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EPIDEMIOLOGY AND SURVEILLANCE

Redefining the Origin and Evolution of Chromosomally Encoded $bla_{CTX\text{-}M/KLU}$ in the Context of a Revised Taxonomy of Genus *Kluyvera*

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ABSTRACT Changes in *Kluyvera* taxonomy may clarify each species contribution for recruitment and dissemination of their relevant β-lactamases. The CTX-M-2 subgroup is linked to *Kluyvera ascorbata*, KLUC to *Kluyvera cryocrescens*, and CTX-M-25 to *Kluyvera georgiana*. The CTX-M-8 subgroup can be linked to *Kluyvera* genomospecies 3 and CTX-M-9 to *Kluyvera* genomospecies 2. *Kluyvera sichuanensis* and *Kluyvera* genomospecies 1 harbor new subgroups. The CTX-M-1 subgroup has a direct counterpart in an isolate proposed as a new genomospecies 5.

KEYWORDS CTX-M evolution, *Kluyvera* genomospecies

CTX-Ms are the most prevalent group of extended-spectrum β-lactamases (ESBLs) among pathogens, representing a global pandemic (1–4). There are ~233 CTX-M variants (http://bldb.eu/) (5), distributed in at least five gene clusters (6).

Because the first chromosomic variants reported in *Kluyvera ascorbata* displayed 95% to 100% amino acid identity with CTX-M-2, they were suggested to be the progenitors of the plasmid-encoded CTX-M-2 subgroup (7). Later, other chromosomally encoded enzymes, such as CTX-M-76 and CTX-M-95, were found already circulating among pathogens (8).

Other chromosomal counterparts of acquired CTX-M β-lactamases were identified in different species of *Kluyvera*. KLUC-1 (from *Kluyvera cryocrescens*) was the proposed ancestor of the CTX-M-1 cluster (9), but other plasmid-encoded enzymes were found to have direct chromosomal counterparts, i.e., CTX-M-3 and CTX-M-37, in an environmental isolate identified as *K. ascorbata* (6), and a *K. cryocrescens* isolate from a urinary tract infection (10). KLUG-1 (now CTX-M-152), the chromosomal β-lactamase from *Kluyvera georgiana*, was proposed as the putative origin of the CTX-M-8 subgroup (11) and the chromosomally encoded KLUY-1-4 (*K. georgiana*) for the CTX-M-9 subgroup (12), whereas the most recent member of this subgroup is the chromosomally encoded CTX-M-213 in *Kluyvera ascorbata* strain KA2 (13). CTX-M-78, a chromosomally encoded β-lactamase also reported in *K. georgiana*, has been suggested as the putative progenitor of the CTX-M-25 subgroup (14).

Even though participation of *Kluyvera* as the origin of antimicrobial resistance (AMR) genes is accepted, each species contribution is unclear because the assigned species from which chromosomal β-lactamases were recovered need to be revisited (15). A previous version of our manuscript included a deep reexamination of this genus taxonomy; however, a publication by Liu et al. (16) proposed a new species, *Kluyvera sichuanensis* sp. nov., and that other isolates whose sequences were available and used in our analysis should correspond to new genomospecies: *K. cryocrescens* L2, as the novel genomospecies 1; *K. georgiana* KA2 as a novel genomospecies 2; *K. georgiana* PO2S7 in the novel genomospecies 3; and

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K. intermedia D51-sc-1712206 as novel genomospecies 4 (ERR2221162). The cited reference sequences corresponding to *Kluyvera* genomospecies 4 could not be found.

(Part of this work was presented at ASM Microbe 2019, San Francisco, CA [15]).

In this study, we performed a genomic analysis comparing $bla_{CTX\text{-}M/KLU}$ genes present in the *Kluyvera* chromosome to revise their association with the new proposed taxons and correspondence to subgroups of CTX-M.

We included 15 isolates of *Kluyvera* sp. recovered from clinical sources (see Table S1 in the supplemental material), preliminarily identified with biochemical tests, 16S rRNA gene sequencing, and matrix-assisted laser desorption ionization–time of flight mass spectrometry (data not shown). Whole-genome sequencing (WGS) was performed in a HiSeq X10 sequencer (Illumina, San Diego, CA, USA); paired forward and reverse reads were used as inputs for *de novo* assembly using the Velvet package (https://www.ebi.ac.uk/~zerbino/velvet/) and 27 genomes from type, reference, and/or representative strains (including WGS assemblies related to *Kluyvera* obtained from NCBI genomes between 20 November 2016 and 25 March 2021) (see Table S2 in the supplemental material).

Sequences were aligned with ClustalX (http://clustalx.software.informer.com/2.1/). The molecular evolution model was estimated with JModelTest2 (http://gihub.com//ddarriba/jmodeltest2/releases), and unrooted phylogenetic trees (1,000 bootstraps) were obtained with PhyML (http://www.atgc-montpellier.fr/phyml/versions.php) and MEGA 7 (17), visualized and edited by FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

WGS data from *Kluyvera* isolates were used to perform digital DNA-DNA hybridization (dDDH) using the genome-to-genome distance calculator (version 2.1; http://ggdc.dsmz.de) (18, 19) and average nucleotide identity (ANI) using the OrthoANIu (https://www.ezbiocloud.net/tools/ani) (20) (Table 1), confronting the isolates with the correspondent type strain (Table S2). Minimal cutoff points of 70% dDDH and 95% OrthoANI values were considered to represent species delineation.

Sequences that were found to be unrelated to the appropriate type or reference strains were submitted to the online TYGS platform (https://tygs.dsmz.de/user_requests/new) to assess their identification at the genus or species level (21). *Kluyvera* species Nf5 and 4105 (from our collection) matched with *Phytobacter diazotrophicus* and therefore were not included in further analyses.

Most isolates could be assigned to the accepted species *K. intermedia*, *K. ascorbata*, *K. cryocrescens*, and *K. georgiana*; the proposed species *K. sichuanensis* sp. nov.; or the proposed genomospecies 1, 2, and 3. The isolate identified preliminarily as *K. cryocrescens* 169 did not match with any of the already established species within the genus. With ANI values and dDDH still compatible within the genus, we propose that this isolate is representative of a new genomospecies 5. This correlates with differences in its chromosomal $bla_{CTX\text{-}M}$ genes and with a preliminary analysis using MLST (data not shown) (15).

A phylogenetic tree of all mature CTX-M enzymes showed that the chromosomally encoded enzymes from *Kluyvera* can be included in at least six clusters (Fig. 1): (i) CTX-M-1 subgroup containing only two variants (CTX-M-3 and CTX-M-37); (ii) CTX-M-2 cluster, including 16 enzymes from *K. ascorbata* isolates (KLUA-1 to -12, CTX-M-76, CTX-M-77, CTX-M-95, and CTX-M-115); (iii) CTX-M-8 subgroup comprising KLUG-1 from *K. georgiana*; (iv) CTX-M-25 subgroup with CTX-M-78 from *K. georgiana* 14751, suggested as the origin of the group (14); (v) the "compact" CTX-M-9 subgroup with the KLUY enzymes from not-well-defined *Kluyvera* species isolates (and CTX-M-213 from the current *Kluyvera* genomospecies 2 strain KA2) (13); (vi) KLUC cluster, including KLUC-1 from *K. cryocrescens* NBRC102467 and four other named KLUC β-lactamases, although from plasmid.

The proposed hybrid enzymes derived from CTX-M-1 and CTX-M-9, their point-mutant derivatives, and other CTX-Ms with extensive amino acid changes, such as CTX-M-45 (formerly, Toho-2, having several frameshifts throughout the original sequence) and CTX-M-151 from a *Salmonella choleraesuis*, could not be assigned to a single group and distort the picture (22–26).

According to the phylogenetic tree of $bla_{CTX\text{-}M/KLU}$ from *Kluyvera* species isolates, most genes are distributed in two clades (Fig. 2).

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TABLE 1 Mean ANI and dDDH values for all strains

Strain[a]	ANI (%)[b]	Estimated dDDH (%)[c]
***Kluyvera ascorbata* ATCC 33433**	**100**	**100**
K. ascorbata 3162	98.65	89.10
K. ascorbata LFC	97.15	86.90
K. ascorbata 4663	98.46	87.90
K. ascorbata 58	98.58	88.10
K. ascorbata 68	98.73	89.60
K. ascorbata 8633	98.37	86.40
K. ascorbata 220	98.44	87.40
K. ascorbata 280	98.51	87.80
K. ascorbata 711	98.52	87.40
K. ascorbata TP1631	99.60	99.92
K. ascorbata Colony 413	94.74	56.40
K. ascorbata 13608	90.99	42.90
K. cryocrescens 169	85.12	29.50
***Kluyvera sichuanensis* 090646**	**100**	**100**
K. ascorbata 13608	97.50	78.60
K. cryocrescens 169	85.42	29.70
***Kluyvera georgiana* ATCC 51603**	**100**	**100**
K. georgiana 14751	97.89	82.30
K. cryocrescens 169	85.08	29.20
***Kluyvera cryocrescens* NBRC102467**	**100**	**100**
K. cryocrescens 1919	99.21	93.50
K. cryocrescens 4701	99.26	94.40
K. cryocrescens SCW13	99.23	94.20
K. cryocrescens NCTC10483	99.18	92.70
K. cryocrescens NCTC12993	99.71	98.70
K. cryocrescens 169	89.89	40.00
***Kluyvera* genomosp. 1_L2**	**100**	**100**
K. cryocrescens 169	89.50	38.20
***Kluyvera intermedia* ATCC 33110**	**100**	**100**
K. intermedia N2-1	99.01	91.70
K. intermedia NCTC12125	99.95	99.90
K. intermedia HR2	99.01	91.70
K. cryocrescens 169	85.85	30.40
***Kluyvera* genomosp. 2_KA2**	**100**	**100**
K. cryocrescens 169	84.71	28.90
***Kluyvera* genomosp. 3_PO2S7**	**100**	**100**
K. cryocrescens 169	85.26	29.20
***Phytobacter diazotrophicus* DSM17806**	**100**	**100**
P. diazotrophicus UAEU22	99.00	91.30
Kluyvera strain 4105	97.75	80.50
Kluyvera sp. Nf5	98.88	90.20

[a]ANI and dDDH values are relative to reference strain, in boldface.
[b]Relative to reference genome (in boldface) and other average of genome nucleotide identity analyzed.
[c]Identity/high-scoring pair length.

The first clade (node A) groups *K. cryocrescens* isolates with strains NBRC102467 (encoding KLUC-1), 4701, 1919 (encoding KLUC-5), NCTC10483 (also encoding KLUC-1), NCTC12993 (containing a single deletion at 133 bp), and SCW13 as a monophyletic group and the remaining isolates (corresponding to *Kluyvera* genomospecies 1 strain L2 and *Kluyvera* genomospecies 5 strain 169, see below) as external branches. True *K. cryocrescens* isolates in this clade carry a chromosomal bla_{KLUC} and share high nucleotide identity. New KLUC-1-derived variants (WP_061284984.1) were found in *K. cryocrescens* 4701 (Ala219Thr mutant) and *K. cryocrescens* SCW13 (a variant with 93% amino acid identity).

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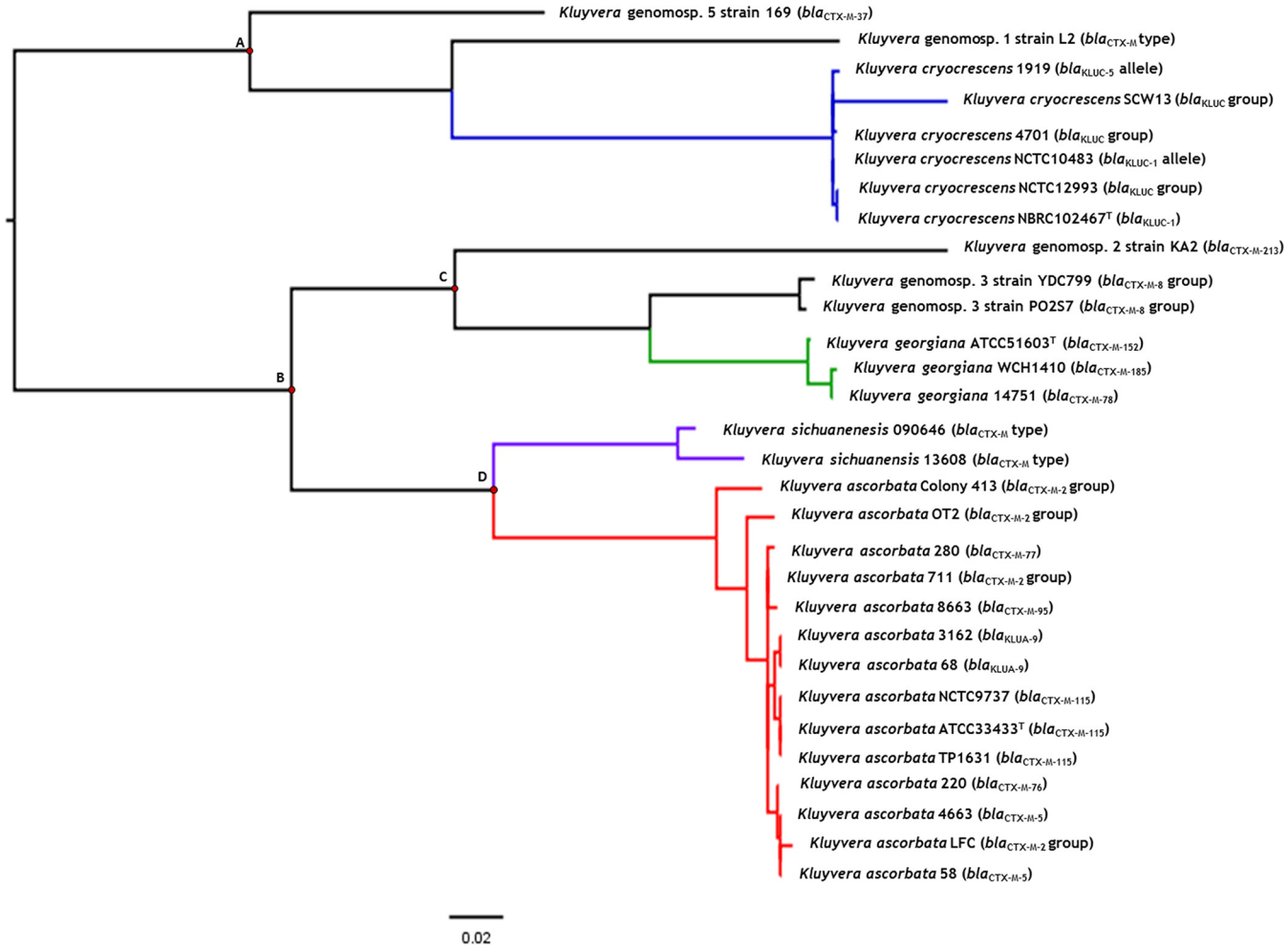

FIG 1 Phylogenetic tree of mature CTX-M enzymes. CTX-M enzymes from *Kluyvera* isolates are shown in red. The widths of the branches are related to bootstrap values.

The second clade (node B) includes two main subclades: (i) node C, containing all *K. georgiana* isolates and *Kluyvera* genomospecies 3 strains (YDC799 and PO2S7), in two separate subclades, and *Kluyvera* genomospecies 2 strain KA2 as an external branch; and (ii) node D, including one subclade containing all *K. ascorbata* isolates as a monophyletic group and a second subclade containing *K. sichuanensis* 090646 and the assigned 13608 strain (deposited as *K. ascorbata*), each encoding new CTX-Ms close to CTX-M-95 (94% and 91% amino acid identity, respectively). Within the *K. ascorbata* group, isolates with new variants (all related to the CTX-M-2 cluster) include *K. ascorbata* 711, encoding a new enzyme having 99% amino acid identity with CTX-M-95 (Leu80Val and Val249Ile; WP_063860092.1); *K. ascorbata* LFC, encoding a new variant having 99% amino acid identity with CTX-M-5 (Asn254Ser; WP_032072602.1); and *K. ascorbata* OT2 and colony 413, encoding a variant having 99% and 97% amino acid identity with KLUA-10 and CTX-M-95, respectively.

Summarizing, the novel $bla_{\text{CTX-M/KLU}}$ genes in the *Kluyvera* taxons include (i) a novel $bla_{\text{CTX-M}}$ (86% nucleotide identity and 91% amino acid identity with the closer relative CTX-M-95) from *K. sichuanensis* 090646 and *K. sichuanensis* 13608 with no plasmidic counterpart; (ii) a new CTX-M-9-like allele, $bla_{\text{CTX-M-213}}$, from *Kluyvera* genomospecies 2 strain KA2 (16); (iii) $bla_{\text{CTX-M-37}}$ ($bla_{\text{CTX-M-1}}$ subgroup) from *Kluyvera* genomospecies 5 strain 169 (deposited as *K. cryocrescens*) (10); (iv) *Kluyvera* genomospecies 1 strain L2, encoding a new CTX-M closer to KLUC and having ≤80% acid identity with the described KLUC enzymes and the CTX-M-1 cluster; and (v) *Kluyvera* genomospecies 3 strain PO2S7, encoding a single-mutant variant (Thr109Ala) related to CTX-M-8. YDC799 sequence could not be analyzed due to the presence of multiple stop codons that suggest sequencing errors.

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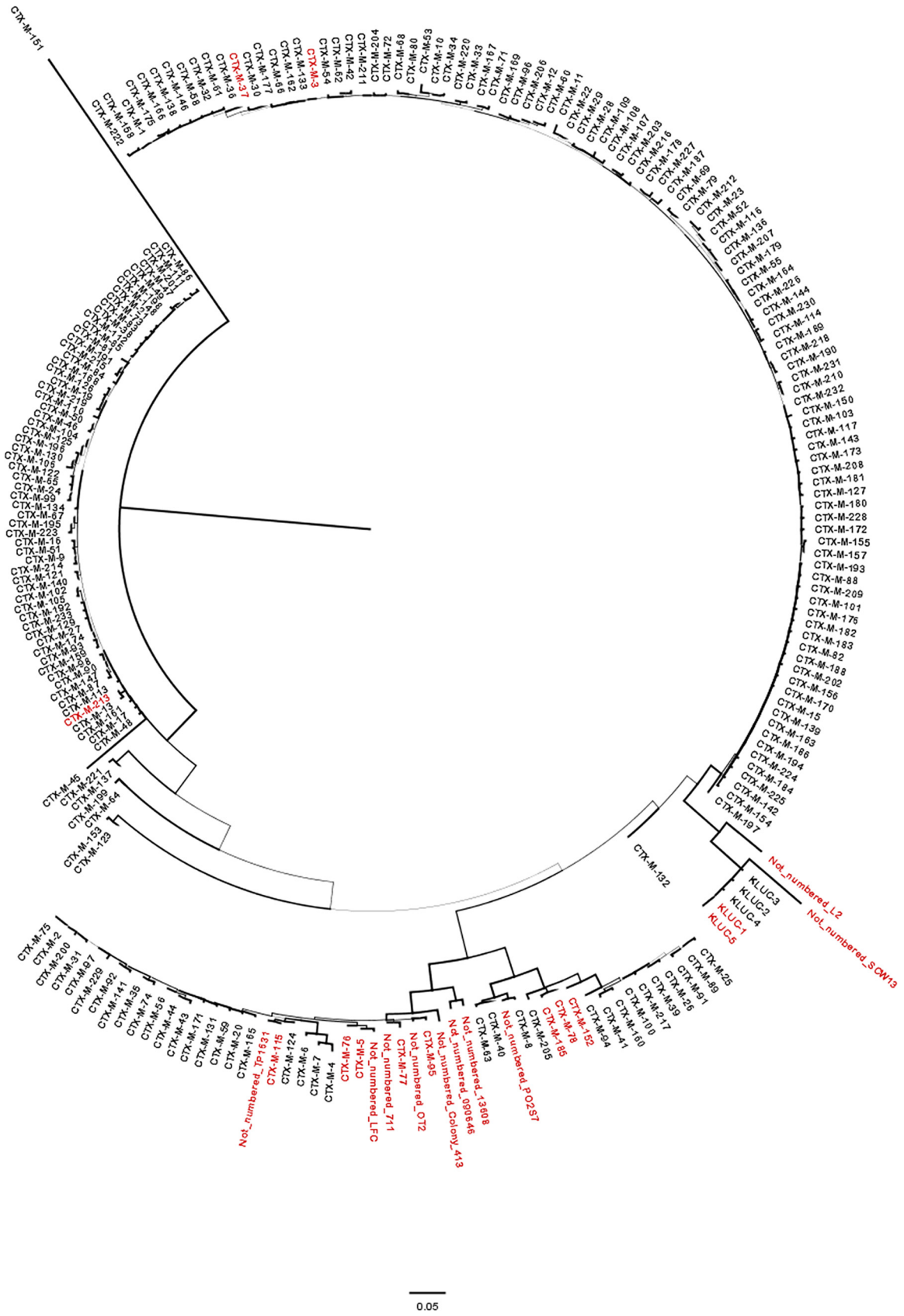

FIG 2 Phylogenetic tree of *bla*$_{CTX-M}$ genes from *Kluyvera* isolates.

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TABLE 2 Proposed CTX-M subfamilies and their corresponding chromosomally encoded progenitors in *Kluyvera* species

Group denomination	Representative plasmid-borne gene	Best-fitting chromosomal gene (ancestor)	Closest plasmid gene (to ancestor)	Species	GenBank accession no.
CTX-M-1	$bla_{CTX-M-3}$	$bla_{CTX-M-3}$	$bla_{CTX-M-3}$	*Kluyvera* sp.[a]	AJ632119
	$bla_{CTX-M-15}$	$bla_{CTX-M-37}$	$bla_{CTX-M-37}$	*Kluyvera* genomosp. 5	FN813246
CTX-M-2	$bla_{CTX-M-2}$	$bla_{CTX-M-5}$/bla_{KLUA-2}	$bla_{CTX-M-5}$	*K. ascorbata*	AJ251722
		$bla_{KLUA-1, 3, 4, 12}$	$bla_{CTX-M-124}$	*K. ascorbata*	AJ272538, AJ427461, AJ427462, AJ427469
		$bla_{CTX-M-115}$	$bla_{CTX-M-115}$	*K. ascorbata*	NZ_JMPL00000000.1
		$bla_{CTX-M-76}$		*K. ascorbata*	NG_049028
		$bla_{CTX-M-77}$		*K. ascorbata*	NG_049029
		$bla_{CTX-M-95}$		*K. ascorbata*	NG_049049
CTX-M-8	$bla_{CTX-M-8}$, $bla_{CTX-M-40}$, $bla_{CTX-M-41}$, $bla_{CTX-M-63}$	Not yet assigned	$bla_{CTX-M-8}$	*Kluyvera* genomosp. 3	NZ_CP050321.1
				Kluyvera genomosp. 3	CP022114.1
CTX-M-25	$bla_{CTX-M-25}$, $bla_{CTX-M-26}$, $bla_{CTX-M-160}$, $bla_{CTX-M-100}$, $bla_{CTX-M-89}$, $bla_{CTX-M-217}$, $bla_{CTX-M-39}$, $bla_{CTX-M-94}$, $bla_{CTX-M-91}$, $bla_{CTX-M-205}$	$bla_{CTX-M-78}$	$bla_{CTX-M-205}$	*K. georgiana*	AM982522
		$bla_{CTX-M-152}$		*K. georgiana*	AF501233
		$bla_{CTX-M-185}$		*K. georgiana*	KX266838
CTX-M-9	$bla_{CTX-M-9}$	$bla_{CTX-M-213}$		*Kluyvera* genomosp. 2	MH094805
	$bla_{CTX-M-14}$	bla_{KLUY-1}		*Kluyvera* sp.[a]	AY623932
KLUC	$bla_{KLUC-1-5}$	bla_{KLUC-1}	bla_{KLUC-1}	*K. cryocrescens*	AY026417
		bla_{KLUC-5} allele	bla_{KLUC-5}	*K. cryocrescens* 1919	(SAMN16845439)[b]
No name[c]	Unknown		New bla_{CTX-M} gene group	*K. sichuanensis*	NZ_JABBJF010000000
				K. sichuanensis 13608	(SAMN16845436)[b]
No name[c]	Unknown		New bla_{CTX-M} gene group	*Kluyvera* genomosp. 1	NZ_LGHZ01000000
Outliers					
CTX-M-151				*S. choleraesuis*	NG_048927
CTX-M-64	$bla_{CTX-M-64}$, $bla_{CTX-M-199}$, $bla_{CTX-M-123}$, $bla_{CTX-M-132}$, $bla_{CTX-M-153}$, $bla_{CTX-M-174}$, $bla_{CTX-M-234}$				
CTX-M-137	$bla_{CTX-M-221}$				

[a]No WGS data available.
[b]BioSample.
[c]Will depend on the number after the first formal deposit in β-lactamase databases.

If we accept that plasmid-borne CTX-M enzymes derive from recruitment into mobile elements of previously native (chromosomal) gene counterparts present in different isolates of *Kluyvera*, it would be expected to have heterogeneity based on the long evolution of these environmental bacteria in different isolated geographic locations. Because different *Kluyvera* species have been able to at least transiently colonize the human/animal gut, selection under antibiotic pressure of promiscuous $bla_{CTX-M/KLU}$ genes is probably the driving force for recruitment. Once existing as plasmidic genes, further diversification may be facilitated by the higher copy number, granted by their location in proper and promiscuous platforms, plasmids, and strains.

Incorporating the recent taxonomic proposals by Liu et al. (16) with our findings that depict even more diversity within the genus with a phylogenetic approach to the different bla_{CTX-M} gene origins, including at least sequences from all the new or better-defined taxons within *Kluyvera*, we provide some clarification about their origin in Table 2.

The CTX-M-1 subfamily may be the most prevalent group present in epidemic plasmids and clones and has been a driver or coselected for multiresistance. The most "popular" enzymes are CTX-M-3 and its derivative CTX-M-15, whose carriers also exhibit resistance to ceftazidime. A 100% identical counterpart to CTX-M-3 was originally proposed as the chromosome origin from an isolate classified (at that time) as *K. ascorbata*. Unfortunately, this strain could not be recovered for full reexamination. The other chromosomal counterpart described so far is CTX-M-37, well embedded in the cloud represented by this subfamily. In this case, the corresponding isolate was identified as *K. cryocrescens* 169, but it should be reclassified as a new genomospecies 5 (*Kluyvera* genomospecies 5 strain 169) (15).

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The CTX-M-2 subfamily is well populated on both sides, plasmidic and chromosomal enzymes. In fact, the proposal of KLUA enzymes as ancestors of this family was initially supported within this subfamily (7), and with direct counterparts 100% identical, more ancestors (e.g., $bla_{CTX-M-76}$, $bla_{CTX-M-77}$, and $bla_{CTX-M-115}$) should be considered directly recruited. Presence of a new bla_{CTX-M} from *K. ascorbata* colony 413, showing 94% nucleotide identity and borderline ANI values compared with the monophyletic *K. ascorbata* clade, should be considered with caution because the deposited genome has only 2,832,874 bp, compared to the ~5,000,000 bp for other *Kluyvera* genomes.

In the case of the very compact CTX-M-9 subfamily, the origin is most likely a new *Kluyvera* genomospecies 2, represented by strain KA2 as a close relative of the indeterminate origin of KLUY enzymes.

Within the CTX-M-8 subfamily, only four plasmid-borne variants are included, with maximum 2.40% nucleotide divergence. *Kluyvera* genomospecies 3 strain PO2S7 is likely to be related to the CTX-M-8 group ancestor.

The CTX-M-25 group includes nine plasmid-borne members; three chromosomal genes from *K. georgiana* ($bla_{CTX-M-78}$, $bla_{CTX-M-152}$, and $bla_{CTX-M-185}$) group better with plasmidic $bla_{CTX-M-205}$ than with other $bla_{CTX-M-25}$ alleles, so perhaps with further sequencing, they may consolidate as a new group, especially if a closer chromosomic counterpart is found for this last group.

Kluyvera genomospecies 1 strain L2 harbors a gene for a new CTX-M subfamily cluster, so far not recruited as clinically relevant plasmidic counterparts (16).

Because some of the previously known "plasmidic" enzymes have a strict identity with chromosomal encoded enzymes (and vice versa), the limits between the nomenclature as KLU or CTX-M (chromosomal or plasmidic, respectively) also produce obscure inconsistencies that should be examined to clarify our understanding of this family volution by providing a univocal single (consensus) name for a single (mature) enzyme sequence.

Data availability. Short reads for all sequenced isolates have been submitted to NCBI under BioProject number PRJNA679803.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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