*bla*_{CTX-M-65} is carried by a Tn1722-like element on an IncN conjugative plasmid of ST131 *Escherichia coli*

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Escherichia coli clinical isolate WCE307 was found to belong to phylogroup B2, O25b and sequence type (ST) 131 and had *bla*_{CTX-M-65}, which was carried by an IncN conjugative plasmid, pWCE307. On pWCE307, the *ISEcp1*Δ-*bla*_{CTX-M-65}-*IS903D-iron*Δ structure was located in a Tn1722-like element flanked by 5 bp direct target repeats. This context was highly similar to that on pKC396, an IncN plasmid from *E. coli* isolate KC396 of ST131 from Germany. The Tn1722-like elements carrying *bla*_{CTX-M-65} on pWCE307 and pKC396 are likely to be hybrids of Tn1722 and Tn5051, resulting from double crossover at the *res* sites and the *tnpA* genes. The left end of the Tn1722-like elements was partially missing. As *ISEcp1* and Tn1722 were both incomplete, *bla*_{CTX-M-65} might have been trapped in this context. A plasmid multilocus sequence typing (pMLST) scheme with five targets, *repN*, *stbB*, *tral*, *traB* and the *korB*–*orfI* spacer region, was developed for IncN plasmid typing. pMLST revealed that the five alleles of pWCE307 and pKC396 were identical, indicating that WCE307 and KC396 are likely to have originated very recently from a common strain, suggesting the international spread of *bla*_{CTX-M-65} mediated by ST131 *E. coli*.

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INTRODUCTION

*bla*_{CTX-M} genes are widespread and have become the dominant gene family encoding extended-spectrum β-lactamases in the world. More than 90 *bla*_{CTX-M} variants have been identified so far (<http://www.lahey.org/Studies/other.asp>), forming a large family. Among them, *bla*_{CTX-M-65} is a recently identified variant of *bla*_{CTX-M-14a} (GenBank accession no. AF252622) (Sidjabat *et al.*, 2009; Yin *et al.*, 2009), and CTX-M-65 differs from CTX-M-14 by two amino acid substitutions, Ala78Val and Ser273Arg, neither of which confers resistance to ceftazidime (Yin *et al.*, 2009). *bla*_{CTX-M-65} has been found in *Escherichia coli* from China (Yin *et al.*, 2009; Yuan *et al.*, 2009), Germany (Cullik *et al.*, 2010), Korea (Lee *et al.*, 2009) and the USA (Sidjabat *et al.*, 2009) and in a *Citrobacter freundii* isolate from China (GenBank accession no. EF394372). During a local surveillance for isolates carrying genes encoding extended-spectrum β-lactamases, *bla*_{CTX-M-65} was identified in an *E. coli* clinical isolate, WCE307, and its genetic context and host plasmid were characterized in detail.

Abbreviations: DR, direct repeat; IRL, left-hand inverted repeat; IRR, right-hand inverted repeat; pMLST, plasmid multilocus sequence typing; ST, sequence type.

The GenBank/EMBL/DDBJ accession number for the sequence of the genetic context of *bla*_{CTX-M-65} on pWCE307 is HM440049.

METHODS

Bacterial isolate. *E. coli* isolate WCE307 was recovered from a blood sample collected from a patient receiving choledochoscopy in West China Hospital, Chengdu, PR China, in October 2005. Species identification and antimicrobial susceptibility testing were performed using the Phoenix automated microbiology system (BD). *bla*_{CTX-M} was screened for using a multiplex PCR (Woodford *et al.*, 2006) and the complete sequence of *bla*_{CTX-M} was obtained by another PCR with primers *ISEcp1IR-F* and *5orf903-R* (Table 1), as described previously (Zong *et al.*, 2008), and sequencing. Primers used in this study are listed in Table 1.

Strain typing. The phylogroup (A, B1, B2 or D) of WCE307 was determined by a multiplex PCR (Clermont *et al.*, 2000). O25 antigen typing for WCE307 was performed by O25b allele PCR (Clermont *et al.*, 2008). Two established PCR methods were used to determine whether WCE307 belonged to sequence type (ST) 131, one targeting a *pabB* fragment specific for the O25b ST131 lineage (Clermont *et al.*, 2009) and another (a duplex PCR) targeting single-nucleotide polymorphisms of the *mdh* and *gyrB* genes seen in ST131 isolates (Johnson *et al.*, 2009).

Mating and replicon typing. Mating was carried out in brain–heart infusion broth (Oxoid) using *E. coli* DH5αRf, a spontaneous rifampicin-resistant mutant of *E. coli* DH5α (Δ*lacZ*), as the recipient. Transconjugants were selected on 4 μg cefotaxime ml^{−1} plus 250 μg rifampicin ml^{−1} and streaked onto CHROMagar Orientation agar (BD) plates to distinguish transconjugants (white colonies) from the clinical isolate of *E. coli* (pink). Plasmid DNA was prepared from the transconjugants by alkaline lysis (Sambrook & Russell, 2001) and subjected to PCR-based replicon typing to determine the incompatibility (Inc) group (Carattoli *et al.*, 2005).

Table 1. Primers used in this study

Primer	Sequence 5'→3'	Target	Amplicon size (bp)	Reference
For mapping				
ISEcp1IR-F	CAATGTGTGAGAAGCAGTCTAAA	<i>ISEcp1</i> , 3' end	1137	Zong <i>et al.</i> (2008)
5orf903-R	CGGTTGATGAGGGCTTTATT	<i>IS903</i>		Zong <i>et al.</i> (2008)
traI_R46-F1	GACAGCAGCATTTACGACGA	<i>traI</i>	1453	This work
CTXM9R	ATTGGAAAGCGTTCATCACC	<i>bla</i> _{CTX-M-9} -like		Clermont <i>et al.</i> (2000)
nuc-F1	CGTGGATAATGTGACCGTTG	<i>nuc</i>	7947	This work
CTXM9F	CAAAGAGAGTGCAACGGATG	<i>bla</i> _{CTX-M-9} -like		Clermont <i>et al.</i> (2000)
For pMLST				
N FW	GTCTAACGAGCTTACCGAAG	<i>repN</i>	660	Carattoli <i>et al.</i> (2005)
N RV	GTTTCAACTCTGCCAAGTTC			Carattoli <i>et al.</i> (2005)
traI_N-F1	GGCTACGACCTGACGTTTTTC	<i>traI</i>	671	This work
traI_N-R1	TCCAGAGGTTTACCGTGTC			This work
traB_N-F1	TCTGGGATAATGACCAAAACG	<i>traB</i>	682	This work
traB_N-R1	ATAAGTGAGCCTTCCCCCATA			This work
stbB_N-F1	TTACAGTGGCAGYGTGGTA	<i>stbB</i>	692	This work
stbB_N-R1	TCTCCCATTAAGCCGCATA			This work
korB-F1	AAATTGGACGCTGGCATTAG	A spacer between <i>korB</i> and <i>orfI</i>	341	This work
ORF1-R1	AAGAGCCGGATTTGATGATG			This work

PCR mapping. The genetic context of *bla*_{CTX-M-65} in pWCE307 was determined by PCR mapping using the structure on plasmid pKC396 (GenBank accession no. HM138653) as the reference. pKC396, a plasmid carrying *bla*_{CTX-M-65}, was obtained from an *E. coli* isolate in Germany (Cullik *et al.*, 2010). The linkage from *bla*_{CTX-M-65} to *traI* and to *nuc* (encoding an endonuclease, see below) on pWCE307 was established by long-range PCR (Fermentas; Fig. 1). Amplicons were sequenced and assembled, generating a 9 kb sequence containing *bla*_{CTX-M-65}.

IncN plasmid multilocus sequence typing (pMLST). To determine the similarity in plasmid backbone between pWCE307 and the completely sequenced IncN plasmids deposited in GenBank, a pMLST method was set up. Completely sequenced IncN plasmids were R46 (GenBank accession no. AY046276), pMUR050 (González-Zorn *et al.*, 2005), pLEW517 (Williams *et al.*, 2006), pMAK2 (GenBank accession no. AB366441), pKP96 (Shen *et al.*, 2008) and plasmids 9 (Gootz *et al.*, 2009) and 12 (Gootz *et al.*, 2009) (Table 2). Very recently, complete sequences of pKC394 (GenBank accession no. HM138652) and pKC396, two IncN plasmids carrying *bla*_{CTX-M-65}, were released. pKC394 is almost identical to pKC396 but has a large section containing an additional *bla*_{CTX-M} variant, *bla*_{CTX-M-1}. In light of the overall similarity, pKC396 mentioned hereafter also represents pKC394.

Five targets were chosen for pMLST as they marked plasmid basic functions (replication, maintenance and conjugation) and were present in all IncN plasmids with complete sequences available from GenBank, but also exhibited significant nucleotide variations. These targets were *repN* (IncN replicon), *stbB* (encoding StbB for plasmid partition), *traI* (encoding a conjugal nickase and helicase), *traB* (encoding an ATPase for conjugation) and a spacer region between *korB* (potentially encoding a transcriptional repressor) and an ORF (named *orfI* in R46) of unknown function.

The sequences of these targets from the completely sequenced IncN plasmids were aligned for primer design (Table 1). PCRs were performed as follows: one cycle of 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min, and extension at 72 °C for 5 min. The amplicons were purified using an OMEGA Cycle Pure kit (Norchross) and sequenced.

RESULTS AND DISCUSSION

WCE307 was found to be phylogroup B2, O25b O-antigen type and ST131. WCE307 had *bla*_{CTX-M-65}, which was carried by an IncN conjugative plasmid, pWCE307.

*bla*_{CTX-M-65} is 'trapped' in a complex Tn1722-like element on pWCE307 and pKC396

In general, the genetic context of *bla*_{CTX-M-65} on pWCE307 and pKC396 was highly similar, with only a minor variation (Fig. 1). As on pKC396, *bla*_{CTX-M-65} was associated with a 398 bp right-end relic of *ISEcp1* including its right-hand inverted repeat (IRR). Downstream of *bla*_{CTX-M-65}, *IS903D* and a truncated gene, *iron* (encoding an outer-membrane iron receptor protein), were identified. This *ISEcp1*Δ-*bla*_{CTX-M-65}-*IS903D*-*iron*Δ structure was part of a Tn1722-like element and was located adjacent to a fragment of *orfI*, which potentially encodes a methyl-accepting chemotaxis protein (Allmeier *et al.*, 1992). Tn1722 contains *tnpA* (encoding transposase), *tnpR* (encoding resolvase) and the *res* (resolution) site in addition to *orfI* and is bound by two 38 bp inverted repeats with three nucleotide differences (Allmeier *et al.*, 1992; Schöffl *et al.*, 1981). Tn1722 in combination with an additional *tet* region conferring resistance to tetracycline comprised Tn1721 (Allmeier *et al.*, 1992).

The *ISEcp1*-*bla*_{CTX-M}-*IS903D*-*iron*Δ structure has been seen in several cases (Fig. 1) associated with *bla*_{CTX-M-14} (Izumiyama *et al.*, 2005), *bla*_{CTX-M-19} (Poirel *et al.*, 2003), *bla*_{CTX-M-24} (Shen *et al.*, 2008) and *bla*_{CTX-M-65} (Cullik *et al.*, 2010), although *ISEcp1* could be incomplete. *bla*_{CTX-M-19}, *bla*_{CTX-M-24} and *bla*_{CTX-M-65} are closely related to *bla*_{CTX-M-14} with only one or two nucleotide differences. It has been proven experimentally, at least for *bla*_{CTX-M-19} (Poirel *et al.*, 2003), that *ISEcp1* is able to recognize part of the *iron* gene sequence

Fig. 1. Genetic context of *bla*_{CTX-M-65} and similar structures. (a) Genetic context of *bla*_{CTX-M-65} on pWCE307. The amplicons for PCR mapping are shown. The *ISEcp1* Δ -*bla*_{CTX-M-65}-*IS903D*-*iron* Δ structure is located in a Tn1722-like element, which is flanked by a 5 bp direct repeat (DR) (TTTAA). *IS26* truncates Tn1722 *tnpA* at one end and Tn5051-like *tnpR* at the other. (b) Genetic context of *bla*_{CTX-M-65} on pKC396, which is almost identical to that in pWCE307 except that *IS26* truncates the Tn5051-like element in *tnpA*. (c) Genetic context of *bla*_{CTX-M-24} on pKP96 (GenBank accession no. EU195449). The *ISEcp1*-*bla*_{CTX-M-24}-*IS903D*-*iron* Δ structure is flanked by a 5 bp DR (taaca) and is inserted into *orfI* of Tn1722. This Tn1722-like transposon flanked by a 5 bp DR (TAACC) is inserted into a region downstream of *fosA* (mediating resistance to fosfomycin). The Tn1722-like element carrying *bla*_{CTX-M-65} is almost identical to that carrying *bla*_{CTX-M-24} except for the missing of part of Tn1722 IRR and *orfI* in the former. (d) The missing part of Tn1722 IRR and *orfI* on pWCE307 and pKC396 compared to that on pKP96. (e) Genetic context of *bla*_{CTX-M-19} in *Klebsiella pneumoniae* ILT-3 (GenBank accession no. AF458080). The *ISEcp1*-*bla*_{CTX-M-19}-*IS903D*-*iron* Δ structure is flanked by a 5 bp DR (taaca) and is inserted into *orfI* of Tn1722 at the exact same position as that in pKP96 except that the *bla*_{CTX-M} variant is *bla*_{CTX-M-24}. This Tn1722-like element carrying *bla*_{CTX-M-19} truncates *sul1* of a class 1 integron but the left part of this Tn1722-like element is not available. (f) Genetic context of *bla*_{CTX-M-14} in pS12 (GenBank accession no. AB180674). *ISEcp1* is truncated by *IS10*, which is truncated by *IS26*. The *ISEcp1*-*bla*_{CTX-M-14}-*IS903D*-*iron* Δ structure is adjacent to an *orfI* remnant of Tn1722 at the exact same position as those in pWCE307, pKC396, pKP96 and ILT-3 except that the *bla*_{CTX-M} variants are different. IRR, Right-hand inverted repeat; IRL, left-hand inverted repeat.

as its alternative IRR and then mobilize *bla*_{CTX-M}, *IS903* and the partial *iron* gene (*iron* Δ) into *orfI* of Tn1722. As this *ISEcp1*-mediated mobilization into the exact same position of Tn1722 might have occurred only once, *bla*_{CTX-M-14} might have diverged to generate variants such as *bla*_{CTX-M-19},

*bla*_{CTX-M-24} and *bla*_{CTX-M-65} in this element. Furthermore, it is likely that Tn1722 serves as a common vehicle carrying *bla*_{CTX-M-14}-like genes, representing an alternative mechanism mediating the mobilization of these genes in addition to the action of *ISEcp1*.

Table 2. pMLST results for IncN plasmids

Plasmid	Accession no.	Host species	Location	ST	repN	<i>traI</i>	<i>traB</i>	<i>stbB</i>	<i>orfI-korB</i>
R46	AY046276	<i>E. coli</i>	–	1	1	1	1	1	1
pMUR050	AY522431	<i>E. coli</i>	Spain	2	1	2	2	1	2
pLEW517	DQ390455	<i>E. coli</i>	USA	3	1	2	3	1	1
pMAK2	AB366441	<i>S. Dublin</i> *	Japan	3	1	2	3	1	1
p12	FJ223605	<i>K. pneumoniae</i>	USA	4	2	3	4	2	3
pKP96	EU195449	<i>K. pneumoniae</i>	China	5	2	4	4	2	3
p9	FJ223607	<i>K. pneumoniae</i>	USA	6	3	4	5	3	4
pKC394	HM138652	<i>E. coli</i>	Germany	7	4	2	3	1	1
pKC396	HM138653	<i>E. coli</i>	Germany	7	4	2	3	1	1
pWCE307	This study	<i>E. coli</i>	China	7	4	2	3	1	1

**S. Dublin*, *Salmonella enterica* subsp. *enterica* serovar *Dublin*.

The *tnpA* gene of this Tn1722-like element on pWCE307 was truncated by IS26 at the exact same location as that on pKC396 (Fig. 1). On the other side, the truncation due to IS26 resulted in a truncated *tnpA* and an intact *tnpR* of a Tn5051-like transposon on pKC396 and a 297 bp *tnpR* remnant on pWCE307 (Fig. 1). Tn5051 is a mercury resistance transposon of the Tn21 subgroup (Mindlin *et al.*, 2001). The *res* sites of the Tn1722-like elements on pKC396 and pWCE307 were hybrids of those of Tn1722 and Tn5051 (Fig. 2), indicating that the *res* sites might serve as a sites for homologous recombination between the two transposons. Nonetheless, a single recombination event at the *res* sites could not explain the presence of part of Tn1722 *tnpA*. It is likely that the Tn1722-like elements carrying *bla*_{CTX-M-65} on pKC396 and pWCE307 were hybrids of Tn1722 and Tn5051, resulting from double crossover, with the *tnpA* genes serving as the recombination site in addition to *res*. The presence of the remnant of Tn5051 *tnpA* on pKC396 also suggests that *tnpA* of the Tn1722-like elements might once have been a hybrid of those of Tn1722 and Tn5051 prior to the insertion of IS26.

Of note, no characteristic 8 bp direct repeats (DRs) were identified flanking IS26 either on pKC396 or on pWCE307. This could have resulted from adjacent deletion by IS26 (Iida *et al.*, 1984) or from insertion of an additional copy of IS26 in a close position followed by homologous recombination between two copies of IS26 (Fig. 3). On pWCE307 and pKC396, IS26 abutted *tnpA*Δ of Tn1722 at the left side with identical boundaries but abutted different structures at its right side. This difference was more likely due to homologous recombination between two copies of IS26 after insertions at different locations (Fig. 3) than to different adjacent deletions due to insertion of a single IS26 at the same location.

The Tn1722-like elements carrying *bla*_{CTX-M-65} on pWCE307 and pKC396 contained an intact IRR and a 21 bp relic of a left-hand inverted repeat (IRL) and was flanked by a 5 bp (TTTAA) DR. The presence of the 5 bp

DR strongly suggests insertion of this Tn1722-like element (Schöffl *et al.*, 1981) into the *fip* (mediating fertility inhibition) and *nuc* spacer region. The left side of the Tn1722-like elements carrying *bla*_{CTX-M-65} on pWCE307 and pKC396 was almost identical to that carrying *bla*_{CTX-M-24} in pKP96 (Shen *et al.*, 2008) but with a deletion. Part of the Tn1722 including a 17 bp internal region of its IRL and a 1046 bp *orfI* fragment together with the 1258 bp 5' part of *ISEcp1* were missing on pWCE307 and pKC396 (Fig. 1). The deletion was due to unknown processes.

Although the mechanism mediating the deletion remains unidentified, this deletion might hinder further mobilization of *bla*_{CTX-M-65}. As its IRL was incomplete and *tnpA* was truncated, this Tn1722-like element could no longer mobilize by transposition. Due to truncation, *ISEcp1* is also unable to mobilize *bla*_{CTX-M-65} to a new location. Therefore, *bla*_{CTX-M-65} might have been trapped in this Tn1722-like element.

The Tn1722-like elements carrying *bla*_{CTX-M-65} on pKC396 and pWCE307 were in complex structures and might have been derived from that carrying *bla*_{CTX-M-24} on pKP96, the proposed prototype structure, following multiple events (Fig. 2): (i) it appears that the Tn1722-like element was inserted into a region between *fip* and *nuc* of the IncN plasmid backbone, generating a 5 bp DR (TTTAA); (ii) *bla*_{CTX-M-65} might have evolved from *bla*_{CTX-M-24} through a point mutation in the Tn1722-like element; the mutation could have been generated before or after insertion of the Tn1722-like element into the *fip*–*nuc* spacer; (iii) part of the left end of the Tn1722-like element including part of *ISEcp1* (Fig. 1d) was then deleted by unknown processes; (iv) double crossover at the *res* sites and *tnpA* genes between Tn1722 and Tn5051 generated a hybrid element with *orfI* and inverted repeats of Tn1722, *tnpR* of Tn5051 and hybrid *res* and *tnpA*; this recombination might have occurred before or after the insertion in the *fip*–*nuc* spacer; (v) subsequently, IS26 was inserted in the Tn1722-like elements carrying *bla*_{CTX-M-65} in *tnpA* on pKC396 or in *tnpR* on pWCE307.

Fig. 2. Formation of the Tn1722-like elements carrying *bla*_{CTX-M-65} on pWCE307 and pKC396. The Tn1722-like elements carrying *bla*_{CTX-M-24} on pKP96 is the proposed prototype. Multiple events might have occurred to form the structures on pWCE307 and pKC396 as follows. (i) Part of Tn1722 including part of its IRL and an *orfI* fragment together with the 5' part of *ISEcp1* may have been deleted by unknown processes. (ii) *bla*_{CTX-M-65} could have evolved from *bla*_{CTX-M-24} due to a point mutation. (iii) Double crossover (shown as crosses) between Tn1722 and Tn5051 at the *res* sites and *tnpA* may have resulted in a hybrid element with *orfI* from Tn1722, *tnpR* from Tn5051 and hybrid *tnpA* and *res*. The complete sequence of Tn5051 is not available and both ends of this transposon are therefore represented by dashed lines. The hybrid *res* sequence is shown. The *res* sequence identical to that in Tn1722 is depicted in bold and that identical to the counterparts in both Tn1722 and Tn5051 is underlined. Otherwise, the *res* sequence is identical to that in Tn5051. (iv) Insertion of IS26 into *tnpA* could have led to the formation of the structures seen in pWCE307 and pKC396. The absence of part of *tnpA* (on both pKC396 and pWCE307) and part of *tnpR* (pWCE307 only) could be explained by the adjacent deletion due to insertion of IS26 or, more likely, by insertion of an additional copy of IS26 in a close position followed by homologous recombination between two copies of IS26 (see Fig. 3).

pWCE307 appears to have the same backbone as pKC396

IncN plasmids have a broad host range and are usually associated with antimicrobial resistance genes. As IncN plasmids have increasingly been detected, it is necessary to establish a rapid and simple method for plasmid typing. pMLST is an attractive method to determine plasmid relatedness with phylogenetic implications and has been employed for typing IncH1- (Phan *et al.*, 2009), IncI1- (García-Fernández *et al.*, 2008) and IncFII-related plasmids (Villa *et al.*, 2010). The pMLST scheme developed here used five targets and was able to categorize the eight completely sequenced IncN plasmids into seven types, as all alleles examined in pLEW517 and pMAK2 were identical.

All of the five alleles in pWCE307 were identical to those in pKC396 (Table 2), suggesting that both plasmids probably shared the same backbone. pWCE307 and pKC396 apparently had a common origin in light of the identical pMLST results and the nearly identical region harbouring

*bla*_{CTX-M-65}. Indeed, the *HpaI* restriction pattern of pWCE307 was highly similar to that of pKC396, which was analysed *in silico* (data not shown).

pWCE307 and pKC396 (type 7) had four alleles (*stbB*, *traB*, *traI* and the *korB*-*orfI* region) identical to those of pLEW517 and pMAK2 (type 2; Table 2). This suggests that plasmids of the two pMLST types were closely related. However, the partial *repN* of pWCE307 and pKC396 was closer to those of plasmid 12 and pKP96 with only one nucleotide variation than to those of pLEW517 and pMAK2 with two variations. Of note, although the Tn1722-like element carrying *bla*_{CTX-M-65} on pWCE307 was highly similar to that carrying *bla*_{CTX-M-24} in pKP96, pWCE307 had a backbone that was apparently different from that of pKP96. None of the five alleles of pWCE307 were identical to those of pKP96. This suggests that *bla*_{CTX-M-24/65} was mobilized by the Tn1722-like element to a different IncN plasmid rather than that a common plasmid carrying *bla*_{CTX-M-24/65} travelled to different strains.

Fig. 3. A proposed model to explain the deletion of part of the Tn1722/5051 hybrid elements on pKC396 and pWCE307. IS26 was inserted into the Tn1722-derived part (in grey) of the hybrid *tnpA*. Another copy of IS26 was inserted into the Tn5051-derived part of the hybrid *tnpA* (left) or into *tnpR* (right). Homologous recombination between the two copies of IS26 could result in the structure of pKC396 or that of pWCE307 and deletion of the intervening part. Alternatively, the structure of pWCE307 may have been derived directly from that of pKC396 with the third copy of IS26 inserted into *tnpR* (bottom). Homologous recombination between IS26 could result in the structure of pWCE307 and deletion of the intervening part.

Conclusions

WCE307 in this study and isolate KC396 from Germany were both of ST131 and, in both cases, *bla*_{CTX-M-65} genes were associated with highly similar genetic contexts on IncN plasmids with probable identical backbones. These common features illustrate that WCE307 and KC396 are likely to have originated from a common ancestor very recently. This suggests the international spread of *bla*_{CTX-M-65} mediated by ST131 *E. coli* and IncN plasmids.

On pWCE307 and pKC396, *bla*_{CTX-M-65} was associated with a truncated *ISEcp1* and was located in Tn1722-like elements, of which the left end was partially deleted by unknown processes. The Tn1722-like elements carrying *bla*_{CTX-M-65} on pWCE307 and pKC396 might have been derived from that carrying *bla*_{CTX-M-24} in pKP96. As *ISEcp1* and Tn1722 were both incomplete, *bla*_{CTX-M-65} might have been trapped in this context. A pMLST scheme with five targets was established for IncN plasmid typing. pWCE307 and pKC396 were close to pLEW517 and pMAK2 with four identical alleles. The apparent differences between pWCE307/pKC396 and pKP96 suggested that *bla*_{CTX-M-24} and *bla*_{CTX-M-65} are carried by two different IncN plasmids rather than a common one.

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