Emergence of a novel *Enterobacter kobei* clone carrying chromosomal-encoded CTX-M-12 with diversified pathogenicity in northeast China

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Dear Sir,

Enterobacter cloacae complex (ECC), comprising at least five species (*Enterobacter cloacae*, *Enterobacter kobei*, *Enterobacter asburiae*, *Enterobacter hormaechei* and *Enterobacter ludwigii*) with different subspecies, has emerged as one of the important nosocomial pathogens in the last decade, responsible for 65%–75% of all infections due to *Enterobacter* species [1]. Recently, some potentially high-risk international clones causing nosocomial infections were revealed in a Europe-wide survey [2]. In this study, we characterized a CTX-M-12-producing ECC clone responsible for severe infections circulating in northeast China.

Three ECC strains were isolated from bronchoalveolar lavage (ECC3018), blood (ECC3026) and abscesses (ECC3047) of three patients admitted to a secondary hospital in a northeast province (Liaoning) in China. The isolates were further identified as *E. kobei* by *hsp60* typing [3]. The three strains exhibited similar antimicrobial profiles determined by the agar dilution method (Table 1). They showed an extended spectrum β-lactamase-positive and AmpC-overexpression phenotype detected as previously reported [2]. Multilocus sequence typing with use of seven loci (*dnaA*, *fusA*, *gyrB*, *leuS*, *pyrG*, *rplB* and *rpoB*) detected a new profile (3-3-110-32-19-16-17) for all isolates, and was assigned as ST591 by the PubMLST database (https://pubmlst.org/ecloacae/).

Isolates were sequenced using Illumina Hiseq2500 (Illumina, San Diego, CA, USA) using 2 × 125-bp pair-end libraries. Genomes were assembled by CLC GENOMIC WORKBENCH v8.0, and annotated by the RAST service (http://rast.nmpdr.org/). To analyse the resistome, genomic sequences were uploaded to RESFINDER (https://cge.cbs.dtu.dk). The three isolates shared an identical resistome comprising 19 genes, including: *aac(3)-IId*, *aac(6′)-II*, *aadA2*, *aadA16*, *aph(3′)-Ic* and *armA* for aminoglycoside resistance; $bla_{CTX\text{-}M\text{-}12}$, $bla_{CARB\text{-}2}$, $bla_{TEM\text{-}1}$ and an unnamed bla_{ACT} gene for β-lactam resistance; and other genes for various drug resistance (*fosA*, *msr*(E), *mph*(A), *mph*(E), *ere*(B), *sul1*, *sul2*, *cmlA1*, *dfrA1*). The genotypes can fully explain the results of susceptibility tests.

The $bla_{CTX\text{-}M\text{-}12}$ is a rare CTX-M gene, and its genetic environment was identical in the three strains (Fig. 1a). An IS*Ecp1* was located 48-bp upstream of $bla_{CTX\text{-}M\text{-}12}$, and the structure was identical to that of *Escherichia coli* isolates (DQ658220) identified in Korea. The transposition unit IS*Ecp1*-$bla_{CTX\text{-}M\text{-}12}$-*orf477* was highly similar to the well-reported typical $bla_{CTX\text{-}M\text{-}15}$ transposition unit with a length of 2971 bp. The unit was integrated into the chromosome, located upstream of a pseudo gene and downstream of a gene encoding an asparaginyl-tRNA synthetase. Two 5-bp direct repeats TATTA were identified adjacent to the flanking left inverted repeat and putative right

TABLE 1. MICs of some antibiotic agents for *Enterobacter kobei* isolates

	ECC3018	ECC3026	ECC3047
Ampicillin	>512	>512	>512
Ampicillin-sulbactam	64/32	32/16	32/16
Ciprofloxacin	0.006	0.006	0.003
Levofloxacin	0.03	0.03	≤0.015
Fosfomycin	32	16	16
Piperacillin	256	256	256
Piperacillin-tazobactam	8/4	8/4	4/4
Amikacin	>256	>256	>256
Gentamycin	128	128	128
Cefepime	4	8	8
Cefotaxime	4	4	4
Cefoperazone-sulbactam	64/32	64/32	32/16
Ceftriaxone	32	16	32
Ceftazidime	1	1	1
Cefoxitin	128	128	128
Cefazolin	>128	>128	>128
Cefuroxime	>128	>128	>128
Meropenem	≤0.015	≤0.015	0.03
Imipenem	0.25	0.25	0.25

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inverted repeat (Fig. 1a), suggesting that ISEcp1 mediated the chromosomal integration of $bla_{CTX\text{-}M\text{-}12}$.

The $bla_{CTX\text{-}M\text{-}12}$ gene was first detected from a *Klebsiella pneumoniae* outbreak clone in Kenya in 2001 [4], and was later identified in *Escherichia coli* and *K. pneumoniae* isolates from Colombia and Korea, respectively [5,6]. To our knowledge, this is the first report of $bla_{CTX\text{-}M\text{-}12}$ identified in *E. kobei*. As the geographic area where our strains isolated is bounded on the south by Korea, it is possible that the emergence of $bla_{CTX\text{-}M\text{-}12}$ may be due to cross-border spread. Further surveillance for $bla_{CTX\text{-}M\text{-}12}$ should be carried out to test this hypothesis. Additionally, among *Enterobacteriaceae*, chromosome-encoded CTX-Ms are frequently found in *Escherichia coli*, *K. pneumoniae* and *Proteus mirabilis*, but are very rare in ECC

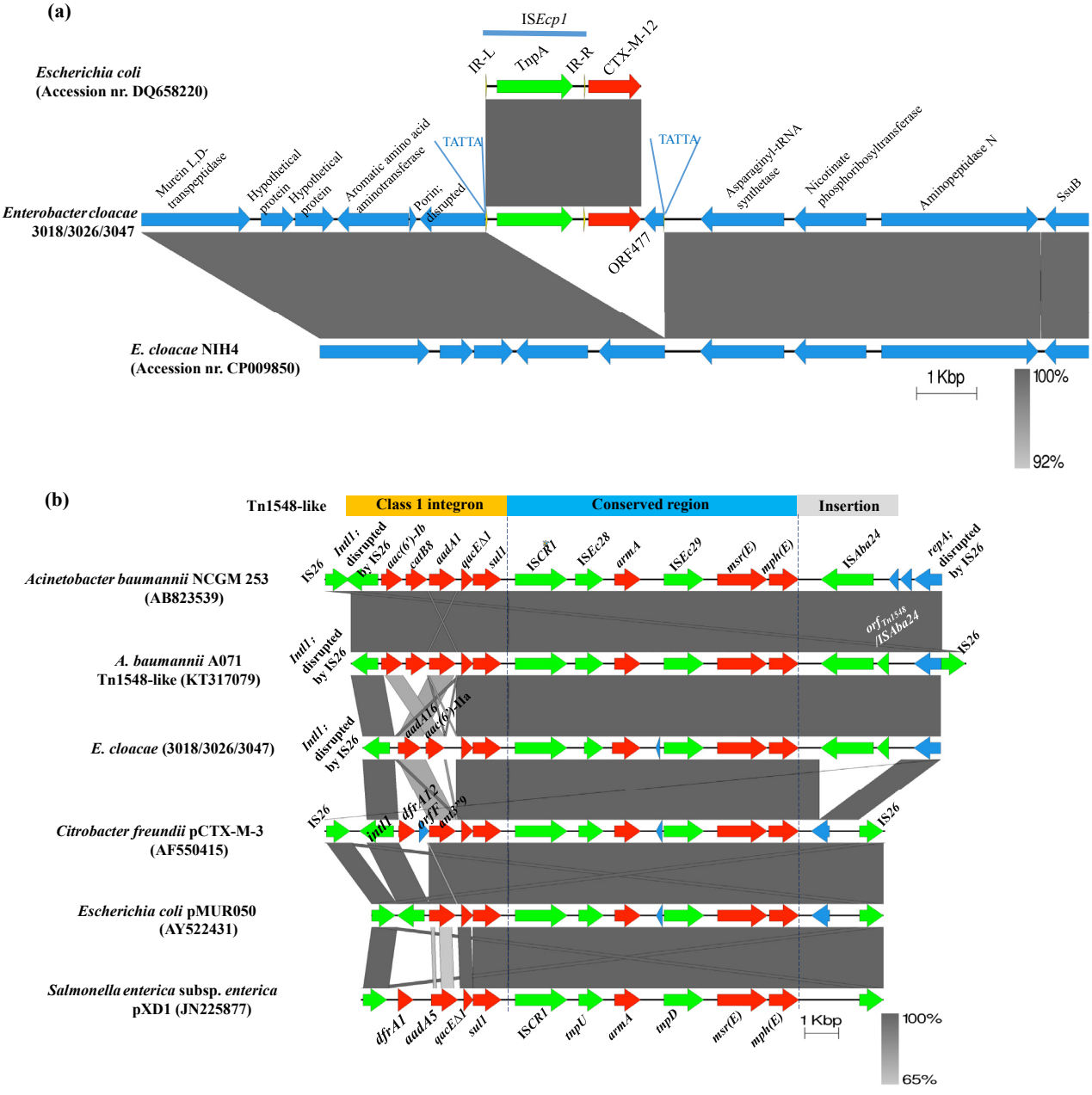

FIG. 1. Schematic diagram of genetic environment surrounding $bla_{CTX\text{-}M\text{-}12}$ and *armA*. Genes are shown in red (resistance genes), green (genes of mobile genetic elements), yellow (inverted repeats) and blue (other genes) arrows. The annotation of hypothetical genes is not shown. (a) The cassette ISEcp1-$bla_{CTX\text{-}M\text{-}12}$-*orf477* was integrated into the chromosome, and its insertion site is shown; (b) the *armA* gene located in a Tn*1518*-like composite transposon.

[7]. Additionally, this is the first evidence of chromosomal integration of $bla_{CTX\text{-}M\text{-}12}$, which had previously been exclusively detected in plasmids. This suggests the role of chromosomal locations in the spread of $bla_{CTX\text{-}M\text{-}12}$.

The three isolates showed high MIC values of amikacin (>256 mg/L). A 16S rRNA methylase gene *armA* was detected in a Tn*1548*-like segment in the isolates responsible for the amikacin resistance (Fig. 1b). This Tn*1548*-like segment consisted of a 3'-end conserved region and a 5'-end class 1 integron. The conserved region was structured as IS*CR1*-IS*Ec28*-*armA*-IS*Ec29*-*msr*(E)-*mph*(E), and the variable region of class 1 integron carried *aac(6')-Il* and *aadA16*. Notably, the region from an IS*26*-disrupted *intI1* gene to an IS*Aba24* was identical to that identified in *Acinetobacter baumannii* strain A071 (KT317079) (Fig. 1b). Both ends of the Tn*1548*-like segment were disrupted by IS*26*, indicating that IS*26* mediated the mobilization of this composite transposon cross species. The concomitance of extended spectrum β-lactamases and 16S rRNA methylases raises clinical concern and may become a major therapeutic threat in the future.

The genetic diversity of the three strains was determined by single-nucleotide polymorphism (SNP) analysis as described previously [8], and they differed by 57 SNPs (see Supplementary material, Table S1). This excludes the possibility of a recent transmission among the three patients, suggesting a clonal dissemination in the region. To investigate whether the genetic differences were associated with alterations of biological function, biofilm formation was tested by microtitre plate assay as described previously [9]. Intriguingly, ECC3018 could form four- to ten-fold more biofilm (24 h 0.12 ± 0.02; 48 h 0.18 ± 0.04) than the other two (24 h 0.012 ± 0.003, 0.011 ± 0.002; 48 h 0.048 ± 0.03, 0.037 ± 0.02) ($p < 0.05$) at 37°C. The discrepancy could be explained by multiple non-synonymous SNPs identified in the genes involved in biofilm formation (see Supplementary material, Table S1), including *barA* encoding a sensory histidine kinase [10], *kefA* encoding a potassium efflux system [11] and *malT* encoding a transcriptional activator of maltose regulon [12]. Additionally, more non-synonymous SNPs than synonymous ones (36 versus 12) were identified in the clone frequently associated with genes involved in metabolism, membrane and pathogenicity. This implicates that the clone underwent positive selections resulting in pathogenicity diversification.

In summary, this study raises the concern that a wide repertoire of resistance mechanism and enhanced pathogenicity detected in the novel *E. kobei* clone increases its epidemic potential, and highlights the necessity of surveillance on the potential high-risk clone in the future.

Nucleotide sequence GenBank Accession numbers

The Whole Genome Shotgun BioProject for *E. kobei* isolates has been deposited at DDBJ/EMBL/GenBank under the accession numbers LYUR00000000, LYUS00000000, LYUT00000000, respectively.

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Transparency declaration

The authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Additional Supporting Information may be found in the online version of this article at http://dx.doi.org/10.1016/j.nmni.2017.01.006.

References

[1] Eugene Sanders WE, Sanders CC. *Enterobacter* spp.: pathogens poised to flourish at the turn of the century. Clin Microbiol Rev 1997;10:220–41.
[2] Izdebski R, Baraniak A, Herda M, Fiett J, Bonten MJM, Carmeli Y, et al. MLST reveals potentially high-risk international clones of *Enterobacter cloacae*. J Antimicrob Chemother 2015;70:48–56.
[3] Hoffmann H, Roggenkamp A. Population genetics of the nomenspecies *Enterobacter cloacae*. Appl Environ Microbiol 2003;69:5306–18.
[4] Kariuki S, Corkill JE, Revathi G, Musoke R, Hart CA. Molecular characterization of a novel plasmid-encoded cefotaximase (CTX-M-12) found in clinical *Klebsiella pneumoniae* isolates from Kenya. Antimicrob Agents Chemother 2001;45:2141–3.
[5] Villegas MV, Correa A, Perez F, Zuluaga T, Radice M, Gutkind G, et al. CTX-M-12 beta-lactamase in a *Klebsiella pneumoniae* clinical isolate in Colombia. Antimicrob Agents Chemother 2004;48:629–31.
[6] Bae IK, Lee YN, Hwang HY, Jeong SH, Lee SJ, Kwak HS, et al. Emergence of CTX-M-12 extended-spectrum β-lactamase-producing *Escherichia coli* in Korea. J Antimicrob Chemother 2006;58:1257–9.

[7] Zhao W-H, Hu Z-Q. Epidemiology and genetics of CTX-M extended-spectrum β-lactamases in Gram-negative bacteria. Crit Rev Microbiol 2013;39:79–101.

[8] Zhou K, Ferdous M, de Boer RF, Kooistra-Smid AMD, Grundmann H, Friedrich AW, et al. The mosaic genome structure and phylogeny of Shiga toxin-producing *Escherichia coli* O104:H4 is driven by short-term adaptation. Clin Microbiol Infect 2015;21. 468.e7–468.e18.

[9] Nyenje ME, Green E, Ndip RN. Evaluation of the effect of different growth media and temperature on the suitability of biofilm formation by *Enterobacter cloacae* strains isolated from food samples in South Africa. Molecules 2013;18:9582–93.

[10] Sahu SN, Acharya S, Tuminaro H, Patel I, Dudley K, LeClerc JE, et al. The bacterial adaptive response gene, *barA*, encodes a novel conserved histidine kinase regulatory switch for adaptation and modulation of metabolism in *Escherichia coli*. Mol Cell Biochem 2003;253:167–77.

[11] Zhang W, McLamore ES, Wu R, Stensberg M, Porterfield DM, Banks MK. Glutathione-gated potassium efflux as a mechanism of active biofilm detachment. Water Environ Res 2014;86:462–9.

[12] Lone AG, Deslandes V, Nash JHE, Jacques M, MacInnes JI. *malT* knockout mutation invokes a stringent type gene-expression profile in *Actinobacillus pleuropneumoniae* in bronchoalveolar fluid. BMC Microbiol 2009;9:195.