Identification of a Progenitor of the CTX-M-9 Group of Extended-Spectrum β -Lactamases from *Kluyvera georgiana* Isolated in Guyana

Adam B. Olson,¹ M. Silverman,³ David A. Boyd,² Allison McGeer,³
Barbara M. Willey,³ V. Pong-Porter,³ N. Daneman,³
and Michael R. Mulvey^{2*}

University of Manitoba, Winnipeg, Manitoba, Canada,¹ National Microbiology Laboratory, Health
Canada, Winnipeg, Manitoba, Canada,² and Mount Sinai
Hospital, Toronto, Ontario, Canada³

Received 13 July 2004/Returned for modification 26 September 2004/Accepted 17 January 2005

Chromosomal β -lactamase genes (*bla*_{KLUY}) from six *Kluyvera georgiana* strains isolated in Guyana were cloned and expressed in *Escherichia coli*. KLUY-1 exhibited 100% amino acid identity with the extended-spectrum β -lactamase CTX-M-14. We also show that a 2.7-kb *Kluyvera* chromosomal region exhibits 99% nucleotide identity to a portion of In60 that includes *bla*_{CTX-M-9}.

Extended-spectrum β -lactamases (ESBLs) mainly belong to the Ambler class A family (6). CTX-M-type ESBLs, characterized by their preference for hydrolysis of cefotaxime (3, 4, 12), have been grouped into five clusters (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25) based on amino acid identities (5). Chromosomal β -lactamases from *Kluyvera ascorbata* (KLUA) (10), *K. ascorbata* strain 69 (*bla*_{CTX-M-3}) (19), *Kluyvera georgiana* DSM 9408 (KLUG-1) (18), and *Kluyvera cryocrescens* (KLUC-1) (8) have been identified. KLUA and KLUG-1 share greater than 99% amino acid identity with the CTX-M-2 and CTX-M-8 groups, respectively, and are thought to be the progenitors for these groups. *K. ascorbata* strain 69 is likely the progenitor for the CTX-M-1 group (19). KLUC-1 may be a progenitor for an as yet unidentified CTX-M group of ESBLs (5).

Bacterial strains. Six strains of *Kluyvera* spp. labeled N03-0457 to N03-0462 were isolated from human rectal swabs taken from nonhospitalized patients in Guyana, South America, in 2002 or 2003. The strains were isolated as part of a study on the carriage of antibiotic-resistant organisms among healthy individuals in the absence of recognized antibiotic pressure (M. Silverman, personal communication). The strains were identified as *Kluyvera* spp. by Vitek. Biochemical analysis and 16S rRNA sequence analysis confirmed the strains as *K. georgiana*. All strains had $\geq 99.6\%$ rRNA nucleotide identity and exhibited 99.5% identity with the rRNA gene of *K. georgiana* ATCC 51603 (accession no. AF04716) (data not shown).

Cloning and sequence analysis of β -lactamase genes. Each *K. georgiana* strain was positive with the CTX-U1–CTX-U2 primer set designed to amplify a fragment from CTX-M-type genes (CTX-U1, 5'-ATGTGCAGYACCAGTAARGTKATGGC; CTX-U2, 5'-TGGGTRAARTARGTSACCAGAAAYC

AGCGG; K is G or T, R is A or G, S is G or C, and Y is C or T) (13). EcoRI-digested DNA was ligated into the EcoRI site of pACYC184 and transformed into *Escherichia coli* DH10B, and clones were selected on ampicillin. Ten clones screened for each *Kluyvera* strain all harbored plasmids with 4.1-kb inserts. The insert from one plasmid for each strain was sequenced (DNA Core Facility, National Microbiology Laboratory), homology searches were carried out using BLAST (1), and open reading frames (ORFs) were analyzed by ORFinder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>).

The size of the EcoRI fragments were 4,102 bp in N03-0457 and N03-0461, 4,100 bp in N03-0458, 4,099 bp in N03-0459 and N03-0460, and 4,098 bp in N03-0462, with identities ranging from 98.4 to 100%. The N03-0457 and N03-0461 sequences were identical. The size differences were due to single nucleotide insertions and/or deletions found in intergenic regions (data not shown). Analysis revealed four ORFs (Fig. 1). The first, *bla*_{KLUY}, was 876 bp and coded for a deduced protein of 291 amino acids with the characteristic sequence elements of Ambler class A β -lactamases (Fig. 2) (11). The amino acid sequences shared 98.3 to 100% amino acid identity with known ESBLs from the CTX-M-9 group of enzymes and were termed KLUY-1 (N03-0458), KLUY-2 (N03-0457 and N03-0461), KLUY-3 (N03-0459 and N03-0460), and KLUY-4 (N03-0462). KLUY-1 showed 100% amino acid identity to CTX-M-14 and 84.5, 80.4, and 80.1% identity with KLUG-1, KLUC-1, and KLUA-1, respectively (15). The results suggest that the *bla*_{KLUY} gene from these *K. georgiana* strains is likely the progenitor of the CTX-M-9 group of ESBLs. Located 0.46 kb downstream of *bla*_{KLUY} is orf3 (209 amino acids), whose product shares 75% amino acid identity to ORF3 proteins coded for by genes found downstream of *bla*_{KLUA} and *bla*_{KLUC} (8, 10). The *K. ascorbata* CIP82.95T orf3 is located 0.43 kb downstream of *bla*_{KLUA}, whereas in *K. cryocrescens* orf3 is located 0.99 kb downstream of *bla*_{KLUC}. Downstream of orf3 was orf339, encoding a putative protein of 112 amino acids that had no significant homology to proteins in GenBank. In *K. ascorbata* CIP82.95T, orf4 is located

* Corresponding author. Mailing address: Nosocomial Infections, National Microbiology Laboratory, 1015 Arlington St., Winnipeg, Manitoba, Canada R3E 3R2. Phone: (204) 789-2133. Fax: (204) 789-5020. E-mail: Michael_mulvey@hc-sc.gc.ca.

FIG. 1. Schematic diagram of the genetic environment of the *bla*_{KLUG-1} gene compared to similar regions as found in the GenBank database. The nucleotide sequences of the junctions of the nearly identical regions found in *K. georgiana* N03-0458 and In60 are shown. Accession numbers are indicated. Orf1 of pCTX-M-3 is a homolog of Orf477 and is not related to Orf1 of *K. ascorbata* strain 69. The *K. georgiana* DSM 9408 *bla*_{KLUG-1} region is not shown, as no ORFs have been detected in the limited flanking sequence available (accession no. AF501233) (18).

198 bp downstream of orf3. We identified a partial ORF 219 bp downstream of *K. cryocrescens* orf3 coding for an orf4-like protein showing 34% amino acid identity to *K. ascorbata* orf4, though this was not annotated in the original GenBank entry (Fig. 1). Located 54 to 55 bp downstream of orf339 on the opposite strand was orf477 (158 amino acids), whose product shares 86% identity to the *K. ascorbata* strain 69 Orf477, found downstream of the chromosomal *bla*_{CTX-M-3}, and to Orf1, found downstream of *bla*_{CTX-M-3} in the *Citrobacter freundii* plasmid pCTX-M3 (accession no. AF550415). We also identified orf477 in the *bla*_{KLUG-1}-orf3 intergenic region, though this was not annotated in the GenBank entry. There is limited sequence available surrounding *bla*_{KLUG-1}, which shares 75 to 77% nucleotide identity with the *bla*_{KLUG-1} genes, with sequence divergence even more extensive in the flanking regions (data not shown). Thus, the *K. georgiana* "group" may encompass multiple subspecies. Further analysis of the *bla*_{KLUG-1} region revealed that a 2.7-kb region shared 99% nucleotide identity to a region of the complex class 1 integron In60 harboring *bla*_{CTX-M-9} (Fig. 1) (20). Examination of the *Kluyvera* sequence revealed a 10-nucleotide direct repeat se-

quence, CACGGCGAGG, that overlaps the end junctions of this region. It seems likely the direct repeat sequences are involved in site-specific mobilization of the *bla*_{KLUG-1} region, possibly in association with ORF513, a putative recombinase (2). The complex integrons InS21 and In35 have a 2.2-kb region downstream of orf513 that harbors *bla*_{CTX-M-2} and orf3 (Fig. 1) (2, 9). Further, the 347 bp of sequence downstream of *bla*_{CTX-M-9a} and *bla*_{CTX-M-13} from plasmids pOZ173 and pOZ175, respectively, share 95.7 to 98.6% nucleotide identity with the region downstream of *bla*_{KLUG-1} genes (Fig. 1) (7). In addition, the 42 bp upstream of *bla*_{CTX-M-14} in pOZ174 is 100% identical to the same region from the *bla*_{KLUG-1} genes (7); however, the *bla*_{CTX-M-14} gene is located downstream of *ISEcp1*. Other *bla*_{CTX-M} genes are found associated with *ISEcp1*, and it is likely that they were mobilized by the insertion element as has been shown for *bla*_{CTX-M-19} (17). Thus, *bla*_{CTX-M} genes from the same cluster may be mobilized by different methods.

Antibiotic susceptibilities. The MICs of the *K. georgiana* strains and the *E. coli* transformants to β -lactams were determined by broth microdilution using recommended National

FIG. 2. Alignment of the amino acid sequences of KLUY-1, KLUY-2, KLUY-3, KLUY-4, CTX-M-9, and CTX-M-14. Dots represent identical amino acids. The vertical arrow indicates a putative cleavage site of the leader peptide. Five elements characteristic of Ambler class A β -lactamases are boxed in grey, and the omega loop is underlined (11).

Committee for Clinical Laboratory Standards methodology (Table 1) (14). In the *E. coli* transformants, significant synergy with clavulanic acid was observed with cefotaxime, as is typical for class A ESBLs (Table 1). MICs for ceftazidime were lower, ranging from 0.5 to 16 μ g/ml, as is characteristic for most CTX-M enzymes (5). The ceftoxitin resistance of N03-0460 is likely due to another β -lactamase and/or another mechanism, as *E. coli* (pKLUY-3a) had a ceftoxitin MIC of 4 μ g/ml. The *bla*_{KLUY} genes are weakly expressed, as found for other chromosomal β -lactamases from *Kluyvera* (8, 10, 18). Once mobilized, however, the plasmidic location and overexpression from a “foreign” promoter lead to the host strains exhibiting the classical CTX-M ESBL phenotype. The *ISEcp1* element has been shown to provide a strong promoter for *bla*_{CTX-M} genes located downstream from it (17), and sequences downstream

of orf513 have been postulated to act as a promoter for adjacent genes as suggested for *bla*_{CTX-M2} (2) and *dfrA10* (16). The resistance profile observed in the *E. coli* transformants is similar to those of plasmid-encoded CTX-M-9 and CTX-M-14 enzymes (15, 20, 21). We have identified the chromosomal β -lactamase of *K. georgiana* as the progenitor of the CTX-M-9 group of ESBLs. Mobilization of the gene may involve Orf513 or *ISEcp1*.

Nucleotide sequence accession numbers. The complete nucleotide sequences of the EcoRI fragments cloned from the *K. georgiana* strains used in this study have been deposited in the GenBank database with the accession numbers AY623932 (N03-0458, *bla*_{KLUY-1}), AY623935 (N03-0461, *bla*_{KLUY-2}), AY623934 (N03-0460, *bla*_{KLUY-3}), AY824948 (N03-0459, *bla*_{KLUY-3}), and AY623933 (N03-0462, *bla*_{KLUY-4}).

TABLE 1. MICs of β -lactams for *K. georgiana* *E. coli* DH10B harboring cloned *bla*_{KLUY} genes, and *E. coli* DH10B

β -Lactam(s) ^a	MIC (μ g/ml) for:												<i>E. coli</i> DH10B
	<i>K. georgiana</i>						<i>E. coli</i> DH10B with cloned <i>bla</i> _{KLUY} genes ^b						
	N03-0458 (KLUY-1)	N03-0457 (KLUY-2a)	N03-0461 (KLUY-2b)	N03-0460 (KLUY-3a)	N03-0459 (KLUY-3b)	N03-0462 (KLUY-4)	pKLUY- 1	pKLUY- 2a	pKLUY- 2b	pKLUY- 3b	pKLUY- 3a	pKLUY- 4	
Cefpodoxime	4	2	2	2	4	2	>16	>16	>16	>16	>16	>16	1
Ceftriaxone	<1	<1	<1	<1	<1	<1	64	>64	64	16	>64	>64	<1
Cefotaxime	0.5	0.5	0.5	<0.25	0.5	0.5	32	>128	32	16	>128	>128	<0.26
Cefotaxime + CLA	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	0.5	<0.25	<0.25	<0.25
Ceftazidime	<0.25	<0.25	<0.25	<0.25	0.5	<0.25	2	8	2	2	16	4	0.5
Ceftazidime + CLA	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	1	<0.25	0.5	<0.25	0.5	0.5
Cefoxitin	8	4	4	64	4	4	4	16	4	8	4	4	8
Aztreonam	<1	<1	<1	<1	<1	<1	4	>16	4	4	>16	16	<1
Meropenem	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25

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

^b Cloned in pACYC184.

We thank Romeo Hizon, David Woodward, Roger Foster, and Jason Moses for excellent technical expertise.

REFERENCES

- Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389–3402.
- Arduino, S. M., P. H. Roy, G. A. Jacoby, B. E. Orman, S. A. Pineiro, and D. Centron. 2002. *bla*_{CTX-M-2} is located in an unusual class 1 integron (In35) which includes Orf513. *Antimicrob. Agents Chemother.* **46**:2303–2306.
- Bauernfeind, A., H. Grimm, and S. Schweighart. 1990. A new plasmidic cefotaximase in a clinical isolate of *Escherichia coli*. *Infection* **18**:294–298.
- Bernard, H., C. Tancrede, V. Livrelli, A. Morand, M. Barthelemy, and R. Labia. 1992. A novel plasmid-mediated extended-spectrum beta-lactamase not derived from TEM- or SHV-type enzymes. *J. Antimicrob. Chemother.* **29**:590–592.
- Bonnet, R. 2004. Growing group of extended-spectrum β -lactamases: the CTX-M enzymes. *Antimicrob. Agents Chemother.* **48**:1–14.
- Bush, K. 2001. New beta-lactamases in gram-negative bacteria: diversity and impact on the selection of antimicrobial therapy. *Clin. Infect. Dis.* **32**:1085–1089.
- Chanawong, A., F. H. M'Zali, J. Heritage, J. H. Xiong, and P. M. Hawkey. 2002. Three cefotaximases, CTX-M-9, CTX-M-13, and CTX-M-14, among *Enterobacteriaceae* in the People's Republic of China. *Antimicrob. Agents Chemother.* **46**:630–637.
- Decusser, J. W., L. Poirer, and P. Nordmann. 2001. Characterization of a chromosomally encoded extended-spectrum class A β -lactamase from *Kluyvera cryocrescens*. *Antimicrob. Agents Chemother.* **45**:3595–3598.
- Di Conza, J., J. A. Ayala, P. Power, M. Mollerach, and G. Gutkind. 2002. Novel class 1 integron (InS21) carrying *bla*_{CTX-M-2} in *Salmonella enterica* serovar Infantis. *Antimicrob. Agents Chemother.* **46**:2257–2261.
- Humeniuk, C., G. Arlet, V. Gautier, P. Grimont, R. Labia, and A. Philippon. 2002. β -Lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types. *Antimicrob. Agents Chemother.* **46**:3045–3049.
- Joris, B., P. Ledent, O. Dideberg, E. Fonze, J. Lamotte-Brasseur, J. A. Kelly, J. M. Ghuyssen, and J. M. Frere. 1991. Comparison of the sequences of class A β -lactamases and of the secondary structure elements of penicillin-recognizing proteins. *Antimicrob. Agents Chemother.* **35**:2294–2301.
- Matsumoto, Y., F. Ikeda, T. Kamimura, Y. Yokota, and Y. Mine. 1988. Novel plasmid-mediated β -lactamase from *Escherichia coli* that inactivates oxymino-cephalosporins. *Antimicrob. Agents Chemother.* **32**:1243–1246.
- Mulvey, M. R., G. Soule, D. Boyd, W. Demczuk, R. Ahmed, and the Multi-provincial *Salmonella* Typhimurium Case Control Study Group. 2003. Characterization of the first extended-spectrum beta-lactamase-producing *Salmonella* isolate identified in Canada. *J. Clin. Microbiol.* **41**:460–462.
- National Committee for Clinical Laboratory Standards. 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard NCCLS M7–A5, 5th ed. National Committee for Clinical Laboratory Standards, Wayne, Pa.
- Pai, H., E. H. Choi, H. J. Lee, J. Y. Hong, and G. A. Jacoby. 2001. Identification of CTX-M-14 extended-spectrum β -lactamase in clinical isolates of *Shigella sonnei*, *Escherichia coli*, and *Klebsiella pneumoniae* in Korea. *J. Clin. Microbiol.* **39**:3747–3749.
- Parsons, Y., R. M. Hall, and H. W. Stokes. 1991. A new trimethoprim resistance gene, *dhfrX*, in the In7 integron of plasmid pDGO100. *Antimicrob. Agents Chemother.* **35**:2436–2439.
- Poirer, L., J. W. Decusser, and P. Nordmann. 2003. Insertion sequence *ISEcp1B* is involved in expression and mobilization of a *bla*_{CTX-M} β -lactamase gene. *Antimicrob. Agents Chemother.* **47**:2938–2945.
- Poirer, L., P. Kampfer, and P. Nordmann. 2002. Chromosome-encoded Ambler class A beta-lactamase of *Kluyvera georgiana*, a probable progenitor of a subgroup of CTX-M extended-spectrum β -lactamases. *Antimicrob. Agents Chemother.* **46**:4038–4040.
- Rodríguez, M. M., P. Power, M. Radice, C. Vay, A. Famiglietti, M. Galleni, J. A. Ayala, and G. Gutkind. 2004. Chromosome-encoded CTX-M-3 from *Kluyvera ascorbata*: a possible origin of plasmid-borne CTX-M-1-derived cefotaximases. *Antimicrob. Agents Chemother.* **48**:4895–4897.
- Sabate, M., F. Navarro, E. Miro, S. Campoy, B. Mirelis, J. Barbe, and G. Prats. 2002. Novel complex *sulI*-type integron in *Escherichia coli* carrying *bla*_{CTX-M-9}. *Antimicrob. Agents Chemother.* **46**:2656–2661.
- Sabaté, M., R. Tarragó, F. Navarro, E. Miró, C. Vergés, J. Barbé, and G. Prats. 2000. Cloning and sequence of the gene encoding a novel cefotaxime-hydrolyzing β -lactamase (CTX-M-9) from *Escherichia coli* in Spain. *Antimicrob. Agents Chemother.* **44**:1970–1973.