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Short Communication

Characterisation of chromosomally-located *bla*$_{CTX-M}$ and its surrounding sequence in CTX-M-type extended-spectrum β-lactamase-producing *Escherichia coli* isolates

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ABSTRACT

Objectives: Although it has been regarded that the CTX-M-type extended-spectrum β-lactamase (ESBL) gene *bla*$_{CTX-M}$ is mainly carried by antimicrobial resistance plasmids, *Escherichia coli* possessing chromosomally-located *bla*$_{CTX-M}$ has been reported in previous studies. This study aimed to characterise the genetic structure of the chromosomally-located *bla*$_{CTX-M}$ transposition unit and its surrounding sequence in ESBL-producing *E. coli* isolated in a Japanese hospital.
Methods: A total of 81 ESBL-producing *E. coli* isolates were studied. The existence of chromosomally-located *bla*$_{CTX-M}$ was confirmed by *S1* nuclease-digested pulsed-field gel electrophoresis (PFGE) and Southern blot hybridisation and by sequencing analysis of the PCR-amplified DNA fragments.
Results: Chromosomally-located *bla*$_{CTX-M}$ was confirmed in 22 (27.2%) of the 81 *E. coli* isolates examined; five and four location types of chromosomally-located *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-15}$ were determined, respectively. Among the 22 *E. coli* isolates, 15 (68.2%) possessed single chromosomally-located *bla*$_{CTX-M}$ gene, probably due to single transposition of a plasmidic *bla*$_{CTX-M}$ to the chromosome. In isolate N0057, the *bla*$_{CTX-M-15}$ transposition unit was transferred from a plasmid into two different chromosomal regions. In addition, 'recurrent' transposition of already existing chromosomally-located *bla*$_{CTX-M-14}$ to another chromosomal region was observed in isolates N0211, N0214, N01127, N1682 and N1753; consequently, these isolates possessed two copies of chromosomally-located *bla*$_{CTX-M-14}$.
Conclusion: Considering that isolates N0211, N0214, N01127, N1682 and N1753 in which the 'recurrent' transposition event occurred were genetically related according to PFGE, these data suggest the possibility of accumulation of *bla*$_{CTX-M}$ on the chromosome in CTX-M-type ESBL-producing *E. coli*.

1. Introduction

CTX-M-type extended-spectrum β-lactamase (ESBL)-encoding (*bla*$_{CTX-M}$) genes have been detected on several transmissible plasmid lineages involved in the distribution of various ESBL-producing bacterial species [1,2]. As one component of the *bla*$_{CTX-M}$ transposition unit, the *bla*$_{CTX-M}$ gene is found along with insertion sequence (IS) elements such as IS*Ecp1* and IS*CR1* as well as additional downstream gene(s) such as *orf477*, IS*903D*, *iroN* and *lamB* [3–6]. *bla*$_{CTX-M}$ transposition units can mediate the transposition of *bla*$_{CTX-M}$ from antimicrobial resistance plasmids to the chromosome of host bacteria as well as transposition among transmissible plasmids [7–14].

Several studies have indicated that *Escherichia coli* possessing chromosomally-located *bla*$_{CTX-M}$ genes were obtained from several settings including hospitals, nursing home residences and asymptomatic healthy individuals [7,8,10,12–14]. Accumulating knowledge regarding the chromosomal location and surrounding sequences of the chromosomally-located *bla*$_{CTX-M}$ genes is important for studying the transmission of CTX-M-type ESBL-producing Enterobacteriaceae. The implications of chromosomally-located *bla*$_{CTX-M}$ in the dissemination of CTX-M-type ESBL-producing *E. coli* remains unclear.

In this study, nine new chromosomal regions with insertion of a *bla*$_{CTX-M}$ transposition unit that has been transposed into clinical *E. coli* isolates producing CTX-M-type ESBLs were determined.

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2. Materials and methods

2.1. Clinical isolates

A total of 81 ESBL-producing *E. coli* isolates obtained from specimens (faeces, sputum, urine and vaginal discharge) at a hospital in Okinawa Prefecture between July 2013 and July 2014 were examined [15].

2.2. Confirmation of chromosomally-located $bla_{CTX\text{-}M}$

Confirmation of chromosomally-located $bla_{CTX\text{-}M}$ was performed following a previously described method [7,8]. *Xba*I-digested DNA from *Salmonella enterica* serovar Braenderup H9812 (American Type Culture Collection, Manassas, VA) was used as a DNA size marker.

2.3. Determination of chromosomal insertion sites of the $bla_{CTX\text{-}M}$ transposition unit

Inverse PCR was performed using PrimeSTAR® Max DNA Polymerase (Takara Bio Inc., Shiga, Japan) following digestion with one of the restriction enzymes *Bgl*II, *Pst*I, *Sal*I, *Hae*III or *Eco*RV following a previously described protocol [7]. For adapter ligation-mediated PCR, 100 ng of DNA sample purified using a PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific K.K., Kanagawa, Japan) was used. Bacterial DNA was digested with *Bgl*II, *Pst*I or *Sal*I and was amplified using Tks Gflex™ DNA Polymerase (Takara Bio Inc.) with the primers listed in Table 1 following ligation of the corresponding adapter. Amplified DNA fragments were subjected to sequencing analysis using a BigDye® Terminator v.3.1 Cycle Sequencing Kit (Thermo Fisher Scientific K.K.) with the primers indicated in Table 1 [7,16].

2.4. GenBank data search

To retrieve previously analysed sequences of chromosomally-located $bla_{CTX\text{-}M}$ genes, a database search of GenBank was conducted on 14 December 2017.

3. Results

3.1. Evaluation of chromosomally-located $bla_{CTX\text{-}M}$

Chromosomally-located $bla_{CTX\text{-}M}$ was detected in 22 (27.2%) of the 81 *E. coli* isolates studied, including 10 (12.3%) $bla_{CTX\text{-}M\text{-}14}$, 11 (13.6%) $bla_{CTX\text{-}M\text{-}15}$ and 1 (1.2%) $bla_{CTX\text{-}M\text{-}27}$. In three *E. coli* isolates possessing $bla_{CTX\text{-}M\text{-}15}$ (N0846, N1243 and N1500) and one *E. coli* isolate possessing $bla_{CTX\text{-}M\text{-}27}$ (N1315), $bla_{CTX\text{-}M}$ was detected both in plasmid and on the chromosome.

3.2. Molecular structure of chromosomally-located $bla_{CTX\text{-}M}$

As shown in Fig. 1, the resulting insertion sites of the $bla_{CTX\text{-}M}$ transposition unit were indicated by genes adjacent to the insertions, namely *terC* type (**LC335827**), *ompN* type (**LC259303**), *yhjQ* type (**LC259304**), *sraG* type (**LC259305**) and *dacD* type (**LC259306**) for $bla_{CTX\text{-}M\text{-}14}$, and *oppA* type (**LC259307**) and *exuR* (**LC335830**) type for $bla_{CTX\text{-}M\text{-}15}$. Two insertion sites of chromosomally-located $bla_{CTX\text{-}M\text{-}15}$ for which adjacent

Table 1
Primers used in this study.

Primer name	Primer sequence (5'→3')	Reference
PCR, sequencing primer		
T7 promoter	GTAATACGACTCACTATAGGGCGA	
SP6 promoter	GGGCGATTTAGGTGACACTATAGC	
CTX-M-U1	ATGTGCAGYACCAGTAARGTKATGGC	[16]
CTX-M-U2	TGGGTRAARTARGTSACCAGAAYCAGCGG	
iroN seqF1	CACCGTTGAAGCAGGCAGTAAATG	This study
IS*903D* gw-seqF1	GCACACCTGGTAATTGATTCCAC	
IS*903D* gw-seqF2	GAGTAATGCGCGGTGGAAATG	
ORF477seqR	CAGTGCGCATAGTCATCGGCAG	
IS*Ecp1* F	GCTCTGCGGTCACTTCATTGG	
IS*Ecp1* R	GCTGTCTGTATTCTGAAGAGTCC	
IS*Ecp1* flanking R	GAGCACTTTTCTTTCACCCAATGG	
KC2*R3	TGAAGTGACCGCAGAGCATGG	
N211 *lamB* seqF	TCAACTGGGATAACGACAACG	
terC type F	GATCACCGAATCCAGGCTAAACAC	
terC type R	GTTCCGCACGTTGATTCTGCTC	
ompN type F	CATAGCTCTGGTCGCCATCT	
ompN type R	GCAACACCGATTAAATGCTCTGG	
yhjQ type F	CATTGACGATTGCCAGCGAGTG	
yhjQ type R	GCTATGCTGGATGACCAGGACTG	
dacD type F	CGAAAGATAATCCGGTGTTTGTCG	
dacD type R	CAGACCATGCACTGTTTCAAAATGC	
oppA type F	GTCGGTGATTCGCAATAGAG	
oppA type R	GACTGTACGCCACATCTTCAGG	
exuR type F	CTGTTCGATGCGCTCTTTCAGAT	
N0057 type F	CTTCCCGTTCTGCTGATAACCCTTC	
N0057 type R	CTGTACAGACATGCTCACTCCTTC	
N0846 type F	GTTGTCGCCATTGTTCGATTAACG	
U1R	GCCATMACYTTACTGGTRCTGCACAT	[7]
U2R	CCGCTGRTTCTGGTSACYTAYTTYACCCA	
Adapter		
Link PstF	GTAATACGACTCACTATAGGGCGATTTAGGTGACACTATAGCGGCCGCAGAGTGCA	This study
Link BgSalF	GTAATACGACTCACTATAGGGCGATTTAGGTGACACTATAGCGGCCGCAGAG	
GW-SalR	P-TCGACTCTGCGG-NH2	
GW-BglR	P-GATCCTCTGCGG-NH2	
GW-PstR	P-CTCTGCGGCCG-NH2	

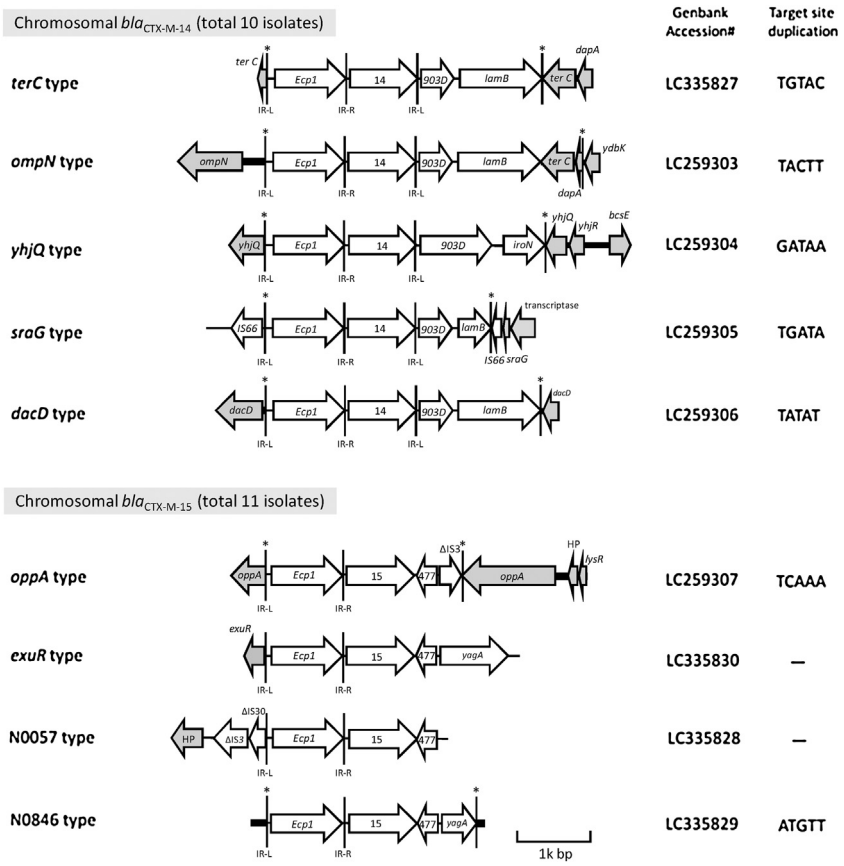

Fig. 1. Schematic diagram of genetic structures surrounding $bla_{CTX-M-14}$ and $bla_{CTX-M-15}$ in *Escherichia coli* isolates. Determined insertion sites of the chromosomally-located bla_{CTX-M} transposition units were indicated by genes adjacent to the insertions, namely *terC* type (**LC335827**), *ompN* type (**LC259303**), *yhjQ* type (**LC259304**), *sraG* type (**LC259305**) and *dacD* type (**LC259306**) for $bla_{CTX-M-14}$, and *oppA* type (**LC259307**) and *exuR* type (**LC335830**) for $bla_{CTX-M-15}$. Two insertion sites of chromosomally-located $bla_{CTX-M-15}$ were indicated by the strain ID, namely 'N0057′ type (**LC335828**) and 'N0846' type (**LC335829**). Thin and thick horizontal lines denote non-coding regions in the plasmid and chromosome, respectively. White and grey arrows denote plasmid and chromosomal genes, respectively. IR-R, right inverted repeat; IR-L, left inverted repeat; IS, insertion sequence; *Ecp1*, IS*Ecp1*; 14, $bla_{CTX-M-14}$; 15, $bla_{CTX-M-15}$; 903D, IS*903D*; 477, ORF477; HP, genes encoding a hypothetical protein.

chromosomal genes were not observed were indicated by the strain ID, namely 'N0057' type (**LC335828**) and 'N0846' type (**LC335829**). These identified bla_{CTX-M} transfer sites were not identical to any previously reported insertion sites listed in the GenBank database.

The most prevalent type was the *oppA* type, which was detected in eight *E. coli* isolates possessing $bla_{CTX-M-15}$ (N0057, N0071, N0165, N0223, N0324, N0327, N0995 and N1384), followed by the *ompN* type and *terC* type (5 isolates). Among the 22 *E. coli* isolates possessing chromosomally-located bla_{CTX-M}, 15 (68.2%) possessed only one chromosomally-located bla_{CTX-M} gene whereas the remainder (7 isolates; 31.8%) possessed two differently transposed bla_{CTX-M} genes on the chromosome. Both the *ompN* type and the *terC* type were detected in five *E. coli* isolates possessing chromosomally-located $bla_{CTX-M-14}$ (N0211, N0214, N1127, N1682 and N1753). Isolate N0057 (*oppA* type and 'N0057' type) and N1243 (*exuR* type and 'N0846' type) harboured two chromosomally-located $bla_{CTX-M-15}$ genes in different chromosomal regions. In this study, we could not determine the exact chromosomal locations in one of each CTX-M-14-type ESBL-producing isolate (N1336), CTX-M-15-type ESBL-producing isolate (N1500) and the CTX-M-27-type ESBL-producing isolate (N1315), in which chromosomally-located bla_{CTX-M} was detected by Southern blotting analysis following *S1* nuclease-digested pulsed-field gel electrophoresis (PFGE).

3.3. Second 'recurrent' transposition of the bla_{CTX-M} transposition unit from chromosome to chromosome

Remarkably, compared with the *terC* type, the *dapA* sequence in the *ompN* type was truncated at the point adjoining 41 bp from the 3′ end, although the downstream sequence (IS*903D*, *lamB* and *terC*) of the *ompN* type was exactly the same as the sequence of the *terC* type (Fig. 1). In this study, the *ompN* type chromosomally-located $bla_{CTX-M-14}$ gene was detected together with the *terC* type chromosomally-located $bla_{CTX-M-14}$ gene. And in *ompN* type, a 5-bp target site duplication downstream of the bla_{CTX-M} transposition unit was observed at a point adjoining the truncated *dapA*. Therefore, these results suggest 'recurrent' transposition of an already chromosomally-located $bla_{CTX-M-14}$ transposition unit (the *terC* type) to another region (the *ompN* type) on the same chromosome (Fig. 2).

4. Discussion

The results of this study suggest that *ompN* type chromosomally-located bla_{CTX-M} genes were transferred from an already translocated *terC* type bla_{CTX-M} transposition unit on the chromosome (Fig. 2). Similar 'from chromosome to chromosome' translocation of the bla_{CTX-M} transposition unit was identified by the GenBank database search. In the genome sequence of *E. coli*

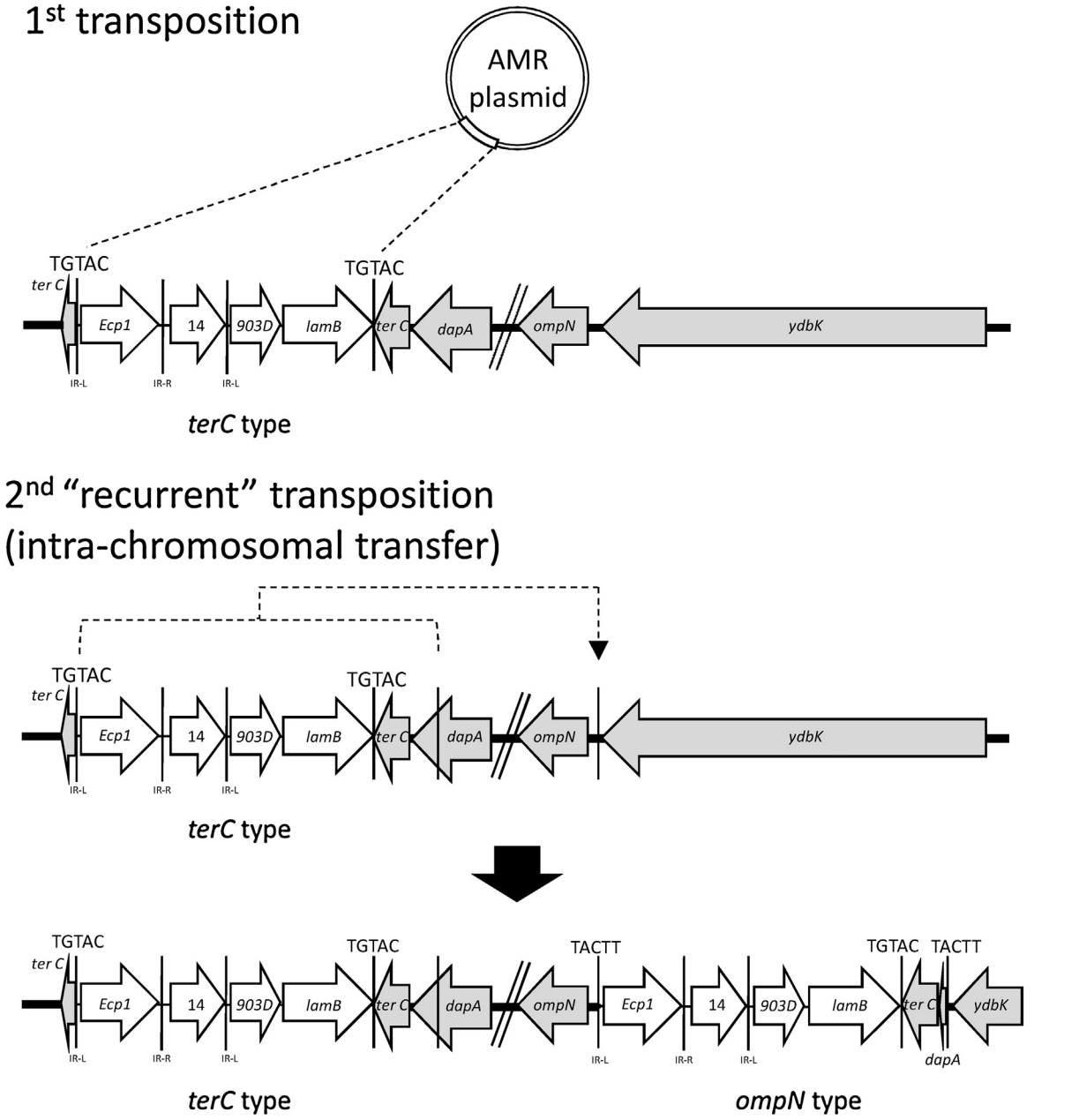

Fig. 2. Schematic diagram of second 'recurrent' transposition of bla_{CTX-M} from chromosome to chromosome. The 5-bp target site duplications resulting from IS*Ecp1* are shown. Thin and thick horizontal lines denote non-coding regions in the plasmid and chromosome, respectively. White and grey arrows denote plasmid and chromosomal genes, respectively. AMR, antimicrobial resistance; IR-R, right inverted repeat; IR-L, left inverted repeat; *Ecp1*, IS*Ecp1*; 14, $bla_{CTX-M-14}$; 903D, IS*903D*.

ST648 isolate (**CP008697.1**), two chromosomally-located $bla_{CTX-M-14}$ transposition units were identified. By identified target site duplications, the first transposition to the chromosome might occur at sequence position 326 771 of the *E. coli* ST648 isolate by leaving a target site duplication (TGGAA) just upstream of IS*Ecp1* and downstream of *iroN*. Then, 'from chromosome to chromosome' transposition might recur at sequence position 499 322 of the *E. coli* ST648 isolate. In this second recurrent transposition, the sequence between the target site duplication (AATGA) was longer than the results in the current study (498 bp); in particular, a downstream additional sequence of the $bla_{CTX-M-14}$ transposition unit in the second recurrent transposition was a long as 7338 bp. There is a possibility that the second transition might be mediated by a mechanism other than transposition by IS*Ecp1*. However, downstream target site duplication (TGGAA) of the first transposition was left in the same region of the second chromosomally-located sequence of the $bla_{CTX-M-14}$ transposition unit on the *E. coli* ST648 chromosome. In any case, further study is needed to explain the detailed mechanism of IS*Ecp1* transition in bacterial cells.

By *Xba*I-PFGE, DNA banding patterns of *E. coli* isolates possessing both the *terC* type and the *ompN* type, which were sporadically collected for 11 months from a hospital, were similar (data not shown). In this study, there were no clear answer regarding how chromosomally-located bla_{CTX-M} can be maintained in *E. coli* isolates, how 'recurrent' transposition of the bla_{CTX-M} transposition unit occurs and how antibiotics effect 'recurrent' transposition of the bla_{CTX-M} transposition unit. The stability an antimicrobial resistance gene in antimicrobial-resistant bacteria can be one of the most important contributing factors to the worldwide distribution of these bacteria. In accordance with this, further precise investigation is important to clarify whether the chromosomally-located bla_{CTX-M} gene contributed to worldwide

spread of CTX-M-type ESBL-producing *E. coli*. This stability may be one contributing factor to the high prevalence of CTX-M-type ESBL-producing *E. coli*, especially in Southeast Asian countries [8,17].

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Competing interests

None declared.

Ethical approval

Not required.

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