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Carbapenem-Resistant Strain of *Klebsiella oxytoca* Harboring Carbapenem-Hydrolyzing β-Lactamase KPC-2

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We investigated a *Klebsiella oxytoca* isolate demonstrating resistance to imipenem, meropenem, extended-spectrum cephalosporins, and aztreonam. The MICs of both imipenem and meropenem were 32 μg/ml. The β-lactamase activity against imipenem and meropenem was inhibited in the presence of clavulanic acid. Isoelectric focusing studies demonstrated five β-lactamases with pIs of 8.2 (SHV-46), 6.7 (KPC-2), 6.5 (unknown), 6.4 (probable OXY-2), and 5.4 (TEM-1). The presence of the bla_{SHV} and bla_{TEM} genes was confirmed by specific PCR assays and DNA sequence analysis. Transformation and conjugation studies with *Escherichia coli* showed that the β-lactamase with a pI of 6.7, *Klebsiella pneumoniae* carbapenemase-2 (KPC-2), was encoded on an approximately 70-kb conjugative plasmid that also carried SHV-46, TEM-1, and the β-lactamase with a pI of 6.5. The bla_{KPC-2} determinant was cloned in *E. coli* and conferred resistance to imipenem, meropenem, extended-spectrum cephalosporins, and aztreonam. The amino acid sequence of KPC-2 showed a single amino acid difference, S174G, when compared with KPC-1, another carbapenem-hydrolyzing β-lactamase from *K. pneumoniae* 1534. Hydrolysis studies showed that purified KPC-2 hydrolyzed not only carbapenems but also penicillins, cephalosporins, and aztreonam. KPC-2 had the highest affinity for meropenem. The kinetic studies revealed that KPC-2 was inhibited by clavulanic acid and tazobactam. An examination of the outer membrane proteins of the parent *K. oxytoca* strain demonstrated that it expressed detectable levels of OmpK36 (the homolog of OmpC) and a higher-molecular-weight OmpK35 (the homolog of OmpF). Thus, carbapenem resistance in *K. oxytoca* 3127 is due to production of the Bush group 2f, class A, carbapenem-hydrolyzing β-lactamase KPC-2. This β-lactamase is likely located on a transposon that is part of a conjugative plasmid and thus has a very high potential for dissemination.

The carbapenems, such as imipenem and meropenem, are used with increasing frequency in the United States and elsewhere for the treatment of multiresistant gram-negative nosocomial pathogens (20, 26). Resistance to carbapenems, while uncommon in enteric organisms, can be mediated by three unique mechanisms. The first mechanism of carbapenem resistance entails the production of large quantities of a chromosomal AmpC cephalosporinase combined with decreased drug permeability through the bacterial outer membrane due to loss or alteration of porins. This has been reported for *Enterobacter cloacae* (25, 39), *Enterobacter aerogenes* (7, 10), *Proteus rettgeri* (39), *Citrobacter freundii* (28), *Escherichia coli* (8, 45), and *Klebsiella pneumoniae* (2, 4). The second mechanism is the production of a β-lactamase that is capable of hydrolyzing carbapenems (5, 41, 47). The third mechanism of resistance, although uncommon, involves changes in the affinity of the target enzymes, the penicillin-binding proteins, for carbapenems (14).

K. pneumoniae carbapenemase-1 (KPC-1) is a class A β-lactamase that is capable of hydrolyzing carbapenems (47). KPC-2 is a closely related enzyme that also hydrolyzes carbapenems and has been reported from isolates of *Salmonella enterica* serotype Cubana (31) and *K. pneumoniae* (32). The amino acid sequence of KPC-2 showed a single amino acid difference, S174G, when compared with KPC-1, a carbapenem-hydrolyzing β-lactamase from *K. pneumoniae* 1534. In this study, a *Klebsiella oxytoca* strain manifesting carbapenem resistance was identified through project ICARE (Intensive Care Antimicrobial Resistance Epidemiology) (1, 19) and analyzed for its mechanism(s) of carbapenem resistance. The results of our study suggest that the carbapenem resistance phenotype of the strain was caused solely by the production of KPC-2 and was not attributable to modifications of the organism's porins. In addition, we provide evidence that the gene encoding this carbapenemase is located on a mobile element related to IS*21*.

MATERIALS AND METHODS

Bacterial strains. The carbapenem-resistant strain *K. oxytoca* 3127 was collected as part of project ICARE (1, 19) from the urine of a hospitalized patient in New York in 1998. Identification of the isolate was confirmed by using standard biochemical tests (16). *E. coli* HB101 [F^- *supE44 lacY1 ara-14 galK2 xyl-5 mtl-1 leuB6* Δ(*mcrC-mrr*) *recA13 rpsL20 thi-1* Δ(*gpt-proA*)*62 hsdSB20* λ^-] (42) was used for electroporation of plasmid DNA isolated from strain 3127 and as a recipient in conjugal mating experiments (42). *E. coli* DH5α (*supE44*

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Δ*lacU169* [ϕ*80 lacZ*Δ*M15*] *hsdR17 recA1 gyrA96 thi-1 relA1*) (*maA-10*) was used for cloning the β-lactamase and plasmid DNA preparation of the clone for DNA sequence analysis (42). *K. pneumoniae* ATCC 13883 (the type strain) was used as a control for porin profiles.

Antimicrobial susceptibility testing. Organisms were tested by broth microdilution using Mueller-Hinton broth (BD Biosciences, Sparks, Md.) and the National Committee for Clinical Laboratory Standards (NCCLS) reference method (33) and by disk diffusion using Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) as described previously by NCCLS (34). Antimicrobial agent powders were obtained from the following sources. Amikacin, amoxicillin, ampicillin, cefotaxime, ceftriaxone, chloramphenicol, gentamicin, piperacillin, tetracycline, and trimethoprim-sulfamethoxazole were from Sigma Chemical Co. (St. Louis, Mo.); aztreonam was from Bristol-Myers Squibb (Princeton, N.J.); ceftazidime and tobramycin were from Eli Lilly (Indianapolis, Ind.); cefoxitin was from Merck (Rahway, N.J.); cefpodoxime was from Pharmacia-Upjohn (Kalamazoo, Mich.); clavulanic acid was from SmithKline Beecham (King of Prussia, Pa.); and tazobactam was from Lederle (Pearl River, N.Y.). All antimicrobial agent-containing disks were obtained from Fisher Scientific (Pittsburgh, Pa.). *E. coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853 (33–35), *E. coli* HB101, and *E. coli* DH5α were used for quality control.

Isoelectric focusing of β-lactamases. Crude cell lysates were prepared by a previously described freeze-thaw procedure (46). Isoelectric focusing (IEF) was performed as described by Matthew and Harris (30). Cell extracts were analyzed by using commercially prepared polyacrylamide gel plates (pH 3.5 to 9.5; Amersham-Pharmacia, Piscataway, N.J.) and electrophoresed to equilibrium using an LKB Multiphor II apparatus (Pharmacia LKB, Piscataway, N.J.). β-Lactamases were visualized by staining the IEF gel with a 0.05% (0.96 mM) solution of nitrocefin (BD Biosciences). The isoelectric points of SHV-46 (8.2), TEM-1 (5.4), OXY-2 (6.4), and KPC-2 (6.7) were estimated by comparison to those of TEM-1 (5.4), SHV-5 (8.2), TEM-3 (6.3), and MIR-1 (8.6).

Examination of porin genes and porin expression. PCR amplifications were performed in a Thermoline Amplitron 1 thermal cycler by using *Taq* polymerase (Pharmacia) with 30 cycles of amplification (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C). The primers used to amplify porin genes were U681 and L1316, which anneal to conserved sequences in porin genes located 215 and 850 bp downstream of the *ompK36* start codon (12), respectively.

Outer membrane proteins (OMPs) were isolated by Sarkosyl extraction of total membrane preparations as described previously (22). Protein concentrations were determined with the bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.) as described by the manufacturer. The proteins were examined on either 8 to 15% sodium dodecyl sulfate-polyacrylamide linear gradient gels or 4 to 12% NuPAGE gels with morpholinepropanesulfonic acid (MOPS) buffer (Invitrogen, Carlsbad, Calif.). For OmpK37 analysis, electrophoresis of OMPs was performed on 11% acrylamide–0.2% bisacrylamide–0.1% sodium dodecyl sulfate gels (12). Samples were boiled for 5 min in Laemmli's sample buffer before electrophoresis. Gels were visualized by staining with Coomassie blue R250.

Western blotting of OmpK35, OmpK36 and OmpK37 was performed as described previously (12, 22). Filters were blocked in 1% bovine serum albumin in phosphate-buffered saline (PBS). After being washed, the filters were incubated with 1:100-diluted anti-OmpK35 or anti-OmpK36 or anti-OmpK37 antibody (12, 22) and then with alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (Sigma; 1:5,000). The filters were developed as previously described (12, 22). All the incubations were carried out at room temperature for 1 h in 1% bovine serum albumin–0.05% Tween 20–PBS and, after incubation with the antiserum, the filters were washed with 0.05% Tween 20–PBS.

Plasmid profile analysis. Plasmid DNA from *K. oxytoca* 3127 was isolated by using the method described by Portnoy et al. (36). Supercoiled plasmid DNAs of pDK9 (165 kb) and R1 (97.6 kb) and the plasmids in *E. coli* V517 (56.7, 5.8, 4.09, 3.15, 2.83, and 2.2 kb) were used as size standards.

Carbapenem inactivation assay. In order to determine whether resistance to imipenem and meropenem was caused by production of a β-lactamase, a disk diffusion bioassay using *E. coli* DH5α was performed as previously described (47). Negative controls for carbapenemase production were *E. coli* HB101 and *K. pneumoniae* ATCC 13883. The positive control was *K. pneumoniae* 1534 (47).

Filter mating. Filter mating studies were performed at both 30 and 37°C (42). *E. coli* HB101 was used as the recipient. The transconjugants were selected on Luria-Bertani (LB) agar containing 30 μg of tetracycline per ml, 2 μg of imipenem per ml, and 120 μg of streptomycin per ml.

Transformation. Plasmid DNA prepared from *K. oxytoca* 3127 via Qiagen plasmid midiprep kit (Qiagen, Chatsworth, Calif.) was electroporated into *E. coli* HB101 as described previously (42). Transformants were selected on LB agar containing 120 μg of streptomycin per ml and 1.5 μg of imipenem per ml.

Cloning of bla_{KPC-2}. The cloning and sequencing of KPC-2 were carried out as described by Yigit et al. (47).

bla_{SHV}-, bla_{TEM}-, and bla_{KPC-2}-specific PCR and DNA sequence analysis. The primers and the PCR conditions used for amplification of bla_{SHV} and bla_{TEM} were those described by Rasheed et al. (40). The bla_{KPC-2} determinant was amplified from the parent strain, *K. oxytoca* 3127, by using the protocol and primers for bla_{KPC-1} described by Yigit et al. (47).

DNA sequencing data were analyzed by using DNASIS for Windows (Hitachi Software Genetic Systems, San Francisco, Calif.). The DNA and protein sequences of the other β-lactamases were obtained from the EMBL and the Swiss-Prot data banks. BLAST and BLASTX programs from the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) were used to identify bla_{KPC-2}.

β-Lactamase purification. The KPC-2 β-lactamase was purified from the *E. coli* DH5α strain containing cloned bla_{KPC-2} (*E. coli* DH5α pBR322-*catI*-bla_{KPC-2}) for kinetic analysis studies. Four 1-liter cultures of tryptic soy broth supplemented with 100 μg of ampicillin per ml were grown overnight at 37°C. Bacteria were harvested by centrifugation and washed with 50 mM phosphate buffer (pH 7.0). The pellets were resuspended in 10 ml of 0.2 M sodium acetate (pH 5.5) and subjected to five freeze-thaw cycles. The lysate was centrifuged at 20,000 × *g*, and the β-lactamase activity of the supernatant was enriched by chromatography through Sephadex G-100 in 50 mM phosphate buffer (pH 7.0). Protein in peak fractions containing nitrocefin-hydrolyzing activity was precipitated with 90% ammonium sulfate; pellets were resuspended in 20 mM morpholineethanesulfonic acid (MES) buffer (pH 6.0)–10% glycerol, and dialyzed in 2 liters of the same buffer at 4°C. The β-lactamase was desalted through a HighTrap desalting column (Amersham-Pharmacia) and eluted from a HiTrap-S cation exchange column in 20 mM MES (pH 6.0)–10% glycerol by a 0 to 0.5 M NaCl gradient. The protein concentration of the HiTrap-S fractions was determined with the Micro Coomassie Plus protein assay (Pierce). The purity of the KPC-2 fractions was determined by scanning densitometry of a Colloidal Blue-stained NuPAGE 10% Bis-Tris gel. Purity of the fractions used for kinetic analysis was >90%.

Kinetic studies. Initial hydrolysis rates were measured on a Shimadzu UV-1601 spectrophotometer at 25°C in 50 mM phosphate buffer (pH 7.0). K_m and V_{max} values were obtained by averaging results from Eadie-Hofstee, Hanes-Woolf, and direct linear plot analyses. Spectrophotometric measurements were determined on several different days, with cephaloridine assayed as a reference each day. The standard error for calculated kinetic parameters was ≤15%. Inhibition of hydrolysis was measured after a 5-min preincubation of enzyme with inhibitor in phosphate buffer (pH 7.0). Nitrocefin at a concentration of 100 μM was the substrate used for the inhibition studies. K_i values were determined by the method of Dixon (11). For comparison, a preparation of KPC-1 purified under the same conditions as KPC-2 was used to determine K_i values.

Gene dosage assays. The gene dosage assays were performed as described by Heritage et al. (21). The plasmid pBR322-*catI* (47) was used for this assay. Transposition of a bla_{KPC-2}-containing element onto pBR322-*catI* should increase the copy number for bla_{KPC-2}, which, in turn, should increase the imipenem MIC because of the gene dosage effect. The overnight culture of *E. coli* DH5α carrying both the 70-kb plasmid and pBR322-*catI* was plated on the LB agar containing 128 μg of imipenem per ml and 40 μg of chloramphenicol per ml. The overnight cultures of *E. coli* DH5α transformant containing only the 70-kb plasmid were used as background controls and were serially diluted and plated on the LB agar containing 128 μg of imipenem per ml.

Nucleotide sequence accession numbers. The nucleotide sequence of bla_{KPC-2} reported in this study will appear under the GenBank accession number AY210886, and that of bla_{SHV-46} will appear under accession number AY210887.

RESULTS

Antimicrobial susceptibility patterns of *K. oxytoca* 3127. The MICs of a variety of antimicrobial agents tested against *K. oxytoca* 3127 are shown in Table 1. The isolate was resistant to imipenem and meropenem, with MICs of 32 μg/ml for each drug. The isolate was also resistant to extended-spectrum cephalosporins and aztreonam. The MICs of both imipenem and meropenem decreased from 32 to 4 μg/ml when tested in the presence of clavulanic acid (4 μg/ml) (Table 1). In the *E. coli* transformants, the imipenem and meropenem MICs decreased by five doubling dilutions from 16 to 0.5 μg/ml and 8 to

TABLE 1. Antimicrobial susceptibility patterns of *K. oxytoca 3127, E. coli* DH5α clone, and *E. coli* HB101 transformant[a]

Antimicrobial agents	*K. oxytoca* 3127 (parent)	*E. coli* DH5α	*E. coli* DH5α (pBR322-*catI*-bla_{KPC-2})	*E. coli* HB101 transformant containing bla_{KPC-2}	*E. coli* HB101
Imipenem	32	≤0.25	16	15	≤0.25
Imipenem-clavulanic acid[b]	4	≤0.25	0.5	0.5	≤0.25
Meropenem	32	≤0.25	8	8	≤0.25
Meropenem-clavulanic acid[b]	4	≤0.25	≤0.25	≤0.25	≤0.25
Ampicillin	>64	2	>64	>64	4
Amoxicillin-clavulanic acid	>32/16	2/1	>32/16	>32/16	2/1
Piperacillin-tazobactam	>128/4	≤1/4	>128/4	>128/4	≤1/4
Aztreonam	>64	≤1	>64	>64	≤1
Ceftazidime	>64	≤2	32	64	≤2
Cefoxitin	>32	2	32	32	4
Cefpodoxime	>16	0.5	>16	>16	≤0.25
Cefepime	>32	≤1	16	>32	≤1
Cefotaxime	>64	≤1	8	64	≤1
Ceftriaxone	>64	≤1	32	64	≤1
Chloramphenicol	8	4	>32	4	4
Gentamicin	8	≤0.25	≤0.25	8	≤0.25
Tobramycin	16	≤0.25	≤0.25	16	≤0.25
Trimethoprim-sulfamethoxazole	>8	≤0.12	≤0.12	≤0.12	≤0.12

[a] Values are MICs and are given in micrograms per milliliter.
[b] Clavulanic acid was tested at a fixed concentration of 4 μg/ml.

≤0.25 μg/ml, respectively, when the carbapenems were tested in combination with clavulanic acid. The MICs of ceftazidime, ceftriaxone, and cefotaxime also decreased by two to four doubling dilutions in the presence of clavulanic acid in the parent strain (data not shown).

Imipenem and meropenem resistance involves production of β-lactamase. IEF of crude extracts of strain 3127 revealed five β-lactamase bands with pIs of 8.2, 6.7, 6.5, 6.4, and 5.4 (Fig. 1A, lane 7). To determine whether resistance to carbapenems could be attributed to production of a β-lactamase, we performed a disk diffusion carbapenem inactivation assay (47). The assay results indicated that a β-lactamase was involved in hydrolysis of imipenem in 3127. The crude cell lysates prepared in the presence and absence of imipenem showed similar zone alterations (data not shown). The presence of EDTA did not inhibit the activity of the β-lactamase, nor did the addition of $ZnCl_2$ enhance the β-lactamase activity against imipenem or meropenem (data not shown).

PCR and DNA sequence analysis of bla_{SHV} and bla_{TEM}. IEF results for *K. oxytoca* 3127 suggested the presence of bla_{TEM} (pI 5.4) and bla_{SHV} (pI 8.2) determinants (Fig. 1A, lanes 2, 3, and 7). PCR analysis using bla_{SHV}- and bla_{TEM}-specific primers confirmed the presence of these genes in strain 3127 (data not shown). DNA sequence analysis identified the genes as bla_{TEM-1} and bla_{SHV-46}. The amino acid sequence of SHV-46 showed three amino acid changes (T195N, G238S, and E240K) when compared to the sequence of SHV-1. The β-lactamase with a pI of 6.4 is presumed to be the chromosomal OXY-2, which has been reported to have a pI ranging from 5.2 to 6.8 (17, 18). Since the IEF of lysates prepared from transformants and transconjugants of strain 3127 did not produce this enzyme (Fig. 1A, lanes 5 and 6, respectively), it is presumed to be chromosomal in origin. The identity of the β-lactamase with a pI of 6.5 carried on the 70-kb plasmid, while consistent with OXA enzymes (9), remains elusive.

Cloning of the bla_{KPC-2} gene from the *E. coli* DH5α transformant. The filter mating results between strain 3127 and *E. coli* HB101 showed that the carbapenem resistance in 3127 was associated with a 70-kb conjugative plasmid that encoded four β-lactamases with pIs of 8.2, 6.7, 6.5, and 5.4 (Fig. 1A, lane 6). Resistance to carbapenems, extended-spectrum cephalosporins, and aztreonam was encoded on the same plasmid (Table 1). The carbapenem MICs for the *E. coli* HB101 transconjugants containing the plasmid encoding bla_{SHV-46}, bla_{TEM-1}, bla_{KPC-2}, and the fourth β-lactamase were similar to those for the parent isolate. The MIC results also suggested that the resistance determinant encoding gentamicin and tobramycin resistance was located on the 70-kb conjugative plasmid. The DNA isolated from a transconjugant was electroporated into E. *coli* HB101 and used for IEF. The IEF results showed that this plasmid encoded SHV-46, TEM-1, and a β-lactamase with a pI of 6.5, in addition to KPC-2 (Fig. 1A, lane 5). The presence of bla_{SHV-46} and bla_{TEM-1} was also confirmed in the transconjugants and transformants by PCR analysis.

To characterize the β-lactamase mediating carbapenem resistance, we cloned a 2.4-kb *Bam*HI fragment encoding KPC-2 in DH5α using pBR322-*catI* as a cloning vector. *E. coli* (pBR322-*catI*-bla_{KPC-2}) encoded a single β-lactamase with a pI of 6.7 as shown by IEF (Fig. 1B, lanes 2 and 3). The antibiogram of the *E. coli* DH5α bla_{KPC-2} clone is shown in Table 1. This demonstrates that bla_{KPC-2} is responsible for the resistance to carbapenems, extended-spectrum cephalosporins, and aztreonam.

Kinetic parameters. The kinetic parameters for the KPC-2 β-lactamase are summarized in Table 2. The KPC-2 enzyme used in these studies was approximately 90% pure. KPC-2 hydrolyzed β-lactams from the penicillin, cephalosporin, carbapenem, and monobactam groups. The highest k_{cat} values were obtained with cephaloridine, which demonstrated k_{cat} values that were approximately seven times higher than those

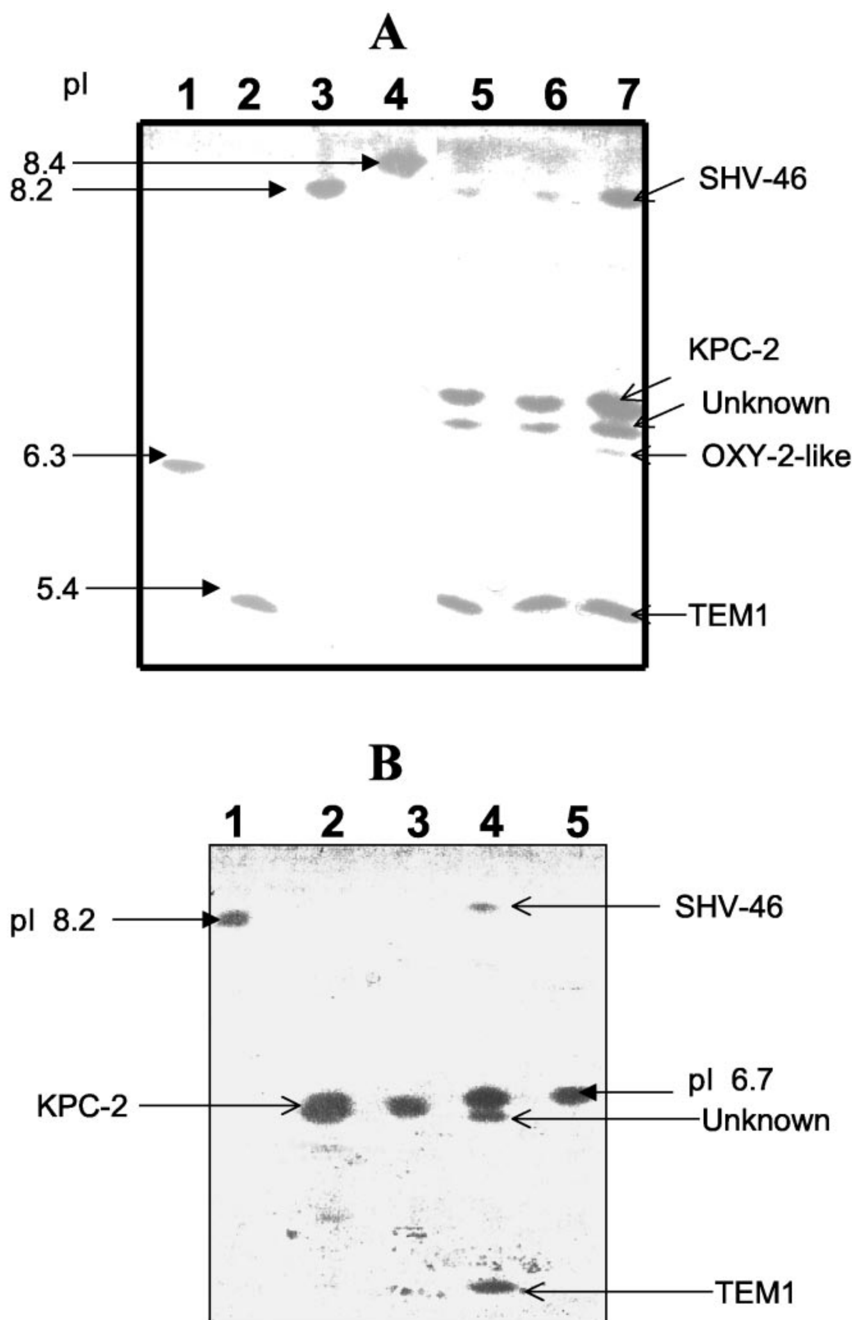

FIG. 1. (A) Isoelectric focusing patterns of cell lysates from carbapenem-resistant strains. The gel was stained with nitrocefin. Lanes 1 to 4, cell lysates prepared from strains producing TEM-3 (pI 6.3), TEM-1 (pI 5.4), SHV-5 (pI 8.2), and MIR-1 (pI 8.4), respectively; lane 5, the imipenem-resistant *E. coli* HB101 transformant containing a 70-kb plasmid from 3127; lane 6, an imipenem-resistant *E. coli* HB101 transconjugant of 3127; lane 7, *K. oxytoca* 3127. (B) Isoelectric focusing patterns of cell lysates prepared from carbapenem-resistant clones of *K. oxytoca* 3127. Lane 1, strain producing SHV-46 (pI 8.2); lanes 2 to 3, clones of strain 3127; lane 4, an imipenem-resistant *E. coli* HB101 transconjugant of K. *oxytoca* 3127; lane 5, *E. coli* DH5α containing the bla_{KPC-1} clone. The pIs of the β-lactamases were calculated by using the known pIs of TEM-1 (5.4), TEM-3 (6. 3), SHV-5 (8.2), KPC-1 (6.7), and MIR-1 (8.4).

for cephalothin or nitrocefin and 2.5 times higher than that for ampicillin. The k_{cat} values for penicillin G, cloxacillin, and aztreonam were similar, approximately 10 to 17 times lower than those for cephaloridine. KPC-2 showed hydrolytic activity against the carbapenems; hydrolysis of imipenem occurred at rates that were approximately 35 times slower than those for cephaloridine. Meropenem had k_{cat} values four times lower than those for imipenem. Hydrolysis rates for cefotaxime and ceftazidime were 24 and 4,416 times lower than the values obtained for cephaloridine. Of the two extended-spectrum cephalosporins tested, cefotaxime had the highest k_{cat} values, which were approxi-

TABLE 2. Hydrolysis parameters of KPC-1 and KPC-2 β-lactamases

Substrate or inhibitor	k_{cat} (s^{-1})		Relative k_{cat}		K_m (μM)		k_{cat}/K_m (μM^{-1} s^{-1})		Relative k_{cat}/K_m	
	KPC-1[a]	KPC-2	KPC-1	KPC-2	KPC-1	KPC-2	KPC-1	KPC-2	KPC-1	KPC-2
Substrates										
Cephaloridine	340	530	100	100	560	500	0.61	1.1	100	100
Cephalothin	75	69	22	13	53	82	1.4	0.84	230	76
Cefotaxime	14	22	4.2	4.2	160	220	0.09	0.10	14	9.1
Cefoxitin	0.26	0.31	0.08	0.06	120	180	0.002	0.002	0.36	0.16
Ceftazidime	0.10	≤0.12	0.03	≤0.02	94	ND[b]	0.001	ND	0.18	ND
Benzylpenicillin	32	51	9.6	9.6	23	27	1.4	1.9	230	170
Ampicillin	110	210	33	40	130	230	0.85	0.91	140	83
Cloxacillin	25	35	7.4	6.6	100	79	0.25	0.44	41	40
Imipenem	12	15	3.7	2.8	81	51	0.15	0.29	26	26
Meropenem	3.0	4.0	0.9	0.75	12	15	0.25	0.27	41	25
Aztreonam	20	30	5.9	5.7	310	360	0.07	0.08	11	7.6
Inhibitors										
Clavulanic acid[c]					1.2	1.5				
Tazobactam[c]					0.23	0.18				
EDTA					>5,000	>5,000				

[a] KPC-1 k_{cat} and K_m data are from reference 47.
[b] ND, not determined.
[c] Inhibitor constant (K_i) is micromolar.

mately 180 times higher than the k_{cat} values for ceftazidime. Of the 12 substrates used in these experiments, cefoxitin and ceftazidime had the lowest hydrolysis rates.

KPC-2 had the highest affinity for meropenem, with a K_m value of 15 μM. Other substrates with low K_m values were nitrocefin, penicillin G, and imipenem, whose K_m values ranged from 22 to 51 μM. Cephaloridine, at 500 μM, had the highest K_m.

Hydrolytic efficiencies, measured by k_{cat}/K_m, revealed that penicillin G was hydrolyzed by KPC-2 approximately two times more efficiently than cephaloridine. Nitrocefin had the highest catalytic efficiency of the substrates tested, with a value 3.3 times that of cephaloridine. The hydrolytic efficiencies for imipenem and meropenem were <30% that of cephaloridine. Cefotaxime was the most efficiently hydrolyzed of the three extended-spectrum cephalosporins tested, with a k_{cat}/K_m value that was approximately 10% that of cephaloridine. The hydrolytic efficiency of cefoxitin was 600-fold lower than that for cephaloridine. The hydrolytic efficiency for ceftazidime could not be determined directly due to extremely slow hydrolysis.

The KPC-2 β-lactamase demonstrated K_i values of 1.5 μM for clavulanic acid and 0.18 μM for tazobactam. These were similar to the values obtained for KPC-1 under the same conditions (Table 2). No inhibition was observed when the enzyme was tested with 5 mM EDTA at pH 7.0.

***bla*$_{\mathbf{KPC\text{-}2}}$ may be located on a mobile element.** The 157-amino-acid partial sequence (GenBank accession number AAO53444.1) encoded in the 863-nucleotide sequence upstream of the *bla*$_{KPC\text{-}2}$ coding region showed a high degree of similarity to several IstB-like proteins: 52% similarity to the IS*21* putative ATP-binding protein (GenBank accession number P15026), 68% to the putative IS*100* transposase from *Yersinia pestis* C092 (GenBank accession number NP_395401), 68% to the putative transposase from *Y. pestis* (GenBank accession number AAC44982), and 98% to the putative transposition helper protein from *S. enterica* subsp. *enterica* serotype Cubana (GenBank accession number AAM10642) (Fig. 2). IstB-like proteins are ATP-binding proteins that contain an ATP- or GTP-binding P-loop motif (http://www.ncbi.nlm.nih.gov). The IstB-like proteins are associated with the IS*21* family of insertion sequences (6, 27). The functions of IstB-like proteins include stimulation of transposase and cointegrase-driven reactions (6). Thus, we investigated whether *bla*$_{KPC\text{-}2}$ was located on an active transposable element.

The 70-kb plasmid from the *E. coli* HB101 transconjugant was electroporated into *E. coli* DH5α containing pBR322-*catI*. Since pBR322-*catI* is a multicopy cloning vector, we hypothesized that it would facilitate the detection of the transposition of *bla*$_{KPC\text{-}2}$ from the 70-kb plasmid by a gene dosage assay (21). The frequency of *bla*$_{KPC\text{-}2}$ transposition was 9.7×10^{-4}, as indicated by the number of colonies with elevated imipenem MICs (i.e., those growing on 128-μg/ml versus 16-μg/ml imipenem). The background rate of colonies containing only the 70-kb plasmid growing on 128-μg/ml imipenem was 5.7×10^{-8}.

Analysis of *K. oxytoca* 3127 OMPs. The MICs of meropenem, ceftazidime, and cefotaxime were lower for the *E. coli* HB101 transformants and *E. coli* DH5α(pBR322-*catI*-*bla*$_{KPC\text{-}2}$) than for the parent *K. oxytoca* 3127. This may be due to species differences among the porins, which are known to increase the MICs of these drugs for *K. pneumoniae* isolates (2, 4, 29). PCR analysis showed that *K. oxytoca* 3127 and *K. pneumoniae* ATCC 13883 (the extended-spectrum cephalosporin-susceptible type strain) both carry all three porin genes, *ompK35*, *ompK36*, and *ompK37* (data not shown). The porin profile of *K. oxytoca* 3127 was compared to that of *K. pneumoniae* ATCC 13883 (Fig. 3), and the expression of the porin genes was examined by Western blotting with polyclonal anti-OmpK35, anti-OmpK36, and anti-OmpK37 antisera. As reported by Hernandez-Alles et al. (22), the presence of OmpK35 and OmpK36 cannot be determined solely by their migration in gels, since in some strains OmpK35 migrates more slowly than OmpK36. This is the case (Fig. 3A) for *K. pneumoniae* ATCC 13883 (lane 3) and *K. oxytoca* 3127 (lane 2),

```
                         90                  100                 110                 120
75   T F Q P G I D R K V V R E L A G L A F V E R S E N V I L L G P P G V G K T H L A  P15026
79   S F N P G V P K A L V Q E L G S L A F V E R A E N V V L I G P S G I G K T H L A  AAM10642
1    - - - - - - - - - - - - - - - - - - - - - R A E N V V L I G P S G I G K T H L A  AAO53444.1
81   T F A T G A P Q K Q L Q S L R S L S F I E R N E N I V L L G P S G V G K T H L A  NP395401
80   T F A T G A P Q K Q L Q S L R S L S F I E R N E N I V L L G P S G V G K T H L A  AAC44982

                         130                 140                 150                 160
115  I A L G V K A V D A G H R V L F M P L D R L I A T L M K A K Q E N R L E R Q L Q  P15026
119  I A L G Y K A A Q A G V K T R F I T A A D L M L Q L S T A Q R Q G R L K E Y F N  AAM10642
20   I A L G Y K A A Q A G V K T R F I T A A D L M L Q L S T A Q R Q G R L K E Y F N  AAO53444.1
121  I A M G Y E A V R A G I K V R F T T A A D L L L Q L S T A Q R Q G R Y K T T L Q  NP395401
120  I A M G Y E A V R A G I K V R F T T A A D L L L Q L S T A Q R Q G R Y K T T L Q  AAC44982

                         170                 180                 190                 200
155  Q L S Y A - R V L I L D E I G Y L P M N R E E A S L F F R L L N R R Y E K A S I  P15026
159  R A I L G P R L L V I D E I G Y L P F G R E E A N L F F Q V V A K R Y E K G A M  AAM10642
60   R A I L G P R L L V I D E I G Y L P F G R E E A N L F F Q V V A K R Y E K G A M  AAO53444.1
161  R G V M A P R L L I I D E I G Y L P F S Q E E A K L F F Q V I A K R Y E K S A M  NP395401
160  R G V M A P R L L I I D E I G Y L P F S Q E E A K L F F Q V I A K R Y E K S A M  AAC44982

                         210                 220                 230                 240
194  I L T S N K G F A D W G E M F G - D H V L A T A I L D R L L H H S T T L N I K G  P15026
199  I L T S N L P F S Q W A D A F A G D T T L T A A M L D R L L H H A H I L T L S G  AAM10642
100  I L T S N L P F S Q W A D A F A G D T T L T A A M L D R L L P - - H I L T L S G  AAO53444.1
201  I L T S N L P F G Q W D Q T F A G D A A L T S A M L D R I L H H S H V V Q I K G  NP395401
200  I L T S N L P F G Q W D Q T F A G D A A L T S A M L D R I L H H S H V V Q I K G  AAC44982

                         250                 260                 270
233  E S Y R L K E K R K A G V L T K N T T P I S D D E M V K S G Q H Q                P15026
239  E S Y R L K D K R K A G V V R K N S K P E                                        AAM10642
138  E S Y R L K D K R K A G V V R K N S K P E                                        AAO53444.1
241  E S Y R L R Q K R K A G V I A E A N - P E                                        NP395401
240  E S Y R L R Q K R K A G V I A E A N - P E                                        AAC44982
```

FIG. 2. Alignment of the partial sequence of the protein encoded upstream of KPC-2 (GenBank accession number AAO53444.1) to IstB-like proteins (6); IS*21* putative ATP-binding protein (GenBank accession number P15026), putative IS*100* transposase from *Y. pestis* C092 (GenBank accession number NP_395401), putative transposase from *Y. pestis* (GenBank accession number AAC44982), and putative transposition helper protein from *S. enterica* subsp. *enterica* serotype Cubana (GenBank accession number AAM10642).

where OmpK35 apparently migrates more slowly than OmpK36. A Western blot produced using anti-OmpK36-specific antisera (Fig. 3B) confirmed the identity of OmpK36 bands for ATCC 13883 (lane 3) and 3127 (lane 2). Anti-OmpK35 antisera are known to cross-react with OmpK36 (22) and reacted, as predicted, with two bands in ATCC 13883 (OmpK35 and OmpK36) (Fig. 3C, lane 3) and with two bands in 3127 (lane 2). These results suggest that OmpK35 was expressed in *K. oxytoca* 3127 but migrated more slowly than the OmpK35 in ATCC 13883. Neither of the two strains appeared to express OmpK37 when tested with OmpK37-specific antisera (data not shown).

DISCUSSION

Carbapenems, such as imipenem and meropenem, are antibacterial agents with activity against many gram-negative, gram-positive, and anaerobic microorganisms. Carbapenems

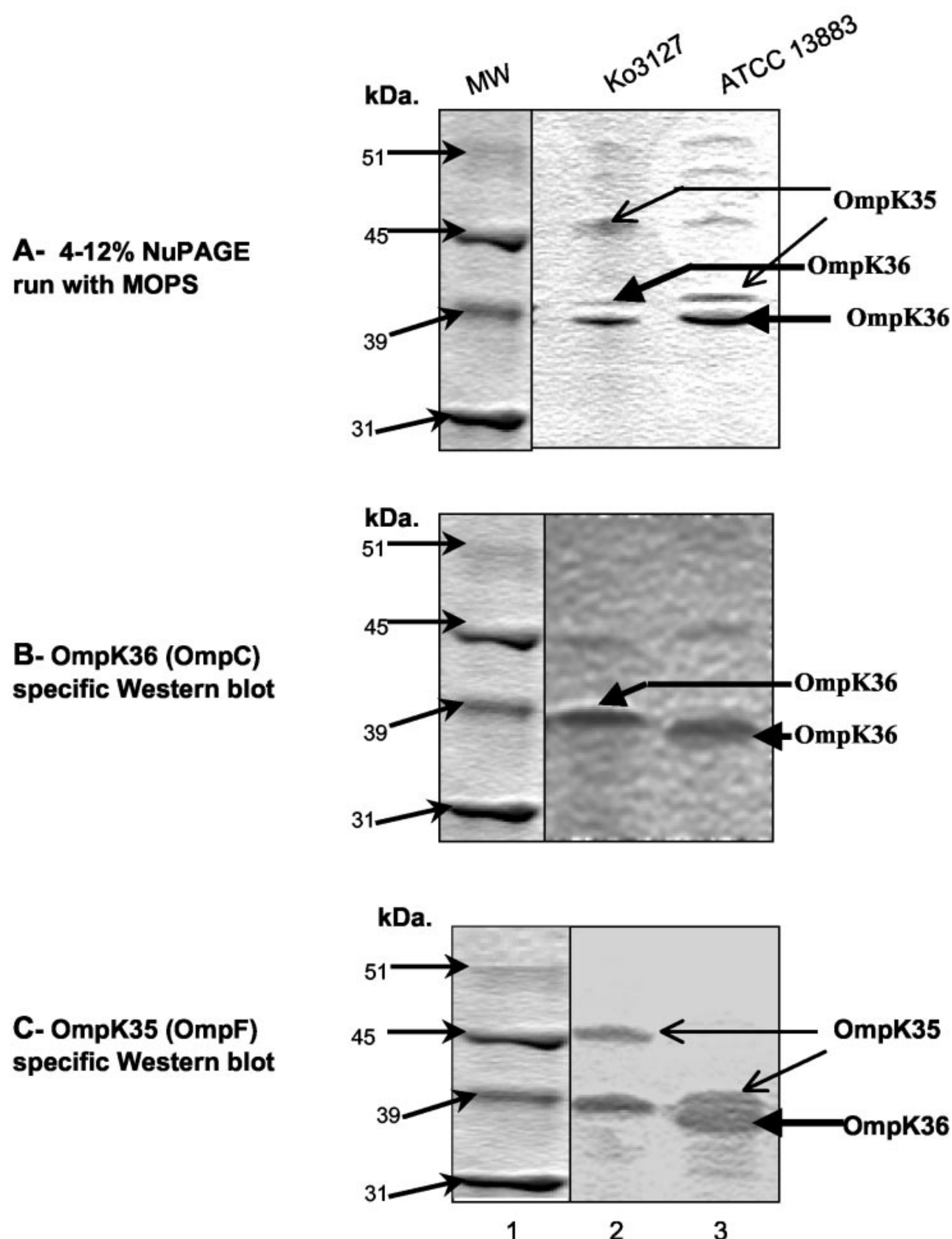

FIG. 3. NuPAGE gel and Western blot analysis of OMPs of *K. oxytoca* 3127 and a carbapenem-susceptible control strain, *K. pneumoniae* ATCC 13883. (A) NuPAGE gel analysis of OMPs. Lane 1, molecular mass markers; lane 2, OMPs prepared from *K. oxytoca* 3127; lane 3, OMPs prepared from *K. pneumoniae* ATCC 13883. (B) Western blot analysis of OMPs performed with anti-OMPK36 antisera. (C) Western blot analysis of OMPs performed with anti-OMPK35 antisera. Molecular mass is indicated in gels to the left of each panel.

are often used to treat infections caused by multidrug-resistant isolates, including strains producing extended-spectrum β-lactamases (ESBLs) (20, 26, 41, 43). However, the recent appearance of β-lactamases capable of hydrolyzing carbapenems, in addition to other mechanisms of carbapenem resistance, makes treating these infections more difficult (4, 20, 26, 41, 47).

Here we describe the appearance of the class A β-lactamase, KPC-2, in a strain of *K. oxytoca.* KPC-2 is closely related to the KPC-1 β-lactamase from *K. pneumoniae* 1534. KPC-2 was initially isolated from *S. enterica* serotype Cubana (31) (GenBank accession number AF481906). KPC-2 differs from KPC-1 by a single amino acid substitution, namely S174G. Our kinetic data show that the S174G substitution did not cause changes in the hydrolytic profile of the enzyme, as the k_{cat}, K_m, and K_i values for β-lactam substrates were similar for KPC-1 and KPC-2 (47).

During the last decade, many hospital outbreaks caused by ESBL-producing *Enterobacteriaceae* spp. have been reported. Most of the ESBL-producing strains carried derivatives of bla_{TEM-1}, bla_{TEM-2}, or bla_{SHV-1} (13, 23, 40). These β-lactamases are typically encoded on large conjugative plasmids, as KPC-2 is (3, 15, 24, 38, 44). More recent reports have high-

lighted the emergence of ESBL-producing strains that are multiply resistant to amikacin, gentamicin, sulfonamides, streptomycin, and trimethoprim (37, 38). The $bla_{\text{KPC-2}}$ determinant of *K. oxytoca* 3127 was located on a 70-kb conjugative plasmid that also encodes SHV-46, TEM-1, and a fourth unidentified β-lactamase. This plasmid also encodes resistance to gentamicin and tobramycin. Although the exact genetic structure has not been determined, the $bla_{\text{KPC-2}}$ determinant is presumably located on a transposable element, as suggested by gene dosage assays. The amino acid sequences inferred from DNA sequencing of the upstream region suggests that the KPC-2-encoding plasmid is 98% identical to the plasmid identified in the *Salmonella* serotype Cubana strain (GenBank accession number AF481906). Thus, the KPC-2 β-lactamase is likely being disseminated among species of *Enterobacteriaceae* through both conjugal plasmid transfer and transposition. Further studies are required to determine the identity and structure of this mobile element and its other resistance determinants.

We searched for alterations in one or more of the three porin proteins described for *K. pneumoniae* strains that are associated with increased MICs for extended-spectrum cephalosporins and carbapenems (12, 22, 29). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of *K. oxytoca* 3127 porin profiles, in combination with the Western blot analysis with OmpK35-, OmpK36-, and OmpK37-specific antibodies, showed that strain 3127 expresses OmpK36 (the OmpC homolog) and a slower-migrating OmpK35 (the OmpF homolog) (Fig. 3B and C). This finding is consistent with the observations of Hernandez-Alles et al., who reported that ESBL-producing *K. pneumoniae* strains can have OmpK35 porins that migrate more slowly than OmpK36 porins (22). Studies by Domenech-Sanchez et al. suggest that the newly identified porin, OmpK37, might be used by carbapenems to gain access to the cell (12); however, this porin is strongly down-regulated under standard laboratory conditions and is often seen only in the absence of OmpK35 and OmpK36 expression. Thus, its contribution to resistance in 3127 remains unclear.

In conclusion, we have isolated and characterized the class A carbapenemase determinant, KPC-2, from a clinical isolate of *K. oxytoca*. The data presented here show that KPC-2 is responsible for the carbapenem resistance of this strain, which, unlike the *K. pneumoniae* strain harboring KPC-1, has no detectable porin alterations.

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