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Characterization of a Chromosomally Encoded Extended-Spectrum Class A β-Lactamase from *Kluyvera cryocrescens*

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A chromosomally located β-lactamase gene, cloned and expressed in *Escherichia coli* from a reference strain of the enterobacterial species *Kluyvera cryocrescens*, encoded a clavulanic acid-inhibited Ambler class A enzyme, KLUC-1, with a pI value of 7.4. KLUC-1 shared 86% amino acid identity with a subgroup of plasmid-mediated CTX-M-type extended-spectrum β-lactamases (CTX-M-1, -3, -10, -11, and -12), the most closely related enzymes, and 77% amino acid identity with KLUA-1 from *Kluyvera ascorbata*. The substrate profile of KLUC-1 corresponded to that of CTX-M-type enzymes.

At present, the genus *Kluyvera* is composed of four enterobacterial species: *Kluyvera ascorbata*, *Kluyvera cryocrescens*, *Kluyvera georgiana*, and *Kluyvera cochleae* (9). *K. ascorbata* is more frequently isolated from clinical specimens, while *K. cryocrescens* is mostly isolated from the environment (water, soil, sewage, and hospital environment) (32). Eighteen detailed cases of human *K. cryocrescens* infections have been reported, with some (but not all) of them occurring in immunocompromised patients (32). The detailed susceptibility of *K. cryocrescens* to β-lactams is not known except for its resistance to ampicillin (1, 32).

We report here on the characterization of a class A β-lactamase from *K. cryocrescens* with a substrate profile extended to expanded-spectrum cephalosporins. Sequence analysis revealed its similarity to several plasmid-mediated CTX-M-type extended-spectrum β-lactamases (ESBLs).

Bacterial strains and plasmid analysis. *K. cryocrescens* reference strain 79.54 was from the strain collection of the Institut Pasteur (Paris, France). Plasmid DNA extractions, performed as described previously (26), failed to identify plasmids.

Cloning and sequence analysis of β-lactamase gene from *K. cryocrescens*. Whole-cell DNA of *K. cryocrescens* 79.54 was extracted as described previously (26), digested with *Sau*3AI, and ligated into the *Bam*HI site of phagemid pBK-CMV (26). Thirty *Escherichia coli* DH10B recombinant clones were obtained after selection on kanamycin- and amoxicillin-containing plates, as described previously (26). One of the recombinant plasmids that had the shortest insert (pKC7954) was retained for further analysis. Its DNA insert (6.1 kb) was sequenced and analyzed as described previously (26).

An open reading frame (ORF) of 932 bp was identified (data not shown). The G+C content of this ORF was 54.9%, which lies within the G+C ratios for enterobacterial genes. Within the deduced protein of this ORF (311 amino acids), named KLUC-1, characteristic elements of Ambler class A β-lactamases were identified (Fig. 1) (17). Isoelectric focusing analysis, performed as reported previously (26), showed that cultures of *K. cryocrescens* 79.54 and *E. coli* DH10B(pKC7954) gave single and identical β-lactamases, each with a pI value of 7.4.

The KLUC-1 β-lactamase was closely related to the CTX-M-1 (MEN-1) subgroup of plasmid-mediated enzymes (CTX-M-1, -3, -10, -11, and -12), sharing 85 to 86% amino acid identity (5, 18, 21). It shared 77% identity with the CTX-M-2 subgroup (CTX-M-2, -5, -6, and -7 and Toho-1) and 76 and 78% identities with CTX-M-8 and -9, respectively (7, 15, 27, 31).

KLUC-1 from *K. cryocrescens* shared only 77% amino acid identity with the chromosomally encoded KLUA-1 β-lactamase from *K. ascorbata* (GenBank accession no. CAB59824), while the two enterobacterial species from which these β-lactamases were obtained are phylogenetically related (10). The amino acid identities of KLUC-1 with the naturally occurring class A β-lactamases from *Klebsiella oxytoca* (3), *Serratia fonticola* (22), *Citrobacter koseri* (formerly *Citrobacter diversus*) (24), *Proteus vulgaris* (23), *Yersinia enterocolitica* (29), and *Klebsiella pneumoniae* (SHV-1) (4) were 72, 72, 71, 63, 57, and 37%, respectively.

A 1,241-bp ORF was identified 498 bp upstream of bla_{KLUC-1} in the same transcription orientation; this ORF codes for a putative 401-amino-acid protein. This protein shared 96% identity with an aspartate aminotransferase from *E. coli* K-12 (6) and with a putative protein whose ORF was found upstream of bla_{KLUA-1} from *K. ascorbata* (GenBank accession no. 272538). Additionally, 90% nucleotide identity was found in these intergenic regions in *K. ascorbata* and *K. cryocrescens*. No upstream-located LysR-type regulator gene was identified, whereas AmpR genes are upstream-located compared to the chromosomally encoded β-lactamase genes of *S. fonticola*, *P. vulgaris*, and *C. koseri* (14, 16, 20).

Another ORF was identified 928 bp downstream of bla_{KLUC-1} in the same transcription orientation; this ORF encoded a putative protein that shared 69% amino acid identity with that identified downstream of bla_{KLUA-1} (GenBank accession no. 272538). No consistent nucleotide identity was found in the immediate downstream region of the β-lactamase genes identified in *K. cryocrescens* and *K. ascorbata*.

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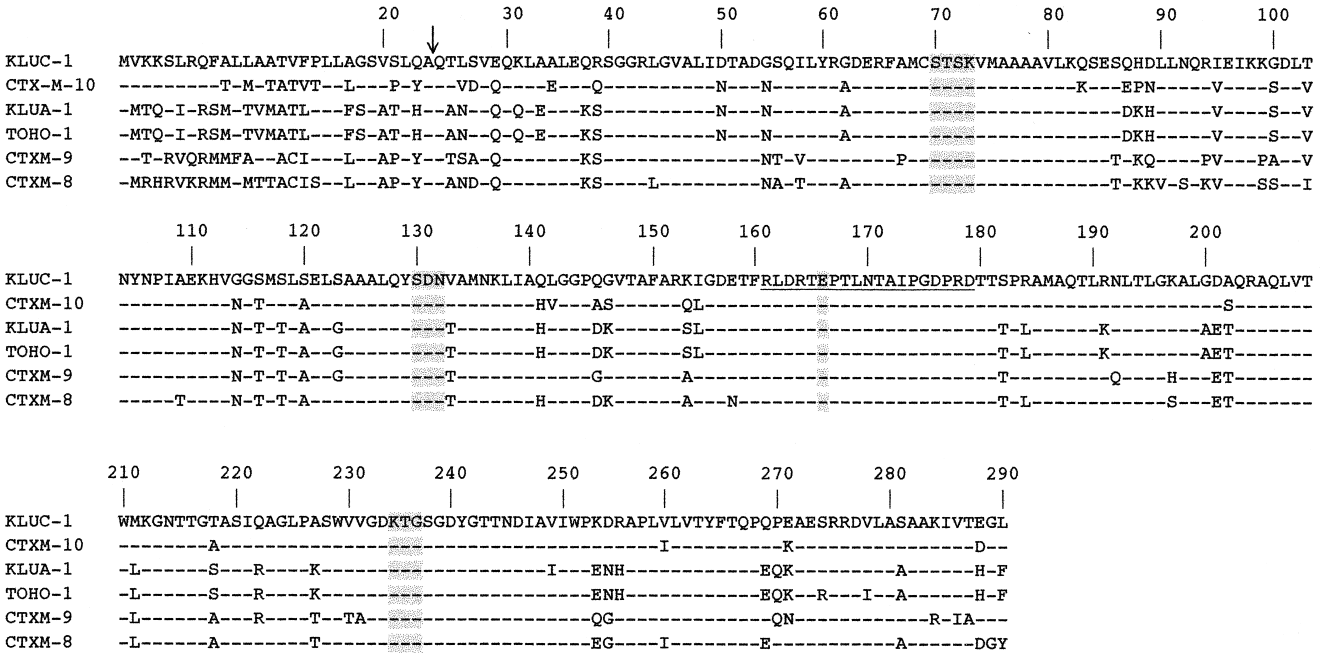

FIG. 1. Alignment of the KLUC-1 amino acid sequence with those of CTX-M-10 from *E. coli* (31), KLUA-1 from *K. ascorbata* (GenBank accession no. CAB 59824), Toho-1 from *E. coli* (14), CTX-M-9 from *E. coli* (27), and CTX-M-8 from *Enterobacter cloacae* (7). The numbering is according to Ambler et al. (2). Dashes indicate identical amino acid residues. The vertical arrow indicates the putative cleavage site of the leader peptide of the mature KLUC-1 β-lactamase. Four structural elements characteristic of class A β-lactamases are shaded (17). The amino acids of the omega loop are underlined.

Since the ORFs located upstream and downstream of the β-lactamase genes from *K. cryocrescens* and *K. ascorbata* shared consistent identities, it is possible that they constituted similar loci. Similarly, AmpC genes in several enterobacterial species are bracketed by nucleotide sequences that encode functionally related proteins (25).

A Southern transfer of a gel containing whole-cell DNA of *K. cryocrescens* 79.54 was performed (28), and the DNA hybridized by PCR with an 846-bp internal fragment of $bla_{\text{KLUC-1}}$ as a labeled probe (28). A positive signal was detected at the chromosomal migration position, further indicating the chromosomal origin of $bla_{\text{KLUC-1}}$ (data not shown).

Susceptibility testing. The MICs of selected β-lactams were determined as described previously (26). *K. cryocrescens* 79.54 was resistant to amoxicillin and ticarcillin and had reduced susceptibility to cephalothin and cefuroxime (Table 1). It was susceptible to the other β-lactam antibiotics tested. Resistance to aminopenicillins has been reported previously for *K. cryocrescens* (1). Once cloned in pBK-CMV (pKC7954) and expressed in *E. coli* DH10B (Table 1), KLUC-1 also conferred resistance or reduced susceptibility to cefotaxime, ceftriaxone, cefpirome, and aztreonam. These activities paralleled the activity of a β-lactamase from crude extracts (25) of a culture of *E. coli* DH10B(pKC7954), which were 40-fold higher than that of a culture of *K. cryocrescens* 79.54 (data not shown). The resistance profile observed for *E. coli* DH10B(pKC7954) corresponded to that conferred by plasmid-mediated CTX-M-type β-lactamases, which do not compromise susceptibility to ceftazidime significantly (31). The addition of clavulanic acid and tazobactam strongly lowered the β-lactam MICs (Table 1).

TABLE 1. MICs of β-lactams for reference strain *K. cryocrescens* 79.54, *E. coli* DH10B(pKC7954), and reference strain *E. coli* DH10B

β-Lactam(s)[b]	MIC (μg/ml)[a]		
	K. cryocrescens 79.54	*E. coli* DH10B (pKC7954)	*E. coli* DH10B
Amoxicillin	64	>512	2
Amoxicillin + CLA	<0.5	8	2
Ticarcillin	128	>512	1
Ticarcillin + CLA	1	8	1
Piperacillin	<1	128	1
Piperacillin + TZB	<1	<1	1
Cephalothin	8	>512	2
Cefuroxime	16	512	2
Cefotaxime	0.06	8	<0.06
Cefotaxime + CLA	<0.06	0.06	<0.06
Cefotaxime + TZB	<0.06	0.12	<0.06
Ceftazidime	<0.06	0.5	<0.06
Ceftazidime + CLA	<0.06	0.12	<0.06
Ceftazidime + TZB	<0.06	0.12	<0.06
Ceftriaxone	<0.06	128	<0.06
Cefepime	<0.06	1	<0.06
Cefepime + CLA	<0.06	0.25	<0.06
Cefepime + TZB	<0.06	0.12	<0.06
Cefpirome	<0.06	4	<0.06
Cefpirome + CLA	<0.06	<0.06	<0.06
Cefpirome + TZB	<0.06	<0.06	<0.06
Cefoxitin	1	4	2
Moxalactam	0.03	0.12	0.12
Aztreonam	0.06	2	0.06
Meropenem	<0.03	<0.06	<0.06
Imipenem	0.06	0.12	0.12

[a] *K. cryocrescens* 79.54 and *E. coli* DH10B(pKC7954) produced the KLUC-1 β-lactamase.

[b] CLA, clavulanic acid at a fixed concentration of 2 μg/ml; TZB, tazobactam at a fixed concentration of 4 μg/ml.

TABLE 2. Kinetic parameters of β-lactamase KLUC-1[a]

Substrate	Relative V_{max}	K_m (μM)	Relative V_{max}/K_m
Benzylpenicillin	100	10	100
Amoxicillin	65	30	25
Ticarcillin	6	5	10
Piperacillin	145	20	70
Cephalothin	1,200	140	100
Cefuroxime	175	30	65
Cefoxitin	<0.1	ND[b]	ND
Cefotaxime	170	110	20
Ceftazidime	0.5	5,700	<0.01
Ceftriaxone	170	45	45
Cefpirome	70	1,950	0.5
Cefepime	70	750	1
Aztreonam	10	150	1
Imipenem	0.01	30	<0.01

[a] V_{max} and V_{max}/K_m values were relative to that of benzylpenicillin, for which the values were set equal to 100. Data are the means of three independent experiments. Standard deviations were within 15%.
[b] ND, not determinable.

These results indicate that KLUC-1 is a clavulanic acid-inhibited ESBL that is likely weakly expressed in *K. cryocrescens*.

The β-lactam resistance pattern conferred by KLUC-1 resembled those conferred by chromosomally encoded β-lactamases of other enterobacterial species: *S. fonticola* (22), *C. koseri*, and *P. vulgaris* (19).

Biochemical analysis of β-lactamase KLUC-1. A culture of *E. coli* DH10B(pKC7954) was grown overnight at 37°C in 2 liters of Trypticase soy broth with amoxicillin (50 μg/ml) and kanamycin (30 μg/ml). The β-lactamase extract was obtained after sonification as described previously (26). It was further purified by a two-step ultrafiltration procedure, as recommended by the manufacturer (Vivaspin, 100,000 MWCOPES and 10,000 MWCOPES; Sartorius, Göttingen, Germany). Partially purified β-lactamase was used for kinetic measurements, performed at 30°C in 100 mM sodium phosphate (pH 7.0) as described previously (25).

The specific activity of the purified KLUC-1 β-lactamase, measured with 100 μM cephalothin as the substrate, was 4.2 U · mg of protein^{-1} with a 10-fold purification factor. KLUC-1 had strong activity against benzylpenicillin, piperacillin, cephalothin, cefuroxime, and ceftriaxone (Table 2). Significant hydrolytic activity was observed against cefotaxime and ceftriaxone, while a very low level of activity was detectable against ceftazidime. For this substrate, high K_m and low relative V_{max} values were observed (Table 2). This substrate profile corresponded to that reported for CTX-M-type enzymes (31).

Inhibition measured as 50% inhibitory concentrations showed that the 50% inhibitory concentrations of clavulanic acid, tazobactam, and sulbactam were low, being 0.05, 0.01, and 0.15 μM, respectively. On the basis of its kinetic parameters, the KLUC-1 enzyme is a β-lactamase that may belong to the 2be group of β-lactamases of the Bush-Jacoby-Medeiros classification (8).

Conclusion. The KLUC-1 β-lactamase from *K. cryocrescens* was mostly related to the subgroup of β-lactamases that comprises CTX-M-1, -3, -10, -11, and -12. However, it was not the direct progenitor of known plasmid-mediated CTX-M enzymes, in contrast to KLUA-1 from *K. ascorbata*, which shares 99% amino acid identity with the CTX-M-2 β-lactamase (C. Humeniuk et al., unpublished data [GenBank accession no. CAB59824]). Like the CTX-M-type enzymes (31), KLUC-1 is a clavulanic acid-inhibited Ambler class A ESBL that possesses a substrate profile that includes extended-spectrum cephalosporins but not ceftazidime. KLUC-1 possesses amino acid residues at key positions that may explain its extended spectrum of hydrolysis. The serine residue at position 237 may contribute significantly to this extended substrate profile, as reported for the β-lactamase of *P. vulgaris* (30). A similar omega loop sequence (residues 161 to 179) is found for KLUC-1 and the sequences of CTX-M-type enzymes such as Toho-1 (Fig. 1). The crystal structure analysis of Toho-1 shows that residue Phe160 suppresses the hydrogen bond between residues Thr160 and Ser/Thr181 that connects the N and C termini of the omega loop in non-ESBLs; this may explain, in part, the expanded substrate profile of Toho-1 (13). Since residue Phe160 was found also in the KLUC-1 sequence, the lack of a hydrogen bond may also increase the flexibility of the omega loop, extending the KLUC-1 substrate profile. Additionally, KLUC-1, like Toho-1 (13), has glycine residues at positions 232, 236, and 238 that may increase the flexibility of the B3 strand, which would make it possible for KLUC-1 to bind to bulky extended-spectrum cephalosporins.

Interestingly, most of the chromosomally encoded class A β-lactamases in members of the family *Enterobacteriaceae* (*S. fonticola*, *P. vulgaris*, *C. koseri*, *K. ascorbata*, and *K. cryocrescens*) have the same substrate profile, which includes amino- and ureidopenicillins, cephalothin, cefuroxime, cefotaxime, and ceftriaxone but not ceftazidime (19). However, KLUC-1 did not confer resistance to expanded-spectrum cephalosporins in *K. cryocrescens*. As reported for the expression of the naturally encoded β-lactamase from *Klebsiella oxytoca*, which is also independent of a LysR-type regulator (11, 12), it is likely that extended-spectrum cephalosporin-resistant *K. cryocrescens* mutants that would contain mutations in the bla_{KLUC-1} promoter region may be selected in vivo. In that case, a strong increase in the level of expression of the KLUC-1 β-lactamase would be expected (as observed when bla_{KLUC-1} was cloned on multicopy vector pBK-CMV [Table 1]), and this would thus confer resistance to extended-spectrum cephalosporins.

Finally, this report further underlines the fact that enterobacterial species are natural producers of either no β-lactamase or the Ambler class A and/or class C β-lactamase.

Nucleotide sequence accession number. The nucleotide sequence of bla_{KLUC-1} and the 6.1-kb insert of recombinant plasmid pKC7954 has been assigned GenBank nucleotide database accession no. AY026417.

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