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Chromosome-Encoded CTX-M-3 from *Kluyvera ascorbata*: a Possible Origin of Plasmid-Borne CTX-M-1-Derived Cefotaximases

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A gene identical to plasmid-borne *bla*$_{\text{CTX-M-3}}$ is present in the chromosome of one *Kluyvera ascorbata* strain. It is associated with a structure including an inverted repeat right and an open reading frame 477-like gene probably involved in the mobilization of *bla*$_{\text{CTX-M-3}}$. Two other *K. ascorbata* strains rendered the previously described *bla*$_{\text{KLUA-9}}$ gene.

In recent years, a constantly growing group of extended-spectrum β-lactamases (ESBLs) has gained the attention of microbiologists worldwide (2, 17). The so-called cefotaximases have stronger activity against cefotaxime or ceftriaxone than against ceftazidime, while the majority of the TEM- or SHV-derived ESBLs could be considered efficient ceftazidime-hydrolyzing enzymes (2).

This group of β-lactamases includes the CTX-M-1 to CTX-M-34 enzymes, Toho-1 to Toho-3, and other β-lactamases, such as UOE- and FEC-derived enzymes (1, 14; accession no. AY267213, AY292654, AJ557142, AY238472, and AY515297). On the basis of their nucleotide and amino acid sequences, these β-lactamases could be clustered into five main groups, CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25, as the first described members of each group (1).

Chromosome-encoded β-lactamases from some *Kluyvera* species were proposed as the source of CTX-M-derived enzymes. The CTX-M-2 subgroup appears to be derived from *Kluyvera ascorbata* KluA-1 (8), and CTX-M-8 subgroup appears to be derived from *Kluyvera georgiana* KluG-1 (11). The CTX-M-1 subgroup, although somehow related, does not seem to have evolved from *Kluyvera cryocrescens* KluC-1 (3).

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Three *K. ascorbata* strains (69 from the environment, 68 from a sputum sample, and 276 from a bile sample) were identified on the basis of a combination of standard biochemical criteria (6, 7), commercial systems (API 20E; BioMérieux, Marcy l'Etoile, France), and sequencing of 16S rRNA genes as a genetic criterion for identification (19). All of the sequences had more than 99.8% identity with each other (GenBank accession numbers AJ627201, AJ627202, and AJ627203) and 98.8 to 99.8% identity with the rRNA gene sequences of *K. ascorbata* that are available in databases, while their identity with those of the other species is lower.

Fragments from genomic DNA partially digested with HindIII (Gibco BRL) were cloned into a pK19 plasmid vector (kanamycin resistant) (13). Recombinant plasmids were introduced by transformation into competent *Escherichia coli* Top 10 F′ cells (Invitrogen), and transformant cells were selected on Luria-Bertani agar plates supplemented with 20 μg of kanamycin per ml and 0.5 μg of cefotaxime per ml. After PCR screening with primers specific for both *bla*$_{\text{CTX-M-1}}$- and *bla*$_{\text{CTX-M-2}}$-type genes (18), three *E. coli* clones were selected i.e., KK68C1, KK276C1 (both detected with *bla*$_{\text{CTX-M-2}}$ primers), and KK69C1 (detected with *bla*$_{\text{CTX-M-1}}$ primers), which were derived from *K. ascorbata* 68, 276, and 69, respectively. Plasmids from the *E. coli* KK68C1 and KK276C1 clones (pKK68C1 and pKK276C1, respectively) contained an insert of about 12 kb, including a *bla*$_{\text{KLUA}}$ gene having >99% nucleotide identity with *bla*$_{\text{KLUA-9}}$ (accession no. AJ427466). This allelic variant showed a single base shift (G831A) with respect to the known *bla*$_{\text{KLUA-9}}$ gene and 100% similarity with the deduced amino acid sequence of the KluA-9 β-lactamase. *E. coli* KK69C1 rendered pKK69C1 with an insert of nearly 10 kb; in this case, the β-lactamase-encoding gene presented strict identity with *bla*$_{\text{CTX-M-3}}$ (accession no. Y10278).

A comparison of the architectures of the flanking regions corresponding to plasmid-encoded versus chromosome-encoded CTX-M-3 is presented in Fig. 1. The proximal upstream 128 bp are identical, including the operator region. More distally, an aspartate aminotransferase-encoding gene can be localized, as in the corresponding *bla*$_{\text{KLUC-1}}$ region (accession no. AY026417). It is noteworthy that the *bla*$_{\text{KLUA-9}}$ upstream region displays a similar architecture (data not shown), suggesting a conserved evolutionary relationship; this is replaced in plasmid-borne *bla*$_{\text{CTX-M-3}}$ by IS*Ecp1* (9). Downstream from *bla*$_{\text{CTX-M-3}}$, there is also an identical region, ending in the inverted repeat right (IRR) of IS*Ecp1*. Three other open reading frames (ORFs) were detected downstream of *bla*$_{\text{CTX-M-3}}$ in *K. ascorbata* 69: an ORF477-like gene (including the IRR) with no known function, a 537-bp ORF (ORF1) encoding a protein with 64% identity with the *E. coli* Z1635-like protein (associated with a genomic pathogenicity island [OI-48]) (16), and a 540-bp ORF (ORF2) encoding a protein with 83% identity

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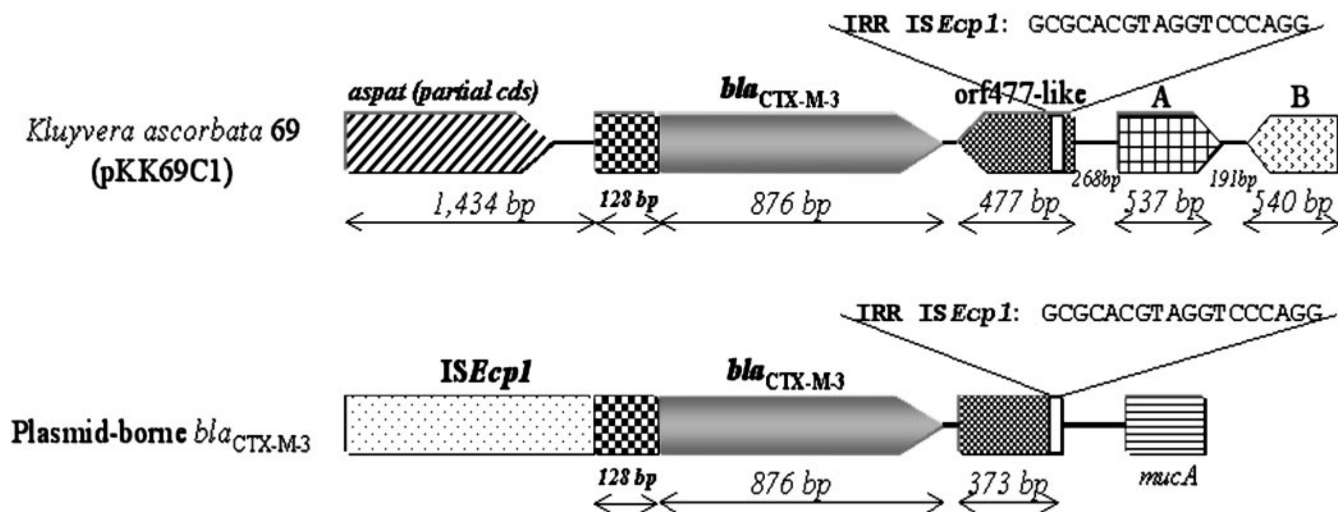

FIG. 1. Schematic representation of the $bla_{\text{CTX-M-3}}$ gene and surrounding regions in *K. ascorbata* 69 (top) compared to those of several members of the family *Enterobacteriaceae* (bottom) (9). The chessboard-like region (128 bp) is a sequence with 100% identity to the upstream region from plasmid-borne $bla_{\text{CTX-M-3}}$ (accession no. AF550415), including the promoter region. The first 373 bp of the downstream region of the *bla* gene are 100% identical to the corresponding sequences from $bla_{\text{CTX-M-3}}$ and $bla_{\text{CTX-M-15}}$ (accession no. AY604722), including the IRR from IS*Ecp1*; in *K. ascorbata*, this IRR is part of an ORF477-like gene (accession no. AY623932). (A) ORF that encodes a protein with 64% identity with the *E. coli* Z1635-like protein (accession no. AY275838). (B) ORF encoding a protein with 83% identity with a putative nucleoprotein from *Y. pestis* (accession no. NP_669893). Block arrows indicate the direction of gene transcription.

with a putative nucleoprotein from *Yersinia pestis* (4). No other element corresponding to an insertion sequence could be detected close to chromosome-encoded $bla_{\text{CTX-M-3}}$.

Antimicrobial susceptibility was determined by agar dilution in accordance with NCCLS guidelines (10). MICs are shown in Table 1. Although wild-type strains were resistant or intermediate to ampicillin (MICs, ≥16 μg/ml) and also to cephalothin (MICs, 16 μg/ml), they remained susceptible to piperacillin, cefoxitin, cefotaxime, ceftazidime, cefepime, and aztreonam. An at least 3-dilution decrease in the β-lactam MICs was detected when clavulanic acid was added to ampicillin, cefotaxime, and ceftazidime. For *E. coli* recombinant clones containing any of the three *Kluyvera* genes, the MICs of β-lactams, except cefoxitin, were higher (also reported by Humeniuk for different KLUAs [8]). It is noteworthy that the cefotaxime MICs for recombinant clones exhibited a 7- to 10-dilution increase compared to those for the corresponding *K. ascorbata* strains, suggesting the necessity of a proper genetic background that is probably not achieved when the gene is part of the *Kluyvera* chromosomal DNA. However, gene expression from a high-copy-number vector could be responsible for this effect.

Crude extracts from all of the *K. ascorbata* strains and the recombinant clones were analyzed by analytical isoelectric focusing with an iodometric overlay system (500 μg of ampicillin per ml as the substrate) to reveal β-lactamase activities (15). Different β-lactamases with known pIs (SHV-2, TEM-1, and CTX-M-2) and pI markers (Pharmacia isoelectric focusing cal-

TABLE 1. MICs for *K. ascorbata* strains and derived clones

Antibiotic(s)	MIC (μg/ml)						
	K. ascorbata strain:			*E. coli* recombinant clone:			*E. coli* TOP 10F′
	68	69	276	KK68C1	KK69C1	KK276C1	
Ampicillin	32	16	16	>512	>512	>512	2
Ampicillin-clavulanate (2:1)	1/0.5	0.5/0.25	0.5/0.25	4/2	4/2	8/4	2/1
Piperacillin	4	4	4	>128	>128	>128	1
Cephalothin	16	16	16	>1,024	1,024	>1,024	2
Cefoxitin	2	2	1	4	4	8	2
Cefotaxime	0.125	0.125	0.063	128	16	64	≤0.016
Cefotaxime-clavulanate[a]	≤0.032	≤0.032	≤0.032	0.25	0.063	0.25	ND[b]
Ceftazidime	0.063	≤0.032	≤0.032	4	1	4	0.125
Ceftazidime-clavulanate[a]	≤0.032	≤0.032	≤0.032	0.125	0.125	0.25	ND
Cefepime	0.016	≤0.008	≤0.008	16	1	16	0.016
Aztreonam	0.063	≤0.032	≤0.032	32	8	32	0.032
Tetracycline	2	1	1	>128	>128	128	>128
Kanamycin	2	2	2	>128	>128	>128	1

[a] Fixed concentration of lithium clavulanate, 4 μg/ml.
[b] ND, not done.

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ibration kit) were focused in parallel as standards. Molecular weights of enzymes were determined by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis with molecular weight standards (Bio-Rad Laboratories). After electrophoresis, gels were renatured with 20 mg of benzylpenicillin solution per ml in 0.05 M phosphate buffer (pH 7.0) (12) prior to β-lactamase detection with the iodometric overlay system. The β-lactamase from *K. ascorbata* 69 has a pI of 8.1 and a molecular mass of 27,200 Da. The enzymes from the other two strains have a pI of 7.7 and a molecular mass of 28,000 Da, in good agreement with the values predicted for the deduced mature proteins (28,038 and 28,117 Da, respectively).

These results firmly support the hypothesis of the ubiquitous *Kluyvera* β-lactamases as the origin of at least some CTX-M-derived cefotaximases. The finding of an enzyme with strict identity with CTX-M-3, included in the CTX-M-1 subgroup, could place the former as the putative origin of this subfamily of β-lactamases. Furthermore, the common architecture of the chromosomal bla_{KLUA} genes seems to be very conserved and located downstream of an aspartate aminotransferase-encoding gene. The association of similar IRR structures with some $bla_{\mathrm{CTX\text{-}M}}$ or bla_{KLU} genes, absent in $bla_{\mathrm{CTX\text{-}M\text{-}2}}$, which is associated with a putative recombination site instead, is interesting (5). It is noteworthy that ORFs located downstream have a GC content different from that expected in the *Kluyvera* chromosome, suggesting that they could be the product of some previous horizontal mobilization. If so, IRR would be a leftover of this event or a preexisting sequence that could have been targeted as convenient for recruiting of the resistance marker into a genetic shuttle.

In the *K. ascorbata* isolates analyzed by Humeniuk et al. (8) and in this study ($n = 15$), nine different enzymes could be detected. If more *Kluyvera* isolates were analyzed, we would probably find more of the CTX-M-n enzymes, so far described as plasmid-borne cefotaximases, as chromosome encoded in hitherto unanalyzed *Kluyvera* strains.

In view of its chromosomal origin, CTX-M-3, instead of CTX-M-1, could be proposed as the ancestor of this subgroup of related enzymes.

Even if most class A ESBLs are the result of specific mutations in genes encoding a narrower-spectrum enzyme extending the activity of the translated protein, the finding of natural chromosome-encoded class A β-lactamase mobilized from genomic to plasmidic backgrounds (after recruitment as gene cassettes or by insertion elements, integrons, or transposons) such as CTX-M-3 could represent an ancestor-to-mutational-derivative evolutionary model in which CTX-M enzymes would be either the ancestor or the derivative part. Diversity within the genus *Kluyvera* for preexisting ESBLs' chromosomal genes is likely to precede their recruitment. Therefore, the term "born cefotaximases" could be coined for preexisting chromosomal β-lactamases, such as CTX-M-3 and CTX-M-5 (designated KluA-2) (1), to differentiate them from other ESBLs that follow the ancestor-to-mutational-derivative model.

Nucleotide sequence accession number. The GenBank accession number for *K. ascorbata* $bla_{\mathrm{CTX\text{-}M\text{-}3}}$ and flanking sequences is AJ632119.

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