 1413B was isolated in May 1984 from a groin wound of a patient hospitalized in California. The patient had undergone portocaval shunt insertion because of alcoholic cirrhosis. The patient received cefazolin preoperatively, and that treatment was continued until postoperative day 15, when he experienced bacteremia caused by *E. cloacae* and *S. marcescens*, both of which were susceptible to cefotaxime. He was begun on treatment with cefotaxime and gentamicin; *E. cloacae* 1413B was isolated 7 days later while he was on this regimen.

E. cloacae 1415B was isolated in November 1984 from a culture of bile from a different patient in the same hospital. The patient had undergone a Whipple procedure for carcinoma of the head of the pancreas in May 1984 and was readmitted in September 1984 for recurrent fever. She received ampicillin, clindamycin, and gentamicin; this was followed by cefazolin, metronidazole, and gentamicin until October 1984, when aspiration of a liver abscess yielded *Escherichia coli* and *Citrobacter freundii*. She was then begun on therapy with cefuroxime and metronidazole; 22 days later *E. cloacae* 1415B plus a *Citrobacter* sp. were isolated during revision of her choledochojejunostomy.

Antibiotics. The antibiotics used in the studies described here were obtained from their respective manufacturers, as indicated: benzylpenicillin, ampicillin, and aztreonam from E. R. Squibb & Sons, Princeton, N.J.; piperacillin and tazobactam from Lederle Laboratories, Pearl River, N.Y.; biapenem from Lederle Japan Ltd., Tokyo, Japan; ticarcillin and clavulanic acid from SmithKline Beecham, Worthing, England; cefoperazone and sulbactam from Pfizer, Groton, Conn.; cefoxitin and imipenem from Merck, Rahway, N.J.; cefuroxime and ceftazidime from Glaxo, Inc., Greenford, United Kingdom; cefotaxime from Hoechst-Roussel, Somerville, N.J.; ceftizoxime from Fujisawa; ceftriaxone from

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TABLE 1. Microbiological activities of β-lactams against imipenem-resistant clinical isolates and laboratory constructs

Antibiotic	MICs (μg/ml) for the following strains with the indicated enzymes:			
	E. cloacae 1413B, TEM-1 type, IMI-1, (AmpC)[a]	*E. cloacae* 1415B, TEM-1 type, IMI-1, (AmpC)	*E. coli* DH5α(pCLL2300), (AmpC)	*E. coli* DH5α(pCLL*imiA1*), IMI-1, (AmpC)
Ampicillin	>256	>256	2.0	>32
Piperacillin	>128	>128	1.0	>128
Piperacillin-tazobactam[b]	>128	>128	1.0	>128
Ticarcillin	>128	>128	2.0	>128
Ticarcillin-clavulanic acid[c]	>128	>128	4.0	>128
Cefazolin	>128	>128	1.0	>128
Cephalothin	>256	>256	ND[d]	ND
Cefamandole	>128	>128	ND	ND
Cefoperazone	>128	>128	ND	ND
Cefoxitin	>128	>128	4.0	16
Cefuroxime	16	16	4.0	>128
Cefotaxime	1.0	0.25	≤0.12	2.0
Ceftazidime	2.0	1.0	0.5	8.0
Ceftizoxime	2.0	4.0	ND	ND
Ceftriaxone	1.0	0.5	≤0.25	16
Moxalactam	≤0.25	≤0.25	≤0.25	1.0
Aztreonam	8.0	16	ND	ND
SCH34343	>128	>128	ND	ND
Imipenem	>32	>32	0.50	>32
Meropenem	4.0	4.0	≤0.06	>32
Gentamicin	1	16	0.25	0.50

[a] (AmpC), assumed to be an AmpC-type β-lactamase.
[b] Tazobactam at a fixed concentration of 4 μg/ml.
[c] Clavulanic acid at a fixed concentration of 2 μg/ml.
[d] ND, not determined.

Roche Laboratories, Nutley, N.J.; cefazolin, cephalothin, cefamandole, and moxalactam from Eli Lilly, Indianapolis, Ind.; SCH34343 from Schering Plough, Bloomfield, N.J.; nitrocefin from BBL, Cockeysville, Md.; and meropenem from Zeneca, Macclesfield, United Kingdom. All solutions were prepared fresh before use.

Antibiotic susceptibility. Susceptibility was measured by the Kirby-Bauer technique in Mueller-Hinton agar or serial dilution with Mueller-Hinton broth in microtiter wells inoculated with 10^5 CFU/ml, or by both methods.

β-Lactamase preparations. Crude extracts for initial studies were prepared by sonication of cells grown overnight in brain heart infusion broth. For induction, the cells were diluted 1:10 in fresh broth containing either cefoxitin (8 μg/ml) or imipenem (4 μg/ml) and were incubated for 4 h before harvesting.

The three β-lactamases from the clinical isolates were separated for initial characterization by chromatofocusing. Crude extracts were passed through a 12- by 50-mm Chromaflex column packed with Polybuffer exchanger PBE94 (Pharmacia). The column was equilibrated with 0.025 M Tris-HCl (pH 8.5) buffer, and enzyme was eluted with Polybuffer PB74. The buffer was diluted 1:10 and was adjusted to pH 4.0. Five milliliters of buffer was added; this was followed by the addition of 7 ml of crude extract (10 mg of protein per ml) and then the Polybuffer PB74. The pH was determined for each 5-ml fraction; β-lactamase activity was estimated with nitrocefin. Pooled fractions were concentrated with a ProDiCon (Bio Molecular Dynamics) apparatus and were dialyzed overnight in cold distilled deionized water. The β-lactamase activities for each fraction were determined spectrophotometrically with 100 μM substrate.

IMI-1 β-lactamase was purified to greater than 95% homogeneity from 2 liters of *E. coli* DH5α(pCLL*imiA1*) (see below) grown in tryptic soy broth. Harvested cells were submitted to five cycles of freezing and thawing, and the supernatant was recovered after centrifugation. The freeze-thaw extract was first eluted from CM-Sephadex C50 in 10 mM phosphate buffer (pH 7.8), with activity monitored by hydrolysis of nitrocefin. Active fractions with imipenem-hydrolyzing activity were then eluted from DEAE-Sephadex A50 in 20 mM Tris HCl (pH 8.0). The purity of the IMI-1 β-lactamase was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% acrylamide. Protein was stained with Coomassie brilliant blue.

IEF. Isoelectric focusing (IEF) of crude extracts was performed by the method of Matthew et al. (11) in a polyacrylamide gel, focused for 18 h at 4°C, and developed with nitrocefin. IEF of purified IMI-1 was performed at 10°C for 2 h on a Multiphor apparatus (LKB-Pharmacia) with prepared PAGplates (pH range, 3.5 to 9.5). IMI-1 activity was detected with a nitrocefin overlay. To check for metallo-β-lactamase activity a filter paper overlay of EDTA (0.1 M) was applied before the application of the nitrocefin. The L1 metalloenzyme from *Stenotrophomonas* (*Xanthomonas*) *maltophilia* 1712 was used as a positive control.

Enzyme kinetics. The kinetic parameters of purified IMI-1 β-lactamase were determined spectrophotometrically in 50 mM phosphate buffer (pH 7.0). K_m and V_{max} values were determined from the steady-state rates obtained at 6 to 10 substrate concentrations. The computer program ENZPACK (Biosoft; Elsevier) used five different plotting programs to calculate the kinetic data. At least two independent kinetic evaluations were performed for each substrate. The average values of the kinetic parameters for each substrate were reported, with the standard deviation of each value being less than 20%.

Enzyme inhibition. Enzyme and inhibitor were preincubated at 25°C in a volume of 50 μl for 10 min before the addition of 100 μM nitrocefin (final volume, 1,000 μl). Initial rates of hydrolysis were monitored spectrophotometrically at 495 nm. The 50% inhibitory concentrations (IC_{50}s) were determined graphically.

Cloning of *imiA1* and *imiR1*. Restriction endonucleases, calf intestinal phosphatase, and T4 DNA ligase were obtained from New England Biolabs and Boehringer Mannheim Biochemicals and were used according to the manufacturer's directions. Basic molecular biology techniques were performed as described by Maniatis et al. (10).

Total DNA was prepared from *E. cloacae* 1413B as described previously (20). The DNA was restricted with *Eco*RI, ligated into appropriately restricted pCLL2300 (21), a cloning vector encoding kanamycin resistance, and used to transform *E. coli* DH5α (Bethesda Research Laboratories). The cells were transformed by standard $CaCl_2$ techniques and plated onto kanamycin (25 μg/ml), and the resulting colonies were pooled. Dilutions of the pooled transformants were plated onto medium containing ampicillin (100 μg/ml), and ampicillin-resistant isolates were identified. β-Lactamase-positive clones were identified by ampicillin resistance or a positive response by penicillinase assay filters, or both (28).

The colonies were lifted off the plates and placed on filter paper impregnated with penicillin G and bromocresol purple (28). Colonies exhibiting undetectable or very weak β-lactamase activity turn purple to green when they are seeded on the indicator filters, while colonies expressing β-lactamase activity turn yellow. The rapidity and intensity of the color change correlate with the level of penicillinase activity (20, 28).

DNA sequence analysis of *imiA1* and *imiR1*. The *imiA1* gene was localized within the cloned restriction fragment by DNA restriction analysis and subcloning. The region of the clone corresponding to the β-lactamase gene was subcloned into pUC118 or pUC119, or both (25). Double-stranded DNA sequence analysis of *imiA1* was performed with the Sequenase kit (U.S. Biochemicals).

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The M13 −40 universal primer and other synthetic oligonucleotides complementary to the determined DNA sequences were used as primers. The complete DNA sequences of both strands were determined. For *imiR1* the DNA sequence was determined for one strand of DNA unless there was difficulty in reading the DNA sequence. In such cases the sequences of both DNA strands were determined. Primers for DNA sequence determination were prepared on the basis of the ECNORRA DNA sequence since the two DNA sequences appeared to be nearly identical. The ECNORRA DNA sequence (accession number Z21956) was obtained from GenBank (3).

DNA and amino acid sequence analyses. Sequence analysis and homology searches were performed with the computer program DNAStar. The predicted amino acid sequence of IMI-1 was compared with those of other proteins in the Swiss Protein data bank (release 80) by using the algorithm of Pearson and Lipman (18).

Nucleotide sequence accession number. The nucleotide sequence data reported here will appear in the GenBank sequence data bank under accession number U50278.

RESULTS

Antimicrobial susceptibility. As indicated in Table 1 the clinical *E. cloacae* isolates were resistant to penicillins, the penicillin-inhibitor combinations, the penem SCH34343, and imipenem. Resistance to the older cephalosporins including cefoxitin was noted. The MICs of meropenem were significantly lower than those of imipenem for the *E. cloacae* isolates, perhaps because of different uptake mechanisms. However, the strains were susceptible to moxalactam and to the aminothiazole-containing expanded-spectrum cephalosporins. Higher MICs were seen in the *E. coli* transformant strain than in the original *E. cloacae* isolates, probably because of a higher copy number of the cloned *imiA1* gene. Both clinical isolates were resistant to tetracycline. Strain 1415B was also resistant to tobramycin and gentamicin as a result of the ANT2″ resistance marker (data not shown). Pulsed-field gel electrophoresis indicated that the two isolates are similar, showing a single band difference (data not shown).

β-Lactamase activities in clinical isolates. Both clinical *E. cloacae* isolates produced three different β-lactamases that were distinguishable on IEF, with pI values of 5.4, 7.05, and 8.1, respectively (Fig. 1). When strain 1413B was induced with imipenem at 4 μg/ml, the imipenem- and SCH34343-hydrolyzing activities were increased 17- and 19-fold, respectively. When cefotaxime or cefoxitin was used as a substrate, no inducible activity was seen after imipenem induction; for these substrates the β-lactamase hydrolysis rates in all samples tested were ≤0.5 nmol/s/mg of protein. Induction of β-lactamase activity by cefoxitin (8 μg/ml) did not result in any significant increase in β-lactamase activity (twofold induction or less) when tested with imipenem, SCH34343, or cefotaxime as substrates. On IEF gels enhanced β-lactamase activity was seen at pIs of 7.05 and 8.1 following induction by imipenem; only the cephalosporinase band at pI 8.1 was enhanced after induction by cefoxitin (Fig. 1). When the crude extract was run on IEF gels, an overlay of the gel with EDTA did not show a reduction in the activities of any of the three bands stained with nitrocefin, although a sample of the L1 metallo-β-lactamase was completely inactivated by this treatment (data not shown).

Chromatofocusing was used to separate the three β-lactamases in the clinical isolate *E. cloacae* 1413B. As seen in Table 2, fractions A and C with pIs of 8.1 and 5.4, respectively, had broad-spectrum cephalosporin-hydrolyzing activities, but they did not hydrolyze imipenem. Only fraction B, containing a β-lactamase with a pI of 7.05, hydrolyzed imipenem at a measurable rate. This enzyme was named IMI-1.

Cloning of the IMI-1 β-lactamase gene from *E. cloacae*. The IMI-1 β-lactamase gene was presumed to be chromosomally encoded because several different plasmid extraction methods yielded no evidence of any plasmids harbored by *E. cloacae*

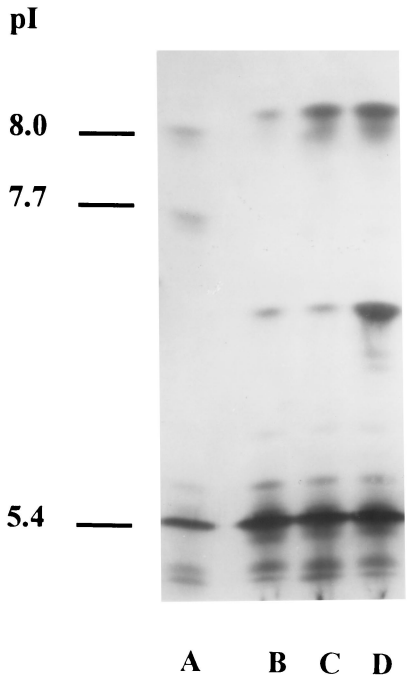

FIG. 1. IEF patterns of imipenem-resistant *E. cloacae* 1413B. Lane A, extract of a marker strain of *E. coli* producing TEM-1 (pI 5.4), OXA-2 (pI 7.7), and AmpC (pI 8.0); lanes B, C, and D, extracts of *E. cloacae* 1413B not induced (lane B), induced by cefoxitin (lane C), and induced by imipenem (lane D).

1413B (data not shown). Chromosomal DNA from *E. cloacae* 1413B was restricted with *Eco*RI and was ligated into pCLL2300 restricted with the corresponding enzyme. The ligation mixtures were transformed into *E. coli* DH5α, and kanamycin-resistant transformants were selected. More than 1.5×10^4 to 2.0×10^4 kanamycin-resistant colonies were obtained. The colonies were scraped from the plates, pooled, and stored frozen. Ampicillin-resistant isolates were selected by plating dilutions of the pooled transformants onto ampicillin (100 μg/ml) with or without kanamycin. Ampicillin-resistant colonies were identified on the plates containing ampicillin at 100 μg/ml. These colonies were screened for β-lactamase activity with the penicillinase assay filters. The majority of the colonies turned pale yellow, indicating a weak penicillin-hydrolyzing activity. A minor percentage, <5% of the population, exhibited a phenotype of strong penicillin-hydrolyzing activity, turning bright yellow within seconds of exposure to the filters. Six to eight representative colonies of each phenotype were selected for further characterization and plasmid content analysis.

The six colonies exhibiting strong penicillin-hydrolyzing activities all contained plasmids with a DNA insert larger than 10

TABLE 2. Activities of chromatofocusing fractions of β-lactamase produced by *E. cloacae* 1413B

β-Lactamase		β-Lactamase activity (nmol/s/mg of protein)		
Fraction	pI	Imipenem	Cephalothin	Cephaloridine
Crude extract	8.1, 7.05, 5.4	0.09	13	21
A	8.1	<0.5	470	240
B	7.05	730	1,250	1,600
C	5.4	<0.5	74	220

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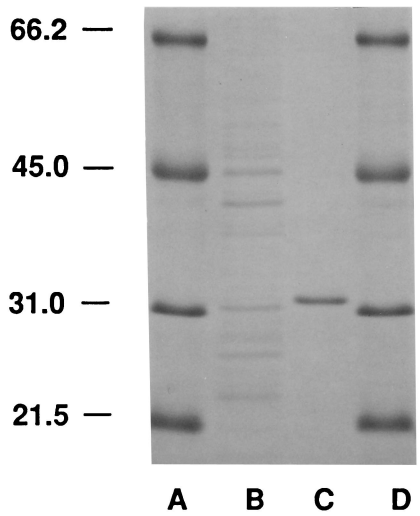

FIG. 2. SDS-polyacrylamide gel of IMI-1 purified from *E. coli* DH5α (pCLL*imiA1*) following two ion-exchange chromatography steps. Lanes A and D, molecular mass standards; lane B, crude freeze-thaw extract from *E. coli* DH5α(pCLL*imiA1*); lane C, purified IMI-1. The gel was stained with Coomassie brilliant blue.

kb. One of these plasmids was selected for further analysis and was designated pCLL*ecbB1*. Analysis of the plasmids extracted from the eight colonies with weak penicillinase activities revealed that all eight plasmids harbored an *Eco*RI fragment of 6.5 kb. Two of the plasmids contained an additional DNA fragment; the fragments were of different sizes in each plasmid. One of these plasmids, harboring only the 6.5-kb *Eco*RI fragment, was selected for further analysis and was designated pCLL*imiA1*. The two representative plasmids were transformed into *E. coli* DH5α and were found to confer the same penicillinase phenotype observed previously.

Restriction analysis of pCLL*imiA1* and pCLL*ecbB1* indicated that the DNA fragments harbored by the two plasmids were distinct and nonoverlapping. IEF analysis of cell extracts from DH5α isolates harboring either pCLL*imiA1* or pCLL*ecbB1* indicated that pCLL*imiA1* encoded a β-lactamase with a pI of 7.05 and that pCLL*ecbB1* encoded a β-lactamase with a pI of 5.4. These pI values corresponded to two of the three *E. cloacae* β-lactamases. Thus, the two plasmids encoded different β-lactamases. The β-lactamase gene harbored by pCLL*imiA1* was designated *imiA1*, and its encoded β-lactamase was designated IMI-1 on the basis of its ability to hydrolyze imipenem (see below). The gene harbored by pCLL*ecbB1* was not fully characterized, but it appeared to be a TEM-type β-lactamase. Imipenem hydrolysis studies of freeze-thaw cellular extracts indicated that extracts prepared from *E. coli* harboring pCLL*imiA1* contained imipenem-hydrolyzing activity, while extracts prepared from cells harboring pCLL*ecbB1* did not. This indicated that the imipenem-hydrolyzing enzyme was encoded on plasmid pCLL*imiA1*.

Biochemical characterization of purified IMI-1. The IMI-1 β-lactamase from the *E. coli* DH5α(pCLL*imiA1*) strain was highly purified by ion-exchange chromatography. Only a single band with a pI of 7.05 appeared on IEF gels with nitrocefin as the substrate. The purified enzyme appeared to be greater than 95% homogeneous on SDS-PAGE, with a molecular mass estimated to be 32,000 Da (Fig. 2).

IMI-1, with broad-spectrum β-lactamase-hydrolyzing activity, recognized cephalosporins, penicillins, monobactams, and carbapenems as substrates (Table 3). Its strongest hydrolytic activity was for the early cephalosporins, including cephalothin and cephaloridine. Although it hydrolyzed cephaloridine with a high k_{cat} value, the affinity for this cephalosporin was much less than that for most other substrates. Hydrolysis rates for penicillins were quite varied, as were the K_m values. The k_{cat} value for ampicillin was five times greater than that for benzylpenicillin, but the affinity was decreased 10-fold. Piperacillin, with a k_{cat}/K_m value that approached that for benzylpenicillin, was the penicillin with the lowest catalytic constant (turnover number), but IMI-1 had the highest affinity for piperacillin compared with its affinity for all other substrates tested. Cefoxitin and the expanded-spectrum cephalosporins, especially ceftazidime, were poor substrates for the IMI-1 enzyme. Imipenem was hydrolyzed by the IMI-1 β-lactamase, with a k_{cat} faster than that of benzylpenicillin. Biapenem and meropenem were more stable to hydrolysis than either benzylpenicillin or imipenem. Aztreonam, with a k_{cat}/K_m value comparable to that of imipenem, also was a good substrate for the IMI-1 enzyme, with a higher k_{cat} value than that of benzylpenicillin.

IMI-1 β-lactamase was inhibited by all the three β-lactamase inhibitors tested. Tazobactam was the strongest inhibitor, with an IC_{50} of 30 nM; this was followed by clavulanic acid, with an IC_{50} of 280 nM. Sulbactam was the least inhibitory, with an

TABLE 3. Kinetic parameters of purified IMI-1 β-lactamase

Substrate	k_{cat} (s^{-1})	Relative k_{cat}	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})	Relative k_{cat}/K_m
Benzylpenicillin	36	100	64	560	100
Ampicillin	190	540	780	240	44
Piperacillin	6.1	17	13	470	84
Cephaloridine	2,000	5,600	1,070	1,900	340
Cephalothin	120	340	130	920	160
Nitrocefin	130	380	37	3,500	630
Cefotaxime	3.4	9.7	190	18	3.2
Ceftazidime	0.0068	0.019	270	0.024	0.0045
Cefoxitin	0.3	0.83	45	6.7	1.2
Aztreonam	51	143	93	550	98
Imipenem	89	250	170	520	93
Biapenem	9.4	27	32	290	52
Meropenem	10	28	26	380	69

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FIG. 3. DNA and amino acid sequence alignment of ECIMIRA and its encoded proteins. The nucleotide sequence of ECIMIRA is shown, with the predicted amino acid sequences of ImiR1 and IMI-1 shown in single-letter code below. The predicted −10, −35, and ribosomal binding sites (RBS) are indicated.

IC_{50} of 1,800 nM. Nonlinear kinetics were observed for clavulanic acid and sulbactam following a 10-min preincubation of enzyme and inhibitor. The enzyme was not inhibited by EDTA at concentrations up to 10 mM.

DNA sequence analysis of *imiA1* and *imiR1*. The β-lactamase gene harbored on pCLL*imiA1* was localized within the 6.5-kb fragment to a 1.9-kb *Eco*RV subfragment. Restriction enzyme digestion analysis indicated that a *Pst*I site was present within *imiA1*. By using this information, *imiA1* was subcloned into pUC118 or pUC119 by using the *Pst*I restriction site to fix one end of the subclone and *Eco*RV to fix the other end. The DNA sequence of *imiA1* was determined by sequencing from within the vector toward the *Pst*I site and directly into *imiA1* by using the M13 −40 universal primer. After the initial primary sequence data were acquired, synthetic primers complementary to the determined DNA sequences were used to complete the DNA sequence determination (Fig. 3). Both strands of *imiA1* and the immediately flanking DNA were sequenced. The DNA sequence of *imiA1* was found to be more than 95% identical to that of ECNORRA. The protein encoded by *imiA1*, IMI-1, shared 97% identity (285 of 292 amino acids) with the β-lactamase, NmcA, encoded on ECNORRA and 70% identity (204 of 292 amino acids) with the β-lactamase Sme-1 (16) (Fig. 4).

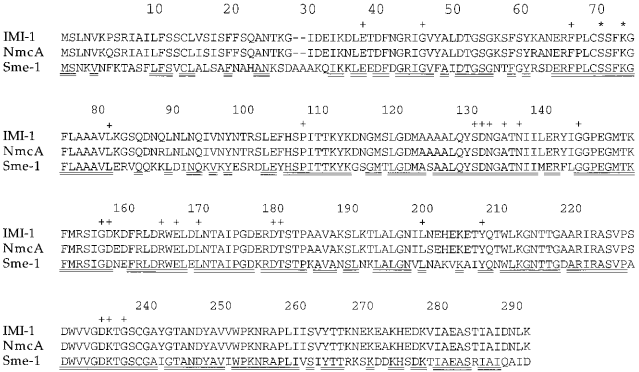

FIG. 4. Amino acid sequence alignment of IMI-1, NmcA, and Sme-1: IMI-1, *E. cloacae* (accession number U50278); NmcA, *E. cloacae* (accession number Z21956) (15); and Sme-1, *S. marcescens* (accession number Z28968) (16). Dashes indicate gaps that were introduced to optimize the alignment. Numbering is according to Ambler et al. (2). The amino acids conserved among IMI-1, NmcA, and Sme-1 are double underlined. The plus signs and asterisks indicate residues that are highly conserved among all class A serine β-lactamases (8, 15). The asterisks indicate the conserved Ser and Lys residues of the Ser-X-X-Lys active-site residues of serine β-lactamases.

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Also encoded on the ECNORRA sequence is a second protein, NmcR, that is proposed to function in a regulatory role in the expression of NmcA (15). To determine if an NmcR counterpart was present upstream of *imiA1*, the DNA sequence of the 1,250 bases preceding the *imiA1* initiation codon was determined. The DNA sequence shared approximately 95% identity with that of the ECNORRA sequence and contained an open reading frame, designated *imiR1*, that corresponded to the NmcR open reading frame. The encoded protein, ImiR1, was 97% identical (286 of 295 amino acids) to the NmcR protein and shared 40% identity with the regulatory protein SmeR (14).

PCR with primers specific to the 3′ coding sequences of *imiR1* and *imiA1* were used to determine if *E. cloacae* 1415B harbored an approximately 1.9-kb DNA fragment encoding ImiR1 and IMI-1. A positive PCR product of the expected size was obtained when *E. cloacae* 1415B chromosomal DNA was used as the source of the template DNA (data not shown). The fragment corresponded in size to that derived by using the *E. cloacae* 1413B ECIMIRA cloned fragment as the template DNA. Restriction fragment length polymorphism analysis of the PCR products with five different enzymes revealed the same restriction pattern with both the *E. cloacae* 1415B and ECIMIRA PCR products (data not shown). This indicates that *E. cloacae* 1415B also harbors the coding sequence for IMI-1 and ImiR1.

DISCUSSION

Two *E. cloacae* strains highly resistant to penem and carbapenem antibiotics were isolated from different patients in the intensive care unit of a California hospital in May and November 1984 at least a year before imipenem was approved for general clinical use in the United States (November 1985). Both strains had the same susceptibility profile for β-lactams, in that they were also resistant to penicillins, cefoxitin, and early cephalosporins but susceptible to expanded-spectrum cephalosporins. Although the aminoglycoside resistance profiles were different, the two strains appeared to be replicates, with a plasmid-encoded ANT2″ marker in isolate 1415B. Both strains produced a pI 7.05 β-lactamase inducible by imipenem but not cefoxitin, in addition to a presumed TEM-1 and a pI 8.1 β-lactamase inducible by both cefoxitin and imipenem, consistent with the type C cephalosporinase produced by *E. cloacae* (7, 13, 24). Of these three enzymes only the non-EDTA-inhibitable pI 7.05 β-lactamase hydrolyzed imipenem.

Until the early 1990s, all carbapenem-hydrolyzing β-lactamases were assumed to be metallo-β-lactamases (4). However, exceptions to this presumed functional-molecular relationship have recently appeared. Two strains of *S. marcescens* that were also resistant to penems and carbapenems were isolated at the London Hospital during 1982 (29), again several years before any penem or carbapenem was approved for general use. A β-lactamase with a pI of 9.7, Sme-1, from these isolates was identified as a carbapenem-hydrolyzing enzyme, but it was initially assumed to be a metallo-β-lactamase (6). More recently, a carbapenem-resistant *E. cloacae* isolate was identified in 1990 in Paris, France. The enzyme produced by this strain, NmcA, was shown to be a class A serine β-lactamase, as was the Sme-1 β-lactamase, on the basis of the nucleotide sequencing of the genes encoding these proteins (15–17). Because of the high degree of sequence homology between IMI-1 and NmcA, IMI-1 may be considered an Nmc-type β-lactamase. Thus, the *E. cloacae* β-lactamases from the United States and France and the *S. marcescens* β-lactamase constitute a novel group of carbapenem-hydrolyzing serine β-lactamases, classified as group 2f in the functional classification based upon imipenem hydrolysis (5), and as a class A serine enzyme in the molecular scheme (1).

Hydrolysis profiles for the three class A carbapenem-hydrolyzing enzymes are similar in certain respects. All the enzymes appear to be primarily cephalosporinases with lesser, but effective, abilities to hydrolyze penicillins and imipenem (17, 29). In contrast to the metallo-β-lactamases that hydrolyze carbapenems but not monobactams (5), these serine enzymes also hydrolyze aztreonam. The differences between the two *E. cloacae* enzymes are evident, in that NmcA appears to hydrolyze cefotaxime and ceftazidime more rapidly than IMI-1. Inhibition characteristics differ, in that both enzymes are inhibited almost equally well by clavulanic acid, but tazobactam is more effective against the IMI-1 enzyme, whereas NmcA is less well inhibited by tazobactam than by clavulanate.

Whereas the two *E. cloacae* enzymes share a high degree of sequence homology and similar regulatory properties, they appear to be quite different from the Sme-1 β-lactamase, the only other imipenem-hydrolyzing β-lactamase in functional group 2f (5). The isoelectric point of the Sme-1 enzyme is significantly higher, pI 9.7 compared with pI values of 6.9 and 7.05 for the NmcA and IMI-1 enzymes, respectively. As one would expect, amino acid sequence homology is much lower for the Sme-1 enzyme compared with those for the two other serine carbapenem-hydrolyzing enzymes. IMI-1 and NmcA share greater than 95% sequence homology with each other, but have only approximately 70% homology with the Sme-1 *S. marcescens* enzyme (Fig. 4).

All the U.S. and French *E. cloacae* strains had β-lactamase activities that were inducible by cefoxitin and imipenem (17). Slight inducibility was seen for the Sme-1 β-lactamase (14). In both sets of strains cephalosporinase as well as imipenem-hydrolyzing activities were inducible, suggesting that both enzymes within the strain are controlled by the same regulatory components. However, in the IMI-1-producing strain, only the cephalosporinase activity was inducible by cefoxitin, but both the cephalosporinase and IMI-1 activities were inducible by imipenem. It is curious that the *S. marcescens* isolates produced a constitutive carbapenem-hydrolyzing enzyme, but an inducible cephalosporinase. Expression of the NmcA β-lactamase is positively regulated by NmcR, the product of a gene whose promoter is overlapping and divergent from bla_{NmcA} (15). As seen in the present study, the regulatory proteins sequenced from the two strains *E. cloacae* NOR-1 and 1413B have sequences that are highly conserved, supporting the observation that these proteins should have similar biological functions. Further studies are necessary to explore the differences in induction properties.

In conclusion, the production of multiple chromosomal-type inducible β-lactamases by individual isolates of the family *Enterobacteriaceae* represents a newly emerging threat. Novel carbapenem-hydrolyzing enzymes are being described in retrospect from clinical populations that were never previously exposed to that class of β-lactam. Although production of these enzymes is rare, it is obvious that these isolates were present previously in the environment and can now be selected when challenged with the appropriate antimicrobial agent. As carbapenems are used more frequently, a larger number of these enzymes can be expected in resistant populations.

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