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

Hesna Yigit,1 Anne Marie Queenan,2 J. Kamile Rasheed,3 James W. Biddle,3 Antonio Domenech-Sanchez,3 Sebastian Alberti,5 Karen Bush,2 and Fred C. Tenover3*

Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, Connecticut 06492; The R. W. Johnson Pharmaceutical Research Institute, Raritan, New Jersey 08869; Division of Healthcare Quality Promotion, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333; and Unidad de Investigacion, Hospital Son Duret, Andreu Doria, Palma de Mallorca 07014, and Area de Microbiologia, Universidad de las Islas Baleares, Ctra. Valldemosa, Palma de Mallorca 07071, Spain

Received 6 May 2003/Returned for modification 14 July 2003/Accepted 31 August 2003

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*<sub>SHV</sub> and *bla*<sub>TEM</sub> 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*<sup>KPC-2</sup> 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 Cuban (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 IS21.

---

*Corresponding author. Mailing address: Division of Healthcare Quality Promotion (G08), Centers for Disease Control and Prevention, 1600 Clifton Rd., Atlanta, GA 30333, Phone: (404) 639-3375. Fax: (404) 639-1381. E-mail: fn1t@cdc.gov.

**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<sup>+</sup> supE44 lacY1 ara-14 gadK2 sul2 mtl-1 lacS8 Δ(mcrC-mrE) recA13 rpsL20 thi-1 Δ(gps-proA462 bsdR82 l×)] (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...
λΔlacU169 [589 lacZAM15] indI17 recA1 gyrA96 thi-1 relA1) (maa-I-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 proteins.

**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, cefepime, 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.); cefazidime and tobramycin were 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 Weatherbee (29). Cell extracts were analyzed by using commercially prepared polyacrylamide gel plates (pH 3.5 to 9.5; Amperose, Amersham Pharmacia Biotech, Piscataway, N.J.). Carboxyfluorescein diacetate (CFDA) and dihydrofluorescein diacetate (DHFDA) were used as size standards. (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 (Valencia, Calif.) was introduced into E. coli DH5α 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 blαKPC-2.** The cloning and sequencing of KPC-2 were carried out as described by Yigit et al. (47).

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

**DNA sequencing data were analyzed by using DNASTAR 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 blαKPC-2.**

**β-Lactamase purification.** The KPC-2 β-lactamase was purified from the E. coli DH5α strain containing cloned blαKPC-2 (E. coli DH5α plR322-catl-blαKPC-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 morpholinethanesulfonic 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 S 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). Km and Vmax 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. Km 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 values.

**Gene dosage assays.** The gene dosage assays were performed as described by Heritage et al. (21). The plasmid pBR322-catl (47) was used for this assay. Transposition of a blαKPC-2-containing element onto pBR322-catl should increase the copy number for blαKPC-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-catl was plated on the LB agar containing 126 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 access numbers.** The nucleotide sequence of blαKPC-2 reported in this study will appear under the GenBank accession number AY210886, and that of blαSHV-1 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
≤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 pl’s 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 ZnCl₂ 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 (pl 5.4) and bla_SHV (pl 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 pl of 6.4 is presumed to be the chromosomal OXY-2, which has been reported to have a pl ranging from 5.2 to 6.8 (17, 18). Since the IEF of lysates prepared from transfectants 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 pl 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 pl’s 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, 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 pl 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 BamHI fragment encoding KPC-2 in DH5α using pBR322-cat1 as a cloning vector. E. coli (pBR322-cat1-bla_KPC-2) encoded a single β-lactamase with a pl of 6.7 as shown by IEF (Fig. 1B, lanes 2 and 3). The antibody 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 cephazolin, which demonstrated k_cat values that were approximately seven times higher than those

### TABLE 1. Antimicrobial susceptibility patterns of K. oxytoca 3127, E. coli DH5α clone, and E. coli HB101 transformant

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>K. oxytoca 3127 (parent)</th>
<th>E. coli DH5α (pBR322-cat1-bla_KPC-2)</th>
<th>E. coli HB101 transformant containing bla_KPC-2</th>
<th>E. coli HB101</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipenem</td>
<td>32</td>
<td>≤0.25</td>
<td>16</td>
<td>≤0.25</td>
</tr>
<tr>
<td>Imipenem-clavulanic acid</td>
<td>4</td>
<td>≤0.25</td>
<td>0.5</td>
<td>≤0.25</td>
</tr>
<tr>
<td>Meropenem</td>
<td>32</td>
<td>≤0.25</td>
<td>8</td>
<td>≤0.25</td>
</tr>
<tr>
<td>Meropenem-clavulanic acid</td>
<td>4</td>
<td>≤0.25</td>
<td>≥0.25</td>
<td>≤0.25</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>&gt;64</td>
<td>2</td>
<td>&gt;64</td>
<td>4</td>
</tr>
<tr>
<td>Amoxicillin-clavulanic acid</td>
<td>&gt;32/16</td>
<td>2/1</td>
<td>&gt;32/16</td>
<td>2/1</td>
</tr>
<tr>
<td>Piperacillin-tazobactam</td>
<td>&gt;128/4</td>
<td>≤1/4</td>
<td>&gt;128/4</td>
<td>≤1/4</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>&gt;64</td>
<td>≥1</td>
<td>&gt;64</td>
<td>≥1</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>&gt;64</td>
<td>≥2</td>
<td>32</td>
<td>≥2</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>&gt;32</td>
<td>≥1</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>Cefpodoxime</td>
<td>&gt;16</td>
<td>0.5</td>
<td>&gt;16</td>
<td>≤0.25</td>
</tr>
<tr>
<td>Cefepime</td>
<td>&gt;32</td>
<td>≤1</td>
<td>16</td>
<td>≥1</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>&gt;64</td>
<td>≤1</td>
<td>8</td>
<td>≤1</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>&gt;64</td>
<td>≤1</td>
<td>32</td>
<td>≤1</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>8</td>
<td>4</td>
<td>&gt;32</td>
<td>4</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>8</td>
<td>≤0.25</td>
<td>≤0.25</td>
<td>≤0.25</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>16</td>
<td>≤0.25</td>
<td>16</td>
<td>≤0.25</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>≥8</td>
<td>≤0.12</td>
<td>≤0.12</td>
<td>≤0.12</td>
</tr>
</tbody>
</table>

* a Values are MICs and are given in micrograms per milliliter.

* b Clavulanic acid was tested at a fixed concentration of 4 μg/ml.
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-
approximately 180 times higher than the \( k_{\text{cat}} \) 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 \( \mu \)M. Other substrates with low \( K_m \) values were nitrocefin, penicillin G, and imipenem, whose \( K_m \) values ranged from 22 to 51 \( \mu \)M. Cephaloridine, at 500 \( \mu \)M, had the highest \( K_m \).

Hydrolytic efficiencies, measured by \( k_{\text{cat}}/K_m \), 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 \( k_{\text{cat}}/K_m \) 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 \( \beta \)-lactamase demonstrated \( K_v \) values of 1.5 \( \mu \)M for clavulanic acid and 0.18 \( \mu \)M 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.

\( \text{bla}_{\text{KPC-2}} \) may be located on a mobile element. The 157-amino-acid partial sequence (GenBank accession number AA053444.1) encoded in the 863-nucleotide sequence upstream of the \( \text{bla}_{\text{KPC-2}} \) coding region showed a high degree of similarity to several IstB-like proteins: 52% similarity to the IS21 putative ATP-binding protein (GenBank accession number P15026), 68% to the putative IS200 transposase from \( Y. \) pseudotuberculosis \( \text{C}092 \) (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. \( S. \) enterica serotype Cubana (GenBank accession number AAC10642) (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 IS21 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 \( \text{bla}_{\text{KPC-2}} \) was located on an active transposable element.

The 70-kb plasmid from the \( E. \) coli HB101 transconjugant was electroporated into \( E. \) coli DH5\( \alpha \) containing pBR322-\( \text{cat} \). Since pBR322-\( \text{cat} \) is a multicopy cloning vector, we hypothesized that it would facilitate the detection of the transposition of \( \text{bla}_{\text{KPC-2}} \) from the 70-kb plasmid by a gene dosage assay (21).

The frequency of \( \text{bla}_{\text{KPC-2}} \) transposition was 9.7 \( \times \) 10\(^{-4} \), as indicated by the number of colonies with elevated imipenem MICs (i.e., those growing on 128-\( \mu \)g/ml versus 16-\( \mu \)g/ml imipenem). The background rate of colonies containing only the 70-kb plasmid growing on 128-\( \mu \)g/ml imipenem was 5.7 \( \times \) 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 DH5\( \alpha \)-\( pBR322-\text{cat} \)-\( \text{bla}_{\text{KPC-2}} \) 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).

### Table 2. Hydrolysis parameters of KPC-1 and KPC-2 â€” lactamas

<table>
<thead>
<tr>
<th>Substrates</th>
<th>( k_{\text{cat}} ) (s(^{-1}))</th>
<th>Relative ( k_{\text{cat}} )</th>
<th>( K_m ) (( \mu )M)</th>
<th>( k_{\text{cat}}/K_m ) (s(^{-1}))</th>
<th>Relative ( k_{\text{cat}}/K_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephaloridine</td>
<td>340</td>
<td>530</td>
<td>100</td>
<td>100</td>
<td>560</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>75</td>
<td>69</td>
<td>22</td>
<td>13</td>
<td>53</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>14</td>
<td>22</td>
<td>4.2</td>
<td>4.2</td>
<td>160</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>0.26</td>
<td>0.31</td>
<td>0.08</td>
<td>0.06</td>
<td>120</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>0.10</td>
<td>0.12</td>
<td>0.03</td>
<td>0.02</td>
<td>94</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>32</td>
<td>51</td>
<td>9.6</td>
<td>9.6</td>
<td>23</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>110</td>
<td>210</td>
<td>33</td>
<td>40</td>
<td>130</td>
</tr>
<tr>
<td>Cefoxacetil</td>
<td>25</td>
<td>35</td>
<td>7.4</td>
<td>6.6</td>
<td>100</td>
</tr>
<tr>
<td>Imipenem</td>
<td>12</td>
<td>15</td>
<td>3.7</td>
<td>2.8</td>
<td>81</td>
</tr>
<tr>
<td>Meropenem</td>
<td>3.0</td>
<td>4.0</td>
<td>0.9</td>
<td>0.75</td>
<td>12</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>20</td>
<td>30</td>
<td>5.9</td>
<td>5.7</td>
<td>310</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>( k_{\text{cat}}/K_m ) (s(^{-1}))</th>
<th>Relative ( k_{\text{cat}}/K_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clavulanic acid†</td>
<td>1.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Tazobactam†</td>
<td>0.23</td>
<td>0.18</td>
</tr>
<tr>
<td>EDTA</td>
<td>&gt;5,000</td>
<td>&gt;5,000</td>
</tr>
</tbody>
</table>

* KPC-1 \( k_{\text{cat}} \) and \( K_m \) data are from reference 47.
† ND, not determined.
‡ Inhibitor constant (\( K_i \)) is micromolar.

\( k_{\text{cat}} \): catalytic constant; \( K_m \): Michaelis constant; \( K_i \): inhibitory constant; \( M \): molar.
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...
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 blaKPC-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 $\beta$-lactamase. This plasmid also encodes resistance to gentamicin and tobramycin. Although the exact genetic structure has not been determined, the blaKPC-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 $\beta$-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.

ACKNOWLEDGMENTS

H. Yigit is a recipient of an American Society for Microbiology-National Center for Infectious Diseases postdoctoral fellowship. We offer special thanks to A. Karls and W. S. Reznikoff for their assistance with the gene dosage assay. We also thank J. Swenson for her help in obtaining the required media and antimicrobial agents for this study, and we thank C. Gowney for help with the KPC-2 purification. R. Clonno and J. Fung-Tomc are also thanked for their support.

The use of trade names is for identification purposes only and does not constitute endorsement by the Department of Health and Human Services or the U.S. Public Health Service.

REFERENCES


