In Vivo Selection of a Chromosomally Encoded β-Lactamase Variant Conferring Ceftazidime Resistance in *Klebsiella oxytoca*

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*Klebsiella oxytoca* clinical isolate A was recovered from the urine of a 55-year-old man with prostatic and urinary tract infections. This isolate displayed a β-lactam resistance phenotype consistent with overproduction of a chromosomally encoded class A β-lactamase and had decreased susceptibilities to all β-lactams except ceftazidime, cephapirin, and carbenicillin. Four weeks after treatment with an antibiotic regimen that included ceftazidime, *K. oxytoca* isolate B, which had a high level of resistance to ceftazidime, was isolated from the urine of the same patient. Isoelectric focusing analysis of the culture extracts of these isolates gave an pI of 5.4 for both isolates. Cloning experiments with the PCR products of the *bla*~*oxy*~ gene resulted in two *Escherichia coli* DH10B recombinant clones with resistance phenotypes mirroring those of the parental isolates. Sequencing analysis revealed that the *bla*~*oxy*~ gene from *K. oxytoca* B had a single nucleotide substitution compared to the sequence of the *bla*~*oxy*~ gene from *K. oxytoca* A, leading to a proline-to-serine substitution at position 167, according to the numbering of Ambler. Biochemical analysis of purified OXY-2-5 showed that it had the ability to hydrolyze ceftazidime. This is the first report of in vivo selection of a *K. oxytoca* isolate that produced a chromosomally encoded β-lactamase conferring resistance to ceftazidime.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** Clinical isolates were identified by using the API 20E system (bioMérieux, Marcy l’Étoile, France), *Escherichia coli* DH10B and rifampin-resistant strain *E. coli* JM109, which was obtained in vitro, were used for cloning and conjugation experiments, respectively (4).

**Plasmid DNA content and conjugation.** Extraction of plasmid DNA from *K. oxytoca* clinical isolates A and B was performed by two different methods, as described previously (5). Direct transfer of the amoxicillin resistance marker into rifampin-resistant strain *E. coli* JM109 was attempted by liquid and solid mating-out assays and by electroporation of a putative plasmid DNA suspension into *E. coli* DH10B (5). Transconjugants and electroporants were selected on Trypticase soy (TS) agar plates containing rifampin (200 µg/ml) and amoxicillin (30 µg/ml) and amoxicillin only, respectively.

**Random amplified polymorphic DNA (RAPD) analysis.** Amplification reactions were performed in a total volume of 50 µl containing 100 µM each deoxynucleoside triphosphates, 0.2 µM ERIC-2 primer (5'-AGTAATGACTGGGTTAOGCC-3'), 25 ng of DNA template, and 2 U of Taq polymerase in PCR buffer (20 mM Tris-HCl [pH 8.3], 50 mM KCl, 3 mM MgCl, 0.001% [wt/vol] gelatin). The PCR mixtures were subjected to amplification in a DNA thermal cycler (GenAmp PCR System 9600; Applied Biosystems, Foster City, Calif.) programmed for 36 cycles of 1 min at 94°C, 1 min at 36°C, and 3 min at 72°C. Amplification products (10-µl samples) were electrophoresed in a 1% agarose gel in Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA [pH 8.2]), stained with ethidium bromide, and photographed while they were on a UV light transilluminator.

**Cloning of β-lactamase genes.** The β-lactamase genes and their promoters from each *K. oxytoca* strain were amplified by PCR with primer 383 (5'-GGG GAT CCA GCC GGC ACC AA-3') and primer S (5'-CGG GCC TGT TCC CGG GTT AA-3'), as described previously (10). Amplification products were obtained by using Pfu DNA polymerase (Promega, Charbonnères-les-Bains, France) and were ligated into phagemid pBK-CMV (Stratagene, Amsterdam, The Netherlands) that had previously been digested with the ScI restriction enzyme (Amersham Pharmacia Biotech, Orsay, France) and were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics, Meylan, France). Recombinant phagemids were transformed into *E. coli* strain DH10B by electrotransformation with a Gene Pulser II apparatus (Bio-Rad, Ivory-sur-Seine, France). Transformsants were selected on TS agar containing ampicillin (100 µg/ml) and kanamycin (30 µg/ml). Recombinant plasmids were purified with the Qiagen plasmid Midi kit (Qiagen, Courtabœuf, France). Both strands of the cloned β-lactamase genes were sequenced with an Applied Biosystems sequencer (ABI 373). The nucleotide and deduced protein sequences were analyzed with soft-
TABLE 1. MICs of β-lactams for *K. oxytoca* A and B and recombinants *E. coli* DH10B(pBK-A), *E. coli* DH10B(pBK-B), and *E. coli* DH10B(pBK-CMV)

| β-Lactam(s) | *K. oxytoca A* | *E. coli DH10B(pBK-A)* | *K. oxytoca B* | *E. coli DH10B(pBK-B)* | *E. coli DH10B(pBK-CMV)* |
|-------------|----------------|-------------------------|----------------|-------------------------|-------------------------|
| Amoxicillin | >512           | >512                    | >512           | >512                    | 2                       |
| Amoxicillin-CLA | >512 | >512 | >512 | >512 | 2 |
| Ticarcillin | >512           | >512                    | >512           | >512                    | 1                       |
| Ticarcillin-CLA | >512 | >512 | >512 | >512 | 1 |
| Piperacillin | >512           | >512                    | >512           | >512                    | 4                       |
| Piperacillin-TZB | >512 | >512 | >512 | >512 | 4 |
| Cephalothin | >512           | >512                    | >512           | >512                    | 0.5                     |
| Cefoxitin | >512           | >512                    | >512           | >512                    | 0.5                     |
| Cefuroxime | >512           | >512                    | >512           | >512                    | 0.5                     |
| Cefotaxime | 32             | 16                      | 32             | 16                      | <0.06                   |
| Ceftazidine | 0.25           | 0.25                    | 512            | 512                     | <0.06                   |
| Aztreonam | 128            | 256                     | 128            | 256                     | 0.06                    |
| Cefepime | 8              | 4                       | 8              | 4                       | 0.06                    |
| Cefpirome | 32             | 16                      | 32             | 16                      | 0.06                    |
| Imipenem | 0.25           | 0.25                    | 0.25           | 0.25                    | 0.06                    |

Nucleotide sequence accession numbers. The nucleotide sequences of the *bla* _OXY-2_ and *bla* _OXY-2-5_ genes from *K. oxytoca* isolates A and B, respectively, had been submitted to the GenBank nucleotide database and have been assigned accession numbers AY303806 and AY303807, respectively.

RESULTS AND DISCUSSION

Isolation of clinical strains. *K. oxytoca* strain A was recovered from a urinary sample from a 55-year-old man who had been admitted to the Albert Chenevier Hospital (Créteil, France) in June 2002 and who had a prosthetic infection. Treatment with ceftazidime was initiated because the antibiotic resistance phenotype of the isolate indicated that it was resistant to co-trimoxazole and fluoroquinolones, was susceptible to all aminoglycosides, and exhibited a β-lactam resistance pattern consistent with overproduction of its chromosomally encoded β-lactamase (Table 1). A control sample was obtained 4 weeks after the onset of treatment and revealed the presence of **K. oxytoca** strain B, which displayed the same antibiotic resistance pattern as isolate A, except for resistance to ceftazidime. The treatment was then replaced by treatment with imipenem, which was administered for 3 weeks. The urine sample recovered at the end of treatment was sterile. No recurrence of prosthetic infection was recorded during the following 6 months.

IEF. Clinical isolates A and B and the *E. coli* DH10B transforms harboring recombinant plasmids each produced a single β-lactamase with a pI of 5.4, consistent with a chromosomally encoded β-lactamase of *K. oxytoca* belonging to the OXY-2 group (10).

Transformation and conjugation assays. Transformation and conjugation assays failed, indicating the very likely chromosomal locations of the *bla* _OXY_ genes.

RAPD experiment. The patterns for *K. oxytoca* strains A and B generated by RAPD analysis were identical (data not shown), thus indicating that the strains were clonally related.

Cloning and sequence analysis of β-lactamase. Cloning experiments yielded recombinant strains *E. coli* DH10B(pBK-A)

were available from the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

Antimicrobial agents and MIC determination. The antimicrobial agents used in this study were obtained from the form of standard laboratory powders and were used immediately after they were solubilized. The agents and their sources have been described elsewhere (4). MICs were determined by an agar dilution technique on Mueller-Hinton agar (Sanofi-Diagnostics Pasteur, Paris, France) with an inoculum of 10⁵ cfu per spot and were interpreted according to the guidelines of the National Committee for Clinical Laboratory Standards (10).

IEF analysis. The purified enzyme and β-lactamase extracts from cultures of clinical isolates and recombinant strains were subjected to analytical isoelectric focusing (IEF) on an ampholine polyacylamide gel with a pH range of 3.5 to 9.5 (Ampholine PAG plate; Amersham Pharmacia Biotech) for 90 min at 1,500 V, 50 mA, and 30 W. The focused β-lactamases were detected by overlaying the gel with a 1 mM nitrocefin solution (Calbiochem, Merck Eurolab SAS, Fontenay-sous-Bois, France).

β-Lactamase purification. Recombinant strain *E. coli* DH10B was grown overnight at 37°C in four liters of TS broth containing amoxicillin (100 μg/ml) and kanamycin (30 μg/ml). The bacterial suspensions were pelleted, resuspended in 40 ml of 100 mM phosphate buffer (pH 7), disrupted by sonication (three times at 50 W for 30 s each time with a Vibra cell 75022 phospholyser [Bioblock, Illkirch, France]), and centrifuged at 20,000 × g for 1 h at 4°C. The β-lactamase extracts were filtered through a 0.45-μm-pore-size filter (Millipore, Saint-Quentin-en-Yvelines, France), dialyzed overnight at 4°C against 20 mM Tris (pH 7.3), and loaded onto a preequilibrated Q-Sepharose column (Amersham Pharmacia Biotech). The enzyme was eluted with a linear NaCl gradient (0 to 1 M) in the same buffer. Eluted fractions with high β-lactamase activities (nitrocefin test) were pooled, dialyzed against 20 mM bis-Tris buffer (pH 6.8), and loaded onto a preequilibrated Q-Sepharose column and eluted with a linear NaCl gradient (0 to 0.5 M). The purified extract was finally dialyzed overnight at 4°C against 100 mM phosphate buffer (pH 7).

Kinetic measurements. Purified β-lactamase OXY-2-5 was used for kinetic measurements (Km and kcat), which were performed at 30°C in 100 mM sodium phosphate (pH 7.0). The rates of hydrolysis were determined with a Pharmacia ULFROSCPEC 2000 spectrophotometer and were analyzed with SWIFT II software (Amersham Pharmacia Biotech). The Km and kcat values were determined by analyzing β-lactam hydrolysis under initial rate conditions by using the Eadie-Hofstee linearization of the Michaelis-Menten equation, as described previously (7). When the Km value was low, Kcat values were determined from initial rates at saturating substrate concentrations ([S]), where [S] is ≥Km.

Various concentrations of clavulanic acid and tazobactam were preincubated with the enzyme for 3 min at 30°C before the rate of cephalothin (100 μM) hydrolysis was tested. The 50% inhibitory concentrations of these inhibitors were determined as the concentration of inhibitor that inhibited hydrolytic activity by 50%. The results were expressed in micromolar units.

MICs (µg/ml) of β-lactams for *K. oxytoca* A and B and recombinants *E. coli* DH10B(pBK-A), *E. coli* DH10B(pBK-B), and *E. coli* DH10B(pBK-CMV)

| β-Lactam(s) | *K. oxytoca A* | *E. coli DH10B(pBK-A)* | *K. oxytoca B* | *E. coli DH10B(pBK-B)* | *E. coli DH10B(pBK-CMV)* |
|-------------|----------------|-------------------------|----------------|-------------------------|-------------------------|
| Amoxicillin | >512           | >512                    | >512           | >512                    | 2                       |
| Amoxicillin-CLA | >512 | >512 | >512 | >512 | 2 |
| Ticarcillin | >512           | >512                    | >512           | >512                    | 1                       |
| Ticarcillin-CLA | >512 | >512 | >512 | >512 | 1 |
| Piperacillin | >512           | >512                    | >512           | >512                    | 4                       |
| Piperacillin-TZB | >512 | >512 | >512 | >512 | 4 |
| Cephalothin | >512           | >512                    | >512           | >512                    | 0.5                     |
| Cefoxitin | >512           | >512                    | >512           | >512                    | 0.5                     |
| Cefuroxime | >512           | >512                    | >512           | >512                    | 0.5                     |
| Cefotaxime | 32             | 16                      | 32             | 16                      | <0.06                   |
| Ceftazidine | 0.25           | 0.25                    | 512            | 512                     | <0.06                   |
| Aztreonam | 128            | 256                     | 128            | 256                     | 0.06                    |
| Cefepime | 8              | 4                       | 8              | 4                       | 0.06                    |
| Cefpirome | 32             | 16                      | 32             | 16                      | 0.06                    |
| Imipenem | 0.25           | 0.25                    | 0.25           | 0.25                    | 0.06                    |

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and *E. coli* DH10B(pBK-B) from *K. oxytoca* isolates A and B, respectively. Sequence analysis of the PCR products obtained after amplification of the blaOXY genes from *K. oxytoca* strains A and B showed that the promoter sequences upstream of the β-lactamase genes were identical. The -35 box of this promoter sequence exhibited 100% DNA identity with the promoter sequence of the β-lactamase gene harbored by isolate A. The first and fifth base pairs, respectively (TATACT instead of GAT GAT, respectively), compared with the wild-type promoter sequence (8). The G-to-T substitution in the first base is known to increase drastically the strength of the promoter of the β-lactamase gene of *K. oxytoca* (8). This result likely indicates that the β-lactamases might have been similarly overproduced in both clinical isolate A and clinical isolate B.

Sequence analysis revealed that the β-lactamase gene harbored by isolate A presented 100% amino acid identity with the OXY-2-5 β-lactamase (10), whereas the amino acid sequence of the β-lactamase of isolate B differed from that of the OXY-2 enzyme by a single substitution of a proline to a serine residue at position 167 (1). Although this substitution has not previously been reported in the OXY β-lactamase of *K. oxytoca*, this substitution has been described at the same Ambler position among CTX-M-type expanded-spectrum β-lactamas (CTX-M-18 and CTX-M-19) and was responsible for resistance to ceftazidime (17).

**Susceptibility testing.** The *E. coli* DH10B transformants that harbored recombinant plasmids showed resistance patterns that mirrored those of the parental strains, thus indicating that β-lactamase production was the main mechanism responsible for the susceptibility patterns of the clinical isolates (Table 1). The addition of clavulanic acid and tazobactam did not restore the activities of the penicillins (Table 1). Since the activity of the OXY-2 β-lactamase is inhibited in vitro by clavulanate and tazobactam (10), the in vivo resistance to inhibitors displayed by isolates A and B was likely related to β-lactamase overexpression. Isolate A had reduced susceptibilities to all cephalosporins except ceftazidime, whereas isolate B was also resistant to this β-lactam (MIC, >512 μg/ml) (Table 1).

**Biochemical analysis of the OXY-2-5 β-lactamase from *K. oxytoca* isolate B.** The specific activity of the purified β-lactamase from *K. oxytoca* isolate B, which was measured with 100 μM of benzylpenicillin as the substrate, was 3,532 U g/mg of protein⁻¹. Its purity was estimated to be >95% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (data not shown).

The OXY-2-5 β-lactamase displayed high catalytic efficiencies toward penicillins (benzylpenicillin, amoxicillin, ticarcillin, piperacillin), narrow-spectrum cephalosporins (cephalothin and cephaloridine), cefuroxime, cefotaxime, and ceftriaxone, which was in agreement with the results of previous studies (2, 6), whereas aztreonam and cefepime were weakly hydrolyzed, with catalytic efficiency values below 50 s⁻¹ mM⁻¹ (Table 2). The catalytic activity and the affinity of OXY-2-5 for ceftazidime remained low, although kinetic parameters could be determined (Table 2). The OXY-2-5 β-lactamase remained unable to hydrolyze a carbapenem (imipenem) and a cephemycin (cefoxitin).

Inhibition studies showed that the 50% inhibitory concentrations of clavulanic acid and tazobactam (1 and 4.2 μM, respectively) for OXY-2-5 were similar to those of OXY-2 (10), thus indicating that the proline-to-serine substitution at position 167 does not modify susceptibility to inhibitors.

**Conclusion.** Another OXY-2 β-lactamase variant has been described (18). It conferred resistance to inhibitors due to a replacement of serine by glycine at Ambler position 130. Here, we reported on an OXY-2 variant that conferred a significant degree of resistance to ceftazidime. The OXY-2-5 β-lactamase differed from OXY-2 by a proline-to-serine substitution at Ambler position 167 and had a higher level of catalytic activity against ceftazidime but an overall loss of activity against other β-lactams. A similar substrate modification has been observed for the CTX-M-18 and CTX-M-19 β-lactamas, which differed by a proline-to-serine substitution at Ambler position 167 (17). These common features may be related to structural

### Table 2. Kinetic parameters of purified β-lactamase OXY-2-5 from *E. coli* DH10B(pBK-B)*a*

| Substrate      | OXY-2-5 | | OXY-2*b* | | Relative $V_{max}$ (%) | | Relative $K_m$ (μM) | | Relative $K_m$ (μM) |
|---------------|---------|---|---------|---|-----------------|---|---------|
| Benzylpenicillin | 120     | 30 | 4,000   | 100 | 47  | 100  | 47  |
| Amoxicillin    | 40      | 605 | 66     | 33  | 66  | 95   |
| Ticarcillin    | 50      | 60  | 830    | 41  | 208 | 20   |
| Piperacillin   | 30      | 15  | 2,000  | 25  | 25  |
| Cephalothin    | 3,400   | 60  | 57,000 | 280 | 285 | 91   |
| Cephaloridin   | 1.60    | 115 | 1,400  | 133 | 250 | 90   |
| Cefuroxime     | 60      | 120 | 500    | 50  | 170 | 7.0  |
| Cefotaxime     | 20      | 115 | 175    | 18  | 1,000 | 7.0  |
| Ceftriaxone    | 50      | 24  | 2,100  | 42  | 395 | 14   |
| Cefotaxime     | 0.2     | 23  | 9      | 0.2 |       |
| Aztreonam      | 7       | 150 | 47     | 6   |       |
| Cefpime        | 60      | 1,300 | 46   | 50  | 1,800 | 5    |
| Cefepime       | 40      | 910 | 44     | 33  | 500  | 4    |

*a* Data are the means of three independent experiments. Standard deviations were within 15%.

*b* Data were obtained from reference 15.

*c* Values are relative to those for benzylpenicillin (taken as 100%).

*d* For compounds with $K_m$ values less than 10 μM, $K_v$ values were determined by using cephalothin as the substrate.
similarities between the CTX-M enzymes and the chromosomally encoded β-lactamases of *K. oxytoca*, as was suggested when the first CTX-M-type β-lactamase was discovered (3).

The in vivo emergence of *K. oxytoca* and CTX-M-producing isolates of the family *Enterobacteriaceae* (17) with acquired resistance to ceftazidime underlines the selective properties of this antibiotic. Even though ceftazidime has been suggested for use in the treatment of infections caused by β-lactamase-overproducing *K. oxytoca* strains (12), it might be safer to use other β-lactams such as carbapenems in light of the present results.

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