

Chromosome-Encoded Ambler Class A β -Lactamase of *Kluyvera georgiana*, a Probable Progenitor of a Subgroup of CTX-M Extended-Spectrum β -Lactamases

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Received 22 April 2002/Returned for modification 30 May 2002/Accepted 10 September 2002

A chromosome-encoded β -lactamase gene, cloned and expressed in *Escherichia coli* from *Kluyvera georgiana* reference strain CUETM 4246-74 (DSM 9408), encoded the extended-spectrum β -lactamase KLUG-1, which shared 99% amino acid identity with the plasmid-mediated β -lactamase CTX-M-8. This work provides further evidence that *Kluyvera* spp. may be the progenitor(s) of CTX-M-type β -lactamases.

Plasmid-mediated CTX-M-type extended-spectrum β -lactamases (ESBLs) are being increasingly reported worldwide in enterobacterial isolates (20). They may be grouped according to their amino acid identity in four clusters: CTX-M-1 (CTX-M-1, -3, -10, -11, -12, -15, and -22), CTX-M-2 (CTX-M-2, -4, -5, -6, -7, and -20 and Toho-1), CTX-M-8, and CTX-M-9 (CTX-M-9, -13, -14/-18, -16, -19, and -21 and Toho-2) (2–4, 6, 10, 11, 13, 15–18, 20) (GenBank accession no. AY080894). These β -lactamases do not hydrolyze ceftazidime at a high level except for CTX-M-15, CTX-M-16, and CTX-M-19 (2, 10, 15). The natural producers of CTX-M enzymes have been identified in rare cases. The chromosome-encoded β -lactamases KLUC-1 from *Kluyvera cryocrescens* (5) and KLUA-1 from *K. ascorbata* (8) share 86 and 99% amino acid identity with the plasmid-borne CTX-M-1 and CTX-M-2 subgroups, respectively.

To elucidate further natural producers of CTX-M-type β -lactamase genes, we studied the β -lactamase content of *K. georgiana*, which belongs to the genus *Kluyvera*, which includes such species as *K. cochleae*, *K. ascorbata*, and *K. cryocrescens* (12). *Kluyvera* spp. have been rarely reported from clinical specimens (19). Detailed susceptibility of *K. georgiana* to β -lactams is not known and a preliminary disk diffusion antibiogram indicated that reference strain *K. georgiana* CUETM 4246-74 displayed a weakly expressed clavulanic acid-inhibited penicillinase phenotype (data not shown).

Cloning and sequencing identified a chromosomally encoded Ambler class A enzyme that shared 99% identity with CTX-M-8, the single representative of one of the four CTX-M clusters.

Bacterial strains and plasmid analysis. *K. georgiana* reference strain CUETM 4246-74 (DSM 9408) has been identified previously (12). The *Escherichia coli* DH10B reference strain was used for cloning experiments. Plasmid DNA extractions,

performed according to several methods as described previously (10, 14), failed to identify a plasmid.

Cloning and sequence analysis of the β -lactamase gene from *K. georgiana*. Whole-cell DNA of *K. georgiana* CUETM 4246-74 was extracted as described previously (14), digested with *Sau*3AI restriction enzyme, and ligated into the *Bam*HI site of pBK-CMV phagemid (14). Ten *E. coli* DH10B recombinant clones were obtained after selection onto Mueller-Hinton plates containing 30 μ g of kanamycin and 50 μ g of amoxicillin per ml. The recombinant plasmid (pKG-2) that had the shortest insert (1,781 bp) was sequenced as described previously (14).

An open reading frame of 876 bp was identified. Within the deduced protein of this open reading frame (291 amino acids), named KLUG-1 (for “*Kluyvera georgiana*”), characteristic elements of Ambler class A β -lactamases were identified (Fig. 1) (9). Computer analysis indicated a putative cleavage site for the mature protein located at the same position compared to that of β -lactamase KLUC-1 of *K. cryocrescens* (Fig. 1) (9). Isoelectric focusing analysis, performed as previously reported (14), showed that homogenates of *K. georgiana* 4246-74 and of *E. coli* DH10B (pKG-2) gave a single and identical β -lactamase with a pI value of 7.6.

The β -lactamase KLUG-1 shared 99% amino acid identity with the plasmid-mediated CTX-M-8 enzyme identified in Brazilian isolates of *Citrobacter amalonaticus*, *Enterobacter cloacae*, and *Enterobacter aerogenes* (3) (Fig. 1). Only one amino acid change (290) was noted between CTX-M-8 and KLUG-1 sequences that was located in the C termini. In addition, two amino acid changes were identified in the leader peptide sequence (Fig. 1). KLUG-1 shared 83 and 77% amino acid identity with the chromosomally encoded β -lactamases KLUA-1 from *K. ascorbata* (8) and KLUC-1 from *K. cryocrescens* (5).

Just upstream of *bla*_{KLUG-1}, a 533-bp DNA fragment was identified from recombinant plasmid pKG-2. Within this fragment, the 389-bp 5'-end located sequence shared 87% identity with DNA sequence upstream of *bla*_{KLUA-1} (8), whereas the 336-bp 5'-end located sequence shared 88% identity with DNA sequence upstream of *bla*_{KLUC-1} (data not shown) (5). This result indicated similarities in the DNA

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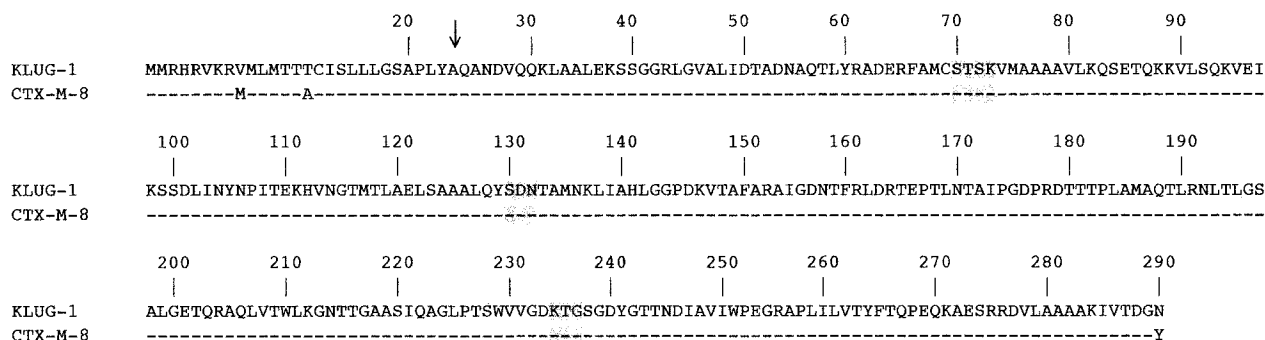


FIG. 1. Alignment of the KLUG-1 amino acid sequence with that of CTX-M-8 from *C. amalonaticus* (3). The numbering is according to the scheme of Ambler et al. (1). Dashes represent identical amino acid residues. The vertical arrow is the putative cleavage site of the leader peptide of the mature β -lactamase KLUG-1. Three structural elements characteristic of class A β -lactamases are boxed in gray (9).

sequences surrounding the β -lactamases genes in some *Kluyvera* species that may constitute similar loci.

A Southern transfer of an agarose gel containing whole-cell DNA of *K. georgiana* CUETM 4246-74 was performed (18) and hybridized with a PCR-generated 800-bp internal fragment of *bla*_{KLUG-1} (primer KLUG-1a, 5'-GATGAGACATG CGTTAAGC-3'; primer KLUG-1b, 5'-CTAATTACCGTCA GTGACG-3') as a labeled probe (15). A positive signal was detected at the chromosomal migration position indicating the chromosomal origin of *bla*_{KLUG-1} (data not shown).

Susceptibility testing. MICs of selected β -lactams were determined as described previously (14). *K. georgiana* CUETM

4246-74 was of intermediate susceptibility to amoxicillin, ticarcillin, and cephalothin (Table 1). It was susceptible to the other β -lactam antibiotics tested. Once cloned in pBK-CMV (pKG-2) and expressed in *E. coli* DH10B (Table 1), KLUG-1 conferred, in addition, resistance or reduced susceptibility to piperacillin, cefotaxime, ceftriaxone, cefepime, cefpirome, and aztreonam. The resistance profile observed for *E. coli* DH10B (pKG-2) corresponded to that conferred by the plasmid-mediated CTX-M-8 enzyme that does not compromise ceftazidime significantly (3). The addition of clavulanic acid and tazobactam lowered the β -lactam MICs for strains expressing KLUG-1. These results indicated that KLUG-1 is a clavulanic acid-inhibited ESBL that is weakly expressed in *K. georgiana*.

Conclusion. This report underlines further that enterobacterial species may be natural producers of Ambler class A ESBLs that are undetectable in these species on the sole basis of analysis of a disk diffusion antibiogram. Thus, it seems possible to select high-level producers via mutations occurring in the promoter sequences as described for the β -lactamase of *K. oxytoca* (7). Although phylogenetically related, the *Kluyvera* species produce different β -lactamases sharing similar substrate profiles. Finally, we identified the potential producer of a subgroup of CTX-M enzymes, CTX-M-8, that shares 83 to 88% identities with the other CTX-M β -lactamases (3).

In many cases, plasmid-encoded *bla*_{CTX-M} genes are located downstream of *ISEcp1*-like insertion sequences (4, 6, 10), whereas an *IS10*-like element was identified upstream of *bla*_{CTX-M-8} gene (GenBank accession no. AF189721). The immediate upstream- and downstream-located sequences of *bla*_{CTX-M-8} and *bla*_{KLUG-1} share consistent identity (Fig. 2). Thus, this *IS10*-like element may be involved in mobilization of the downstream-located β -lactamase gene.

The reason why *Kluyvera* species may be a source of plasmid-mediated CTX-M enzymes has to be investigated, whereas *Kluyvera* strains are rarely isolated in clinical microbiology.

Nucleotide sequence accession number. The nucleotide sequence reported here has been assigned to the GenBank nucleotide database under accession no. AF501233.

This work was funded by a grant from the Ministère de l'Éducation Nationale et de la Recherche (UPRES-EA), Université Paris XI, Faculté de Médecine Paris-Sud, Paris, France.

TABLE 1. MICs of β -lactams for *K. georgiana* reference strain 4246-74, *E. coli* DH10B (pKG-2), and *E. coli* DH10B reference strain

β -Lactam(s) ^a	MIC (μ g/ml) ^b		
	<i>K. georgiana</i> CUETM 4246-74	<i>E. coli</i> DH10B (pKG-2)	<i>E. coli</i> DH10B
Amoxicillin	4	512	2
Amoxicillin + CLA	1	8	2
Ticarcillin	16	>512	1
Ticarcillin + CLA	1	256	1
Piperacillin	2	512	1
Piperacillin + TZB	0.25	2	1
Cephalothin	4	>512	2
Cefuroxime	4	>512	2
Cefotaxime	0.25	32	<0.06
Cefotaxime + CLA	0.06	0.25	<0.06
Cefotaxime + TZB	0.06	0.12	<0.06
Ceftazidime	0.12	1	<0.06
Ceftazidime + CLA	<0.06	<0.06	<0.06
Ceftazidime + TZB	<0.06	<0.06	<0.06
Cefepime	0.12	16	<0.06
Cefepime + CLA	<0.06	0.12	<0.06
Cefpirome	0.12	16	<0.06
Cefpirome + CLA	<0.06	0.12	<0.06
Cefpirome + TZB	<0.06	0.06	<0.06
Cefoxitin	0.06	0.5	0.25
Moxalactam	<0.06	0.12	0.06
Aztreonam	0.06	16	0.06
Imipenem	0.06	0.12	0.12

^a CLA, clavulanic acid at a fixed concentration of 2 μ g/ml; TZB, tazobactam at a fixed concentration of 4 μ g/ml.

^b *K. georgiana* CUETM 4246-74 and *E. coli* DH10B (pKG-2) produced β -lactamase KLUG-1.

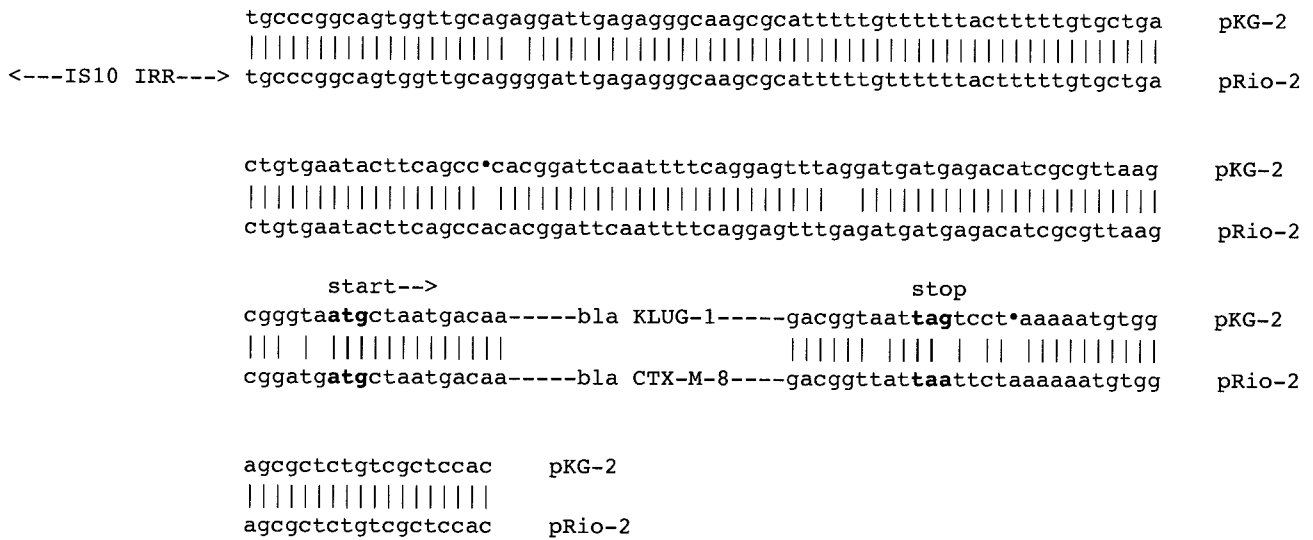


FIG. 2. Comparison of the surrounding DNA sequences of *bla*_{KLUG-1} and *bla*_{CTX-M-8} of recombinant plasmid pKG-2 and natural plasmid pRio-2, respectively. Boldfaced nucleotides indicate start and stop codons of β -lactamase genes. Vertical bars indicate identical nucleotides, whereas dots represent deleted nucleotides. IS10 IRR refers to the inverted repeat right of an IS10-like element.

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