Genetic Characterization of Resistance to Extended-Spectrum β-Lactams in *Klebsiella oxytoca* Isolates Recovered from Patients with Septicemia at Hospitals in the Stockholm Area

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Two β-lactamase gene regions were characterized by DNA sequencing in eight clinical isolates of *Klebsiella oxytoca*. The bla_{OXY-2a} region encoded a β-lactamase nearly identical to OXY-2 (one amino acid residue substituted) and conferred aztreonam and cefuroxime resistance on the *K. oxytoca* isolates. Overproduction of OXY-2a was caused by a G-to-A substitution of the fifth nucleotide in the −10 consensus sequence of bla_{OXY-2a}. The bla_{OXY-1a} was identified in a susceptible strain, and the OXY-1a enzyme differed from OXY-1 by two amino acid residues.

Since the early 1980s, isolates of *Klebsiella oxytoca* have been recognized as clinically significant and an indication for therapy (17). Clinical isolates of *Klebsiella* spp. resistant to cefotaxime, ceftazidime, or aztreonam have been increasingly reported (6, 12, 14). These bacteria can develop resistance to the newer cephalosporins and aztreonam by acquisition of plasmid-located extended-spectrum β-lactamases (ESBLs) (7, 18, 25) or by mutations giving rise to the hyperproduction of chromosomal β-lactamases (15). In wild-type *K. oxytoca*, chromosomal β-lactamases are constitutively produced at low levels which are sufficient to affect the organism's susceptibility to ampicillin, amoxicillin, carbenicillin, and ticarcillin (17). Overproduction of these β-lactamases, however, confers resistance to penicillins, cephalosporins, and aztreonam (8); in most cases this overproduction results from a mutation in the promoter region of the β-lactamase gene (9, 11). Molecular cloning and DNA sequencing have shown that the chromosomal β-lactamase genes in *K. oxytoca* can be divided into two main groups: bla_{OXY-1} and bla_{OXY-2} (11). These two categories of β-lactamase genes show 89.7% homology in DNA sequences and belong to functional group 2be of the ESBLs (4) and Ambler class A (1).

In previous studies (27, 28), 11 clinical isolates of *K. oxytoca* were found to be resistant to aztreonam and cefuroxime (MIC of >16 μg/ml) but susceptible to cefotaxime, ceftazidime, and imipenem (MICs of <4 μg/ml). The bacteria isolated in these previous studies belonged to three subgroups based on their plasmid profiles (27, 28). By isoelectric focusing, a single, common β-lactamase with a pI of 5.25, designated KH, was observed in the strains. The resistance could not be transferred to *Escherichia coli* XAC and C600 by transformation and conjugation, suggesting a chromosomal location for the β-lactamase gene. Furthermore, the substrate profile of the KH β-lactamase exhibited the hydrolysis of aztreonam characteristic of the enzymes of the *K. oxytoca* OXY family (10, 28). The aim of the present study was to characterize the genetic determinant for the ESBL produced by the *K. oxytoca* isolates recovered from hospitalized patients in the Stockholm area.

Seven of the previously reported cefuroxime- and aztreonam-resistant *K. oxytoca* isolates (27, 28) were further investigated in the present study. One susceptible strain of *K. oxytoca* isolated during the same period from a patient in Karolinska Hospital was also included. *E. coli* DH5α was the host for the cloning experiment. Plasmid pACYC177 was used to construct cloning vector pLSK (low copy number, single cloning site, and kanamycin resistance) (Table 1).

All standard nucleic acid techniques were carried out essentially as described by Ausubel et al. (2) and Sambrook et al. (20). Plasmid DNA was prepared by use of the Wizard Plus Minipreps or Midipreps DNA purification system (Promega, Madison, Wis.). Chromosomal DNA from the *K. oxytoca* isolates was prepared according to the procedure of Wilson (26). The quantity of recovered DNA was measured with a GeneQuant RNA-DNA calculator (Pharmacia Biotech, Ltd., Cambridge, England). Plasmid DNA was introduced into *E. coli* by transformation with CaCl$_2$-treated *E. coli* DH5α, as recommended by Ausubel et al. (2). The DNA sequence was determined by the dideoxy-chain termination method (21) with an automated DNA-sequencing system (model 377; PE/ABI, Foster City, Calif.). Nucleotide and deduced amino acid sequences were analyzed with the GCG program (Genetics Computer Group, Madison, Wis.) and Lasergene software (DNASTAR, Madison, Wis.).

Primer oligonucleotides are shown in Table 2. To detect the bla_{OXY-1} gene, primers C and D were used to generate a 668-bp amplicon. Primers L and M were employed to detect bla_{OXY-2}, producing a 723-bp PCR fragment. Primers KHUBI and KHDBI were designed based on bla_{OXY-2} (11) and bla_{RBI} (13) sequences, and these primers were used to amplify the region covering the complete KH β-lactamase gene for cloning. Primers CYC7BI1 and CYC7BI2 were designed and employed to construct plasmid pLSK from plasmid pACYC177 (19). The 5′ nucleotides of the primers KHUBI, KHDBI, CYC7BI1, and CYC7BI2 were modified to create a *Bam*HI restriction site (Table 2) in order to ligate the PCR products.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Strains		
E. coli DH5α	*recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1* φ80 *dlac* ZΔM15	Bethesda Research Laboratories
K. oxytoca		
KH11	Az^r Xm^r, clinical isolate	27, 28
KH26	Az^r Xm^r, clinical isolate	27, 28
KH55	Az^r Xm^r, clinical isolate	27, 28
KH78	Az^r Xm^r, clinical isolate	27, 28
KH93	Az^r Xm^r, clinical isolate	27, 28
KH103	Az^r Xm^r, clinical isolate	27, 28
NBL63	Az^r Xm^r, clinical isolate	27, 28
KH66	Az^s Xm^s, clinical isolate	This study
Plasmids		
pACYC177	Ap^r Kan^r, cloning vector	19
pLSK	Kan^r, PCR fragment of Kan^r and Rep in pACYC177	This study
pLSKSW-7	pLSK, 1.06-kb PCR fragment from *bla* of KH11	This study
pLSKSW-8	pLSK, 1.06-kb PCR fragment from *bla* of KH66	This study
pKH11	Plasmid from *K. oxytoca* KH11 (subgroup 2)	27, 28
pKH78	Plasmid from *K. oxytoca* KH78 (subgroup 1)	27, 28
pNBL63	Plasmid from *K. oxytoca* NBL63 (subgroup 3)	27, 28

[a] Ap, ampicillin; Az, aztreonam; Kan, kanamycin; Xm, cefuroxime; Rep, replication origin; r, resistant; s, susceptible.

The PCR was made with a PCR reagent kit according to the standard reaction recommended by the manufacturer (Perkin-Elmer Cetus, Branchburg, N.J.). Therefore, 100 ng of the chromosomal DNA from each of the *K. oxytoca* isolates and 40 pmol of each primer were included in a 100-μl reaction mixture. PCR amplification was performed in a DNA Thermal Cycler 480 (Perkin-Elmer Cetus) with the following temperature profiles: 94°C for 5 min; 30 cycles of 94°C for 1 min, 60 (for bla_{OXY-1}) or 55°C (for bla_{OXY-2}) for 1 min, 72°C for 1 min, and 72°C for 5 min; and holding at 4°C (16).

The bacterial strains were grown in Luria-Bertani medium (Difco Laboratories, Detroit, Mich.) with aeration at 37°C. Kanamycin (Sigma, St. Louis, Mo.) was added at 50 μg/ml for selection and maintenance of pLSK plasmids. MICs of cefuroxime, aztreonam, cefotaxime, ceftazidime, and imipenem for the *K. oxytoca* isolates and *E. coli* transformants were determined by a microdilution method (27).

To determine whether the gene for KH β-lactamase is located on a chromosome or a plasmid, one strain from each plasmid subgroup of the *K. oxytoca* isolates was studied. Chromosomal and plasmid DNA preparations from strains KH78 (group 1), KH11 (group 2), and NBL63 (group 3) were used as templates for PCR amplification. Under the recommended conditions, PCR products of 0.68 kb for bla_{OXY-1} (primer pair C-D) and 0.72 kb for bla_{OXY-2} (primer pair L-M) were obtained from the chromosomal DNA templates of strains KH11, KH78, and NBL63. However, no PCR product was obtained for bla_{OXY-1} (primer pair C-D) or for bla_{OXY-2} (primer pair L-M) when the plasmid DNA preparations of these strains were used as templates.

The region including the entire KH β-lactamase gene was amplified from the chromosomal DNA of the *K. oxytoca* isolates by using primers KHUBI and KHDBI. All PCR products from strains KH11, KH66, KH78, and NBL63 were sequenced. The 1,062-bp nucleotide sequence containing the β-lactamase gene from strain KH11 and the deduced amino acid sequence are shown in Fig. 1. The 867-bp nucleotide sequence (positions 147 to 1016) encoded a β-lactamase of 289 amino acid residues. The nucleotide sequence of this β-lactamase was nearly identical (99.8%) to that of the wild-type OXY-2 β-lactamase from *K. oxytoca* SL911 (11). The amino acid sequence of the β-lactamase was different from that of OXY-2 by only one residue; Ala-13 (ABL Ala-10) (1) was absent in the enzyme from strain KH11. On the basis of this high degree of similarity, KH β-lactamase was redesignated OXY-2a, and the KH determinant was redesignated bla_{OXY-2a}. The bla_{OXY-2a} gene was preceded by a promoter, in which TTGTCA and GATAAT were the −35 and −10 consensus sequences, respectively, and by a

TABLE 2. Oligonucleotides for detection and amplification of bla_{OXY} regions

Primer	Sequence	Position[a]	Reference (accession no.)
C	5′-GCGTAGCGCTGATTAACACG-3′	494–513	9 (Z30177)
D	5′-CCTGCTGCGGCTGGGTAAAA-3′	1143–1162 (comp)	9 (Z30177)
L	5′-CAGATCTCGAGAAGCGTTCA-3′	421–440	11 (Z49084)
M	5′-ACCTCTTTGCGGTTTTTCGC-3′	1125–1144 (comp)	11 (Z49084)
KHUBI	5′-CCA<u>GGATCC</u>TGGCGAGACACTATAAGC-3′[b]	174–193	11 (Z49084)
KHDBI	5′-CCT<u>GGATCC</u>AGAGTGCAGAGTGTTGCA-3′	994–1012 (comp)	13 (D84548)
CYC7BI1	5′-CAA<u>GGATCC</u>AGGTGAAGATCCT-3′	680–701	19 (X06402)
CYC7BI2	5′-TCG<u>GGATCC</u>ACCAGAATGACATC-3′	3133–3158 (comp)	19 (X06402)

[a] Comp, complementary strand of DNA sequence.
[b] Underlined sequences indicate *Bam*HI restriction sites.

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1    CCAGGATCCTGGCGAGACACTATAAGCAGGCGCTGGAAGGACAAATATCGAATTGATGAT    60
                                                              +1
61   GGAATTCCATCATCAATAAATTGCTTGTCAAAATAGCGGGAGTCGCAGATAATCCGCTGC   120
                            -35              -10
121  GACTTATCACTCTCAAGGAATCAGAAATGATAAAAAGTTCGTGGCGTAAAATTGCAATGC   180
(1)               S.D.    M  I  K  S  S  W  R  K  I  A  M  L   (12)
                          OXY-2a
181  TAGCCGCCGTTCCGCTGCTGCTGGCGAGCGGCGCACTGTGGGCCAGTACCGATGCTATCC   240
(13)  A  A  V  P  L  L  L  A  S  G  A  L  W  A  S  T  D  A  I  H  (32)
241  ATCAGAAGCTGACAGATCTCGAGAAGCGTTCAGGCGGCAGGTTGGGCGTGGCGCTAATCA   300
(33)  Q  K  L  T  D  L  E  K  R  S  G  G  R  L  G  V  A  L  I  N  (52)
301  ACACGGCAGATAATTCTCAAATCTTATATCGGCGACGAGCGTTTTGCCATGTGCAGCA    360
(53)  T  A  D  N  S  Q  I  L  Y  R  G  D  E  R  F  A  M  C  S  T  (72)
361  CCAGTAAAGTGATGGCCGCCGCCGGTATTAAAACAGAGCGAAAGCAATAAAGAGGTGG    420
(73)  S  K  V  M  A  A  A  A  V  L  K  Q  S  E  S  N  K  E  V  V  (92)
421  TAAATAAAAGGCTGGAGATTAACGCAGCCGATTTGGTGGTCTGGAGTCCGATTACCGAAA   480
(93)  N  K  R  L  E  I  N  A  A  D  L  V  V  W  S  P  I  T  E  K  (112)
481  AACACATCTCCAGAGCGGAATGACGCTGGCTGAGCTAAGCGCGGCGACGCTGCAATATAGCG   540
(113) H  L  Q  S  G  M  T  L  A  E  L  S  A  A  T  L  Q  Y  S  D  (132)
541  ACAATACGGCGATGAATCTGATCATCGGCTACCTTGGCGGGCCGGAAAAAGTCACCGCCT   600
(133) N  T  A  M  N  L  I  I  G  Y  L  G  G  P  E  K  V  T  A  F  (152)
601  TCGCCCGCAGTATCGGCGATGCCCACCTTTCGTCTCGATCGTACGGAGCCCACGCTGAATA   660
(153) A  R  S  I  G  D  A  T  F  R  L  D  R  T  E  P  T  L  N  T  (172)
661  CCGCCATCCCCGGGCGATGAGCGTGATACCAGCACGCCGCTGGCGATGGCTGAAAGCCTAC   720
(173) A  I  P  G  D  E  R  D  T  S  T  P  L  A  M  A  E  S  L  R  (192)
721  GCAAGCTGACGCTTGGCGATGCGCTGGGCGAACGCAACGCGCCCAGTTAGTCACCTGGC   780
(193) K  L  T  L  G  D  A  L  G  E  Q  Q  R  A  Q  L  V  T  W  L  (212)
781  TGAAAGGCAATACCACCGGCGGGCAAAGCATTCGCGCGGGCCTGCCTGAAAGCTGGGTGG   840
(213) K  G  N  T  T  G  G  Q  S  I  R  A  G  L  P  E  S  W  V  V  (232)
841  TCGGCGATAAAACCGGCGCCGGAGATTACGGCACCACCAATGATATTGCGGTTATCTGGC   900
(233) G  D  K  T  G  A  G  D  Y  G  T  T  N  D  I  A  V  I  W  P  (252)
901  CGGAAGATCACGCTCCGCTGGTATTAGTCACCTACTTTACCCAGCCGCAGCAGGATGCGA   960
(253) E  D  H  A  P  L  V  L  V  T  Y  F  T  Q  P  Q  Q  D  A  K  (272)
961  AAAACCGCAAAGAAGTGTTAGCCGCAGCGGCAAAAATCGTGACCGAAGGGCTTTAAGCCG  1020
(273) N  R  K  E  V  L  A  A  A  A  K  I  V  T  E  G  L  *       (289)
1021 GGAACAGGCCCGTTCTGCAACACTCTGCACTCTGGATCCAGG                     1062
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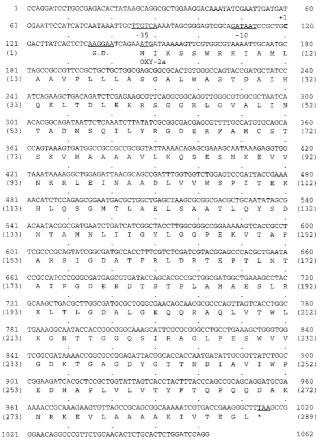
FIG. 1. Nucleotide sequence of the bla_{OXY-2a} region of *K. oxytoca* KH11. Numbering starts at the 5' end of primer KHUBI and ends at the complementary nucleotide of the 5' end of primer KHDBI. The start codon and consensus sequences are underlined. The stop codon is designated with an asterisk. The deduced polypeptide sequence is indicated. S.D., Shine-Dalgarno site.

putative Shine-Dalgarno sequence (AAGGAA). The nucleotide and amino acid sequences of the β-lactamase genes and proteins of strains KH78 and NBL63 were identical to those of strain KH11. The β-lactamase from the susceptible *K. oxytoca* strain KH66 was highly similar to wild-type OXY-1 in amino acid sequence (99.3% identity), and the DNA equences encoding the two proteins were also very similar (98.9% identity) (9). The major difference between the proteins was that Leu-262 (ABL Leu-261) and Glu-278 (ABL Glu-277) (1) in OXY-1 were respectively substituted with Pro-262 (ABL 261) and Lys-278 (ABL 277) in KH66. Accordingly, the KH66 protein was designated OXY-1a.

The promoter regions from each of the eight *K. oxytoca* isolates were sequenced. The promoters for the β-lactamase genes in all the isolates but KH66 had the same consensus sequence as that for bla_{OXY-2a} in the strain KH11. Contrasting with the promoter sequences of wild-type bla_{OXY-2} (TTGTCA for −35 and GATAGT for −10), the bla_{OXY-2a} promoters had a substitution (G→A) of the fifth base in the −10 consensus sequence. The promoter of bla_{OXY-1a} in KH66 was identical to that of wild-type bla_{OXY-1} (9).

The DNA fragment containing the replication origin and kanamycin resistance gene in plasmid pACYC177 was PCR amplified by using the primers CYC7BI1 and CYC7BI2. After digestion with *Bam*HI, the fragment was ligated with *Bam*HI digests of PCR-generated bla_{OXY-2a} and bla_{OXY-1a} regions from *Klebsiella* strains KH11 and KH66 to form recombined

plasmids pLSKSW-7 and pLSKSW-8, respectively. The two plasmids were then transformed into *E. coli* DH5α for the expression of resistance to β-lactam antibiotics. The *E. coli* (pLSKSW-7) transformant and *K. oxytoca* KH11 showed similar antibiograms which indicated resistances to aztreonam and cefuroxime and intermediate susceptibilities or susceptibilities to cefotaxime, ceftazidime, and imipenem (Table 3). The *E. coli* (pLSKSW-8) transformant and *K. oxytoca* KH66 similarly displayed no significant resistances to the β-lactam antibiotics tested (Table 3).

In the present study, by PCR-based cloning and sequencing techniques, the chromosomally encoded OXY-2a was confirmed to be responsible for β-lactam resistance in the *K. oxytoca* isolates recovered from patients in the Stockholm area during 1987 (27, 28). The high identities of the OXY-2a coding regions and promoter sequences in the *K. oxytoca* strains suggest a common origin for the β-lactam-resistant *K. oxytoca* isolates. The mutation observed in the bla_{OXY-2a} promoters may have resulted in the overexpression of this β-lactamase, thereby conferring resistance to β-lactam antibiotics on *K. oxytoca*. Similar findings have been reported by others (3, 8, 9, 17, 22–24).

Of the two substitutions observed in OXY-1a, one of them (Pro-261) is located in a hydrophobic pocket and the other (Lys-277) is quite close to the homologous active site (Asp-276) of TEM β-lactamases (5).

In this study, the primers specific to OXY-1 and OXY-2 cross-amplified. This may have been caused by our use of a large amount of template DNA and the high homology of the two genes. This cross-amplification suggests that PCR conditions should be carefully established. The parallel PCRs for bla_{OXY-1} and bla_{OXY-2} should be set with the intention of differentiating the two genes. On the other hand, PCR might be performed with one of the primer pairs, and the PCR product might be directly sequenced to identify the genes.

The effort to construct a recombinant plasmid by ligating two PCR-generated DNA fragments was successful and demonstrated a simple, fast, and straightforward approach for cloning.

Nucleotide sequence accession numbers. The nucleotide sequences described in this paper have been submitted to EMBL-GenBank under accession no. Y17714 for bla_{OXY-2a} and no. Y17715 for bla_{OXY-1a}.

TABLE 3. Antibiotic susceptibility of *K. oxytoca* strains and *E. coli* transformants

Strain	MIC (μg/ml) of[a]:				
	IPM	CAZ	ATM	CTX	CXM
K. oxytoca					
KH11	0.5	1.0	>64	2.0	>64
KH26	1.0	0.5	>64	2.0	>64
KH55	1.0	1.0	>64	2.0	>64
KH78	1.0	1.0	>64	2.0	>64
KH93	1.0	1.0	>64	4.0	>64
KH103	1.0	1.0	>64	4.0	>64
NBL63	1.0	1.0	>64	4.0	>64
KH66	0.5	0.25	0.25	<0.064	4.0
E. coli					
DH5α/pLSKSW-7	0.5	2.0	>64	8.0	>64
DH5α/pLSKSW-8	0.25	0.25	1.0	0.125	8.0
DH5α/pLSK	0.25	0.125	0.25	0.125	8.0
ATCC 25922	0.25	0.25	0.125	4.0	

[a] IPM, imipenem; CAZ, ceftazidime; ATM, aztreonam; CTX, cefotaxime; CXM, cefuroxime.

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