Escherichia coli モデル株を用いたbla_<CTX-M-14> のプラスミドから染色体への転位の観察

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Characterization of $bla_{CTX\text{-}M\text{-}14}$ transposition from plasmid to chromosome in *Escherichia coli* experimental strain

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ABSTRACT

Mostly, $bla_{CTX\text{-}M}$ is found on transferable plasmids as a component of the $bla_{CTX\text{-}M}$ transposition unit containing an insertion sequence, IS*Ecp1*, which exists on the upstream region of $bla_{CTX\text{-}M}$s. Several recent studies conducted in clinical and community settings have reported the presence of chromosomally located $bla_{CTX\text{-}M}$ in extended spectrum β-lactamase (ESBL)-producing bacterial isolates. In this study, we observed the frequency and molecular nature of the IS*Ecp1*-mediated transposition of $bla_{CTX\text{-}M\text{-}14}$ from a plasmid to a chromosome by using an experimental strain of *Escherichia coli*. We determined 102 different chromosomal transposition sites of $bla_{CTX\text{-}M\text{-}14}$ in 126 *E. coli* isolates following five independent screening procedures. The characterization of the 102 different chromosomal transposition sites of $bla_{CTX\text{-}M\text{-}14}$ observed in this study revealed the presence of 5-bp direct repeat (DR) sequences and identical left terminal inverted sequences in 80 *E. coli* isolates. However, 5′-flanking sequences of the right terminal DR sequences in the 80 *E. coli* isolates were highly diverse, and consensus sequences of the right terminal inverted repeat sequences were not observed. In case of our *E. coli* experimental strain, the frequency of the IS*Ecp1*-mediated transposition of $bla_{CTX\text{-}M\text{-}14}$ from a plasmid to a chromosome was determined to be 0.51% (SD = 0.37). Collectively, the molecular nature of IS*Ecp1* could plausibly be a factor contributing to the high detection rates of *E. coli* possessing chromosomally located $bla_{CTX\text{-}M\text{-}14}$ in both clinical and community settings.

1. Introduction

CTX-M-type extended spectrum β-lactamase (ESBL)-producing bacteria have been reported in both clinical and community settings (Coelho et al., 2010; Fabre et al., 2009; Hamamoto et al., 2016; Harada et al., 2012; Hirai et al., 2013; Mahrouki et al., 2012). CTX-M type ESBL is mainly classified into five groups (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25) and minor variants based on the similarity of amino acid residues (Bonnet, 2004; Rossolini et al., 2008). $Bla_{CTX\text{-}M}$ has mostly been found on transferable plasmids that are known to be spread among bacteria from the family Enterobacteriaceae by plasmid conjugation. Among the several insertion sequence elements involved in mobilizing $bla_{CTX\text{-}M}$, the insertion sequence IS*Ecp1*, which exists on the upstream region of $bla_{CTX\text{-}M}$, is known as one of the most important elements (Bou et al., 2002; Chanawong et al., 2002; Karim et al., 2001; Lartigue et al., 2003). IS*Ecp1*, which is bracketed by a left terminal inverted repeat sequence (IRL) and right terminal inverted repeat sequence (IRR), is located upstream of $bla_{CTX\text{-}M}$. IS*Ecp1* mobilizes itself and its downstream genes including $bla_{CTX\text{-}M}$ by means of spacer sequences of variable lengths; it serves as a $bla_{CTX\text{-}M}$ transposition unit by recognizing an IRL and IRR, or an alternative IRR sequence (IRRalt) (Lartigue et al., 2006; Partridge, 2011). The promoter sequence lying upstream of the IRR, and several variations of spacer sequences between the IRR and ATG start codon of $bla_{CTX\text{-}M}$ have been observed, and the promoter sequence and the length of the spacer sequence are important for high expression levels of $bla_{CTX\text{-}M}$ (Ma et al., 2011).

Using the conjugal plasmid transfer method, Lartigue et al. have demonstrated that the frequency of the transfer of $bla_{CTX\text{-}M\text{-}2}$ from the chromosome of *Kluyvera ascorbata* to a transferable plasmid via naturally occurring IS*Ecp1B*-mediated transposition was (6.4 ± 0.5) ×

Abbreviations: **ESBL**, Extended spectrum β-lactamase; **DR**, Direct repeat; **IRL**, Left terminal inverted repeat sequence; **IRR**, Right terminal inverted repeat sequence; **IRRalt**, Alternative IRR sequence; **PFGE**, Pulsed-field gel electrophoresis; **KAN**, Kanamycin; **CTX**, Cefotaxime; **SPT**, Spectinomycin; **IPTG**, Isopropyl β-D-1-thiogalactopyranoside; **AHT**, Anhydrotetracycline; AMR, antimicrobial resistance

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10^{-7} (Lartigue et al., 2006). In addition, previous studies have reported high detection rates of *E. coli* possessing chromosomally located $bla_{CTX\text{-}Ms}$ among *E. coli* possessing $bla_{CTX\text{-}M\text{-}14}$ in samples collected from asymptomatic, healthy Thai subjects (56.9%) (Hamamoto et al., 2016), *E. coli* possessing $bla_{CTX\text{-}Ms}$ in clinical specimens (*i.e.*, feces, sputum, urine, and vaginal discharge) collected from an Okinawa prefectural hospital (27.2%) (Hamamoto and Hirai, 2018), and *E. coli* possessing $bla_{CTX\text{-}M\text{-}15}$ in samples collected from nursing home residences in Japan (95.5%) (Hirai et al., 2013). The genetic comparisons in these studies have highlighted the IS*Ecp1*-mediated transposition of $bla_{CTX\text{-}Ms}$ from plasmids to the chromosomes of host bacterial cells. However, the factors that contribute to the higher detection rates of these bacterial strains possessing chromosomally located $bla_{CTX\text{-}M}$ are yet to be identified, regardless of clinical or community settings. Therefore, we established an experimental strain of *E. coli* to observe the frequency of the transfer of $bla_{CTX\text{-}M\text{-}14}$ from the $bla_{CTX\text{-}M\text{-}14}$ plasmid, which harbored the $bla_{CTX\text{-}M\text{-}14}$ transposition unit, to the chromosome via IS*Ecp1*-mediated transposition, and analyze the molecular nature of the IS*Ecp1*-mediated transposition of $bla_{CTX\text{-}M\text{-}14}$.

2. Materials and methods

2.1. Construction of the plasmids

PrimeSTAR Max DNA Polymerase (Takara Bio Inc., Shiga, Japan) was used for the construction of the plasmids. The nucleotides used for the plasmid construction are listed in Table S1. Firstly, three DNA fragments containing either the pMW219 (Nippon Gene Co LTD., Toyama, Japan) plasmid backbone structure, *tetR*, or kanamycin (KAN) selection cassette were amplified to construct the pMW219-*tetR* plasmid (4756 bp). The DNA region (3395 bp) of pMW219 that contained the plasmid backbone structure was amplified using suitable primers (p1 and p2). The DNA fragment containing *tetR* (657 bp) was amplified using suitable primers (p3 and p4) and the pwtCas9-bacterial plasmid (#44250, Addgene, Cambridge, MA) as the template. The DNA fragment containing the KAN selection cassette (749 bp) was amplified using suitable primers (p5 and p6) and the pCR-Blunt II plasmid (Thermo Fisher Scientific K.K., Tokyo, Japan) as the template. The three purified DNA fragments were circularized using the In-Fusion HD Cloning Kit (Takara Bio Inc.). Then, the DNA fragment containing the plasmid backbone structure of pMW219-*tetR* was amplified using suitable primers (p7 and p8), and then ligated with the $bla_{CTX\text{-}M\text{-}14}$ transposition unit-containing DNA fragment, which was amplified using suitable primers (p9 and p10) and the genomic DNA of ESBL-producing *E. coli* KC12 (**AB915399**) as the template. Consequently, the $bla_{CTX\text{-}M\text{-}14}$ plasmid (**LC377573**) containing the $bla_{CTX\text{-}M\text{-}14}$ transposition unit, which comprised IS*Ecp1*, $bla_{CTX\text{-}M\text{-}14}$, IS*903D*, and *iroN,* was obtained (Fig. 1).

For the efficient removal of the $bla_{CTX\text{-}M\text{-}14}$ plasmid from the experimental *E. coli* strain, we utilized the CRISPR/Cas9 system targeting the $bla_{CTX\text{-}M\text{-}14}$ plasmid; the target sequence for the CRISPR/Cas9-mediated removal of the $bla_{CTX\text{-}M\text{-}14}$ plasmid was searched between a DnaA-binding site and five RepA-binding sites, which ranged between the 482nd and 738th nucleotide of the pMW219 plasmid (**LC377573**). The plasmid pCas (#62225, Addgene) was digested with the restriction enzymes ApaI and BglII. The DNA fragment obtained as a consequence (928 bp), which contained the *lac* promoter and guide RNA sequence, was subcloned into the pCR-Blunt II plasmid via the ApaI and BamHI sites. Then, a SpeI site was introduced just downstream of the guide RNA sequence by PCR using suitable primers (p11 and p12). The consequently obtained plasmid, p*lac*-gRNA, was used for cloning the guide RNA sequence required for the CRISPR/Cas9-mediated targeting of the $bla_{CTX\text{-}M\text{-}14}$ plasmid. The guide RNA sequence for the CRISPR/Cas9 gene targeting process was selected using the CHOPCHOP website (http://chopchop.cbu.uib.no/). To introduce the selected target sequence (5′-AGTGAGTTATACACAGGGCT-3′), which is located between the 477th and 496th nucleotide of pMW219 (**LC377573**), PCR was performed using suitable primers (p13 and p14) and the p*lac*-gRNA plasmid. The amplified DNA fragment was circularized using the In-Fusion HD Cloning Kit.

The DNA fragment (4991 bp) containing the Cas9 gene was amplified using suitable primers (p15 and p16) and the pwtCas9-bacterial plasmid as the template. The amplified DNA fragment was connected with the plasmid backbone structure of the pCDF Duet-1 plasmid (Merck KGaA, Darmstadt, Germany), followed by amplification using suitable primers (p17 and p18). Then, the DNA fragment containing the *lac* promoter and the guide RNA sequence required for the CRISPR/Cas9-mediated targeting of the region of the pMW219 plasmid that is essential for its replication (934 bp) was cleaved by digestion with the restriction enzymes ApaI and NheI, and subcloned into the ApaI and XbaI sites of the pCDF Duet-1-Cas9 plasmid. Finally, the CRISPR/Cas9 effector plasmid (**LC377574**) was constructed (Fig. 1).

2.2. Mutagenesis of the gene encoding ISEcp1 transposase

PCR-mediated mutagenesis using suitable primer pairs (p19 and p20, and p21 and p22) was performed to obtain a DNA fragment encoding mutant IS*Ecp1* transposase (the Y319A and R322A mutant). The DNA fragment produced consequently was utilized for the preparation of the IS$Ecp1^{Y319A,\ R322A}$-$bla_{CTX\text{-}M\text{-}14}$ plasmid. For the preparation of the ΔIS*Ecp1*-$bla_{CTX\text{-}M\text{-}14}$ plasmid, PCR was performed using suitable primers (p23 and p24) and the $bla_{CTX\text{-}M\text{-}14}$ plasmid to remove the coding sequence of the IS*Ecp1* transposase. The amplified DNA fragment was circularized using the In-Fusion HD Cloning Kit.

2.3. Selection and establishment of the experimental strain of E. coli

Transformation of ECOS competent *E. coli* cells (Nippon Gene Co LTD) with the $bla_{CTX\text{-}M\text{-}14}$ plasmid and the CRISPR/Cas9 effector plasmid was performed following the manufacturer's instructions. The transformants were screened on LB agar supplemented with 30 μg/ml of KAN, 2 μg/ml of cefotaxime (CTX), and 50 μg/ml of spectinomycin (SPT). *E. coli* transformant clones that were resistant to KAN, CTX and SPT were subjected to evaluation to ascertain whether CRISPR/Cas9 activity was observed after the addition of 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) (Takara Bio Inc.) and 20 ng/mL of anhydrotetracycline (AHT) (Sigma-Aldrich, St. Louis, MO), followed by incubation overnight at 37 °C. After induction of CRISPR/Cas9, growth of *E. coli* transformants in LB broth supplemented with 50 μg/ml of SPT, but not in LB broth supplemented with 30 μg/ml of KAN and 50 μg/ml of SPT were confirmed. The confirmed *E. coli* transformants in which CRISPR/Cas9 activity was observed were used as *E. coli* experimental strain after incubation in LB broth supplemented with 30 μg/ml of KAN, 2 μg/ml CTX and 50 μg/ml of SPT for overnight.

2.4. Determination of the frequency of the transfer of the $bla_{CTX\text{-}M\text{-}14}$ transposition unit

The selected *E. coli* experimental strain was incubated in LB broth supplemented with 1 mM of IPTG and 20 ng/mL of AHT at 37 °C for 3 h to activate the CRISPR/Cas9 system. The treated *E. coli* cells were inoculated on LB agar containing 2 μg/ml of CTX and 50 μg/ml of SPT or on LB agar without any antibiotic. After incubation at 37 °C overnight, several colonies that had grown on the LB agar containing 2 μg/ml of CTX and 50 μg/ml of SPT were subjected to S1 nuclease pulsed-field gel electrophoresis (PFGE) and Southern blot hybridization analysis. The S1 nuclease-PFGE and Southern blot hybridization analysis were performed to detect the chromosomally located $bla_{CTX\text{-}M\text{-}14}$, in accordance with a previous study (Hirai et al., 2013). Digoxigenin-labeled probes used in the Southern blot hybridization were prepared using the DIG Probe Synthesis Kit (Sigma-Aldrich) with one of the following primer combinations: p25 and p26 ($bla_{CTX\text{-}M\text{-}14}$ detection probe) or p27 and

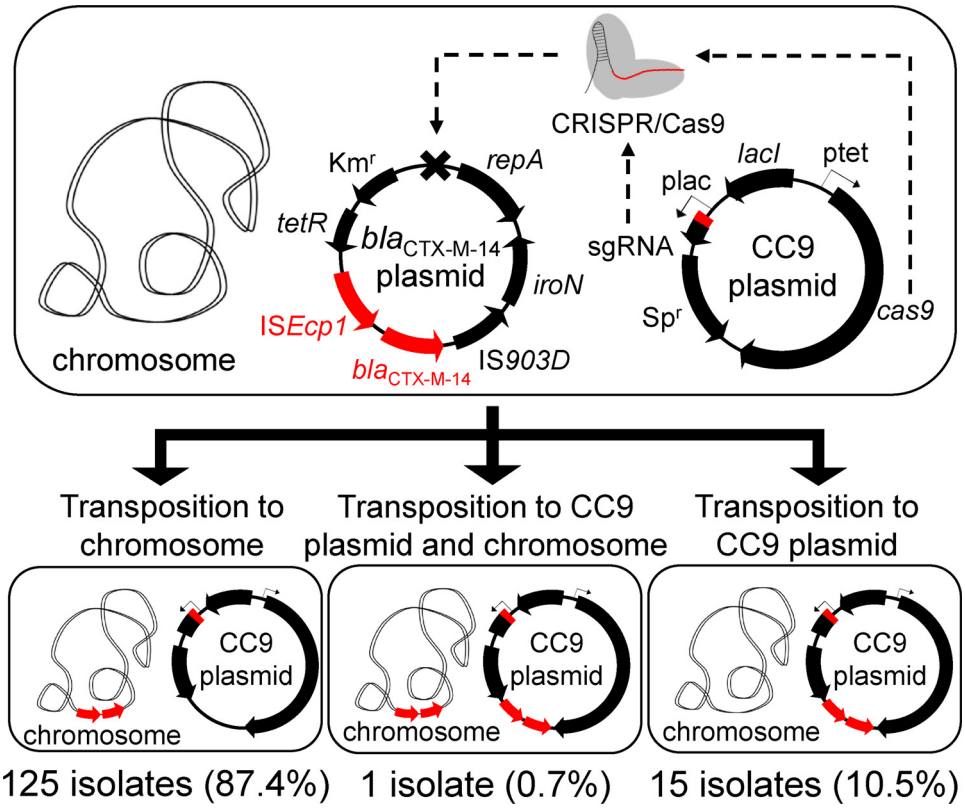

Fig. 1. Overview of the screening of *E. coli* possessing chromosomally located $bla_{CTX-M-14}$. Competent *E. coli* cells were transformed with the CRISPR/Cas9 effector plasmid (CC9 plasmid) and $bla_{CTX-M-14}$ plasmid. DNA fragment containing IS*Ecp1*, $bla_{CTX-M-14}$, IS*903D*, and *iroN* (**AB915399**) was used in $bla_{CTX-M-14}$ plasmid. After the induction of the CRISPR/Cas9 system, the location of the $bla_{CTX-M-14}$ transposition units (i.e., whether they were located either on the chromosome or the CC9 plasmid) was confirmed by Southern blot hybridization analysis, followed by S1 nuclease-PFGE. Transposition units of $bla_{CTX-M-14}$ located on the CC9 plasmid were confirmed by sequencing the upstream region adjacent to the IRL of the $bla_{CTX-M-14}$ transposition units. Chromosomally located $bla_{CTX-M-14}$ transposition units were detected in 126 CTX-resistant *E. coli* isolates, including one isolate possessing $bla_{CTX-M-14}$ located on both the chromosome and the CC9 plasmid. In the 15 CTX-resistant *E. coli* isolates, $bla_{CTX-M-14}$ transposition units were detected on the CC9 plasmid. In some experiments, IS*Ecp1*$^{Y319A,\ R322A}$-$bla_{CTX-M-14}$ plasmid and ΔIS*Ecp1*-$bla_{CTX-M-14}$ plasmid which express mutant versions of IS*Ecp1* transposase were used instead of the $bla_{CTX-M-14}$ plasmid. However, IS*Ecp1* transposases dependent transposition of $bla_{CTX-M-14}$ from a plasmid to a chromosome was not observed.

p28 (16S rRNA gene detection probe).

The frequencies of the transfer of the $bla_{CTX-M-14}$ transposition unit from the $bla_{CTX-M-14}$ plasmid to the chromosome were calculated by the following formula: Transfer frequency (%) = (the number of CTX- and SPT-resistant *E. coli* clones in which chromosomally location of $bla_{CTX-M-14}$ was confirmed by S1-PFGE and Southern blotting hybridization)/(the total number of *E. coli* colonies estimated to have grown on LB agar without antibiotics). The total number of *E. coli* colonies estimated to have grown on LB agar without antibiotics was obtained based on the number of colony-forming units, which was calculated by the serial dilution method. The standard deviations were calculated based on the frequencies of the transfer of the $bla_{CTX-M-14}$ transposition unit from the $bla_{CTX-M-14}$ plasmid to the chromosome observed in five independent screening procedures.

2.5. Characterization of the chromosomal locations of the $bla_{CTX-M-14}$ transposition unit and its surrounding genes

Genomic DNA was extracted from the *E. coli* isolates possessing chromosomally located $bla_{CTX-M-14}$ using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific K.K.), according to the manufacturer's instructions. Two hundred ng of the extracted genomic DNA was digested with HaeIII (Takara Bio Inc.) and self-ligated using the Ligation high reagent (Toyobo Co., LTD., Tokyo, Japan). PrimeSTAR Max DNA Polymerase (Takara Bio Inc.) was used for inverse PCR, which was performed using suitable primers (p29 and p30) and 1 ng of the processed genomic DNA. The amplified DNA fragments containing the region lying upstream of the $bla_{CTX-M-14}$ transposition unit were sequenced using the BigDye® Terminator v.3.1 Cycle Sequencing Kit (Life Technologies Japan).

In order to amplify the downstream region adjacent to the $bla_{CTX-M-14}$ transposition unit, adapter ligation-mediated PCR using the Tks Gflex DNA Polymerase (Takara Bio Inc.) was performed. The adapter was prepared by annealing the oligonucleotides (p31 and p32), and subsequently ligated with the HaeIII-digested DNA using the Ligation high reagent (Toyobo Co., LTD.). The first round of the PCR was performed using 1 ng of the adapter-ligated DNA and suitable primers (p33 and p34). The amplified DNA fragments obtained after the first round of PCR were diluted 1:100 and subjected to a second round of PCR. Primer p35 and one of the following primers: p36, p37, p38, p39, p40, or p41 were used for the second round of the PCR. The amplified DNA was sequenced using the BigDye® Terminator v.3.1 Cycle Sequencing Kit.

The determined chromosomal locations of the $bla_{CTX-M-14}$ transposition unit were mapped onto the genome of the *E. coli* NEB 5-alpha laboratory strain (CP017100) (Anton and Raleigh, 2016), which is derived from the *E. coli* K-12 DH5α strain, using the CGView Server (Grant and Stothard, 2008). The genes interrupted by the $bla_{CTX-M-14}$ transposition unit were characterized by performing the Gene Ontology (GO) analysis using the Blast2GO software (Gotz et al., 2008). The similarity of the IRRalt among the different transformants was analyzed using the GENEIOUS R11 software (Biomatters Ltd., Auckland, New Zealand).

2.6. GenBank data search

The chromosomal locations of the $bla_{CTX-M-14}$ transposition unit determined in this study were subjected to BLAST search for evaluating whether identical chromosomal locations of the $bla_{CTX-M-14}$ transposition unit have already been reported (last access date: May 2, 2019).

3. Results

3.1. Evaluation of the E. coli experimental strain

As shown in Fig. 1, we established an *E. coli* experimental strain to characterize IS*Ecp1*-mediated $bla_{CTX-M-14}$ transposition occurring from the $bla_{CTX-M-14}$ plasmid to the chromosome of *E. coli*. After the treatment of the $bla_{CTX-M-14}$ plasmid with the CRISPR/Cas9 system, in which the CC9 plasmid served as the effector plasmid, the location of the transposed $bla_{CTX-M-14}$ transposition unit, i.e., whether it was on the chromosome and/or on the CC9 plasmid, was determined.

First, we evaluated whether the IS*Ecp1*-mediated transposition of the $bla_{CTX-M-14}$ transposition unit from the $bla_{CTX-M-14}$ plasmid to the chromosome was observed in the *E. coli* experimental strain. In the IS*4* family, including the members IS*4*, IS*10*, and IS*50*, the Y-(2)-R-(3)-E-(6)-(K) signature (the YREK-motif) is known to be highly conserved and play an essential role for DNA strand transposition following DDE catalytic reaction (De Palmenaer et al., 2008; Klenchin et al., 2008). IS*Ecp1* is also a DDE transposase belonging to the IS*1380* family (Siguier et al., 2006). Our preliminary consideration implied that the YREK-motif, especially, Y and R, appears to be highly conserved among the IS*4* and IS*1380* families. It has been previously demonstrated that in IS*50* with Y319A and R322A mutations of the YREK motif, the strand transfer activity was notably defective (Klenchin et al., 2008). For evaluating the IS*Ecp1*-mediated transposition in our *E. coli* experimental strain, we firstly ascertained whether transposition of $bla_{CTX-M-14}$ was dependent on IS*Ecp1* transposase activity. We constructed two plasmids such as the IS*Ecp1*$^{Y319A,\ R322A}$-$bla_{CTX-M-14}$ plasmid which express IS*Ecp1* null-activity mutant and the ΔIS*Ecp1*-$bla_{CTX-M-14}$ plasmid which lack IS*Ecp1* coding sequence. *E. coli* experimental strains with the CRISPR/Cas9 effector plasmid and one of $bla_{CTX-M-14}$ plasmids including the IS*Ecp1*$^{Y319A,\ R322A}$-$bla_{CTX-M-14}$ plasmid and the ΔIS*Ecp1*-$bla_{CTX-M-14}$ plasmid, were used for evaluation of transposition activity (Fig. 1). After induction of CRISPR/Cas9 system to remove $bla_{CTX-M-14}$ plasmids, we examined total 154 *E. coli* transformants including 84 and 70 *E. coli* CTX- and SPT-resistant transformants which were randomly selected from experiments using IS*Ecp1*$^{Y319A,\ R322A}$-$bla_{CTX-M-14}$ plasmid and ΔIS*Ecp1*-$bla_{CTX-M-14}$ plasmid, respectively. These 154 *E. coli* transformants selected from control experiment using IS*Ecp1* transposase mutants were also resistant to KAN, a selective marker for the $bla_{CTX-M-14}$ plasmid. However, chromosomally located $bla_{CTX-M-14}$ transposition unit were not confirmed by S1-PFGE and Southern blot hybridization. These results suggested that imperfect removal of the $bla_{CTX-M-14}$ plasmid occurred after CRISPR/Cas9 induction in the 154 *E. coli* transformants and that transposition of $bla_{CTX-M-14}$ from plasmid to chromosome by transposition mediated by IS*Ecp1* transposase mutants were not observed in the both cases. These data suggested that $bla_{CTX-M-14}$ transposition was dependent on activity of IS*Ecp1* transposase in our experimental strain, indicating that our *E. coli* experimental strain was useful for observing the IS*Ecp1*-mediated transposition of the $bla_{CTX-M-14}$ transposition unit.

Next, for the further evaluation of our *E. coli* experimental strain, we preliminarily evaluated 26 CTX-resistant *E. coli* transformants expressing IS*Ecp1* obtained after the first round of screening. Chromosomally located $bla_{CTX-M-14}$ transposition units were detected in case of all these 26 CTX-resistant *E. coli* transformants. Subsequently, we tested the KAN susceptibility of these 26 isolates to determine whether the *E. coli* transformants lost KAN resistance after the elimination of the $bla_{CTX-M-14}$ plasmid. However, some of the *E. coli* transformants showed weak or moderate growth on LB agar supplemented with KAN. Therefore, regardless of the KAN susceptibility, we examined CTX-resistant *E. coli* transformants expressing IS*Ecp1*; these were obtained after five independent screening procedures.

3.2. Frequency of the transfer of the $bla_{CTX-M-14}$ transposition unit from the plasmid to the chromosome via ISEcp1-mediated transposition

A total of 143 CTX-resistant *E. coli* transformants expressing IS*Ecp1*, which were obtained after five screening procedures, were examined to ascertain whether the $bla_{CTX-M-14}$ transposition unit had been transposed from the $bla_{CTX-M-14}$ plasmid to the chromosome. In addition to the Southern blot hybridization analysis and S1 nuclease-PFGE, whether $bla_{CTX-M-14}$ was located on the CRISPR/Cas9 effector plasmid was confirmed by sequencing the upstream region adjacent to the $bla_{CTX-M-14}$ transposition unit. Consequently, as shown in Fig. 1, the presence of chromosomally located $bla_{CTX-M-14}$ was confirmed in 126 isolates (88.1 %), including one isolate (0.8%) that possessed $bla_{CTX-M-14}$ on both the chromosome and the CRISPR/Cas9 effector plasmid. On the other hand, the transposition of the $bla_{CTX-M-14}$ transposition unit to the CRISPR/Cas9 effector plasmid was detected in 15 isolates (10.5%). In the remaining two isolates (1.4%), no transposition of the $bla_{CTX-M-14}$ transposition unit, i.e., transposition neither to the plasmid nor the chromosome, was detected. Consequently, the frequencies of the transfer of $bla_{CTX-M-14}$ from the $bla_{CTX-M-14}$ plasmid to the chromosome were determined through five independent screening procedures (mean = 0.51% and SD = 0.37).

3.3. The genetic structure of chromosomally located $bla_{CTX-M-14}$ and its surrounding genes

The chromosomal locations of $bla_{CTX-M-14}$ in the 126 *E. coli* isolates in which chromosomally located $bla_{CTX-M-14}$ was detected were determined by sequencing the upstream region adjacent to the chromosomally located $bla_{CTX-M-14}$ transposition unit. In most of the 126 *E. coli* isolates (88.9%; 112 isolates), only one chromosomal location was observed. In three and two *E. coli* isolates possessing chromosomally located $bla_{CTX-M-14}$, two and three different chromosomal locations of $bla_{CTX-M-14}$, respectively, were observed. We could not determine the transposition site of chromosomally located $bla_{CTX-M-14}$ in the remaining nine *E. coli* isolates. Finally, 102 different transposition sites were determined on the *E. coli* DH5α chromosome (Fig. 2A and Table S2). The 102 location sites of $bla_{CTX-M-14}$ that were identified were not identical to any previously reported insertion site that was deposited in the GenBank database. Among the 102 $bla_{CTX-M-14}$ sites, 73 were inserted into coding regions (Fig. 2A and Table S2), of which 66 were inserted into coding regions associated with GO terms (Table S2). In addition, as shown in Fig. 2A and B, enrichment of $bla_{CTX-M-14}$ insertion sites was observed particularly in positions from the 3700000th to the 4200000th nucleotide (CP017100).

For further characterization, the genetic structure of the downstream region of the chromosomally located $bla_{CTX-M-14}$ transposition unit, i.e., features such as 5-bp direct repeat (DR) sequences and IRRalts, was determined in 80 of the 102 identified insertion sites (Fig. 3 and Table S2). In the remaining 22 insertion sites, the structure of the downstream region of the chromosomally located $bla_{CTX-M-14}$ transposition unit could not be identified because of technical limitations associated with the sequencing process. Identical IRLs and various IRRalts were observed in these $bla_{CTX-M-14}$ transposition units transposed by IS*Ecp1* (Figs. 3A and S1). The IRRalts observed in the 80 insertion sites of chromosomally located $bla_{CTX-M-14}$ were sporadically found within the sequence present downstream of $bla_{CTX-M-14}$ (Fig. 3B). The AT ratios at each nucleotide position adjacent to the IRLs and IRRalts of the chromosomally located $bla_{CTX-M-14}$ transposition units are shown in Fig. 3C; this figure suggests that the nucleotide sequences of DRs and its adjacent sequences tended to be AT rich.

4. Discussion

We evaluated whether our *E. coli* experimental strain was suitable for the characterization of the IS*Ecp1*-mediated transposition of the $bla_{CTX-M-14}$ transposition unit from a plasmid to a chromosome. Transposition of $bla_{CTX-M-14}$ from a plasmid to a chromosome was not observed to be mediated by IS*Ecp1* transposase mutants. On the other hand, the transposition of $bla_{CTX-M-14}$ was observed in 141 of the 143 (98.6%) CTX-resistant *E. coli* transformants expressing IS*Ecp1*. These data clearly indicated that our experimental *E. coli* strain was suitable for the characterization of the IS*Ecp1*-mediated transposition of the $bla_{CTX-M-14}$ transposition unit from a plasmid to a chromosome. Experiments using IS*Ecp1* transposase mutants showed that after treatment with the CRISPR/Cas9 system, all *E. coli* transformants showed KAN resistance, a selective marker for the $bla_{CTX-M-14}$ plasmid; this suggested that sensitivity to KAN may not be efficient enough as an index for chromosomally located $bla_{CTX-M-14}$ transposition units in our

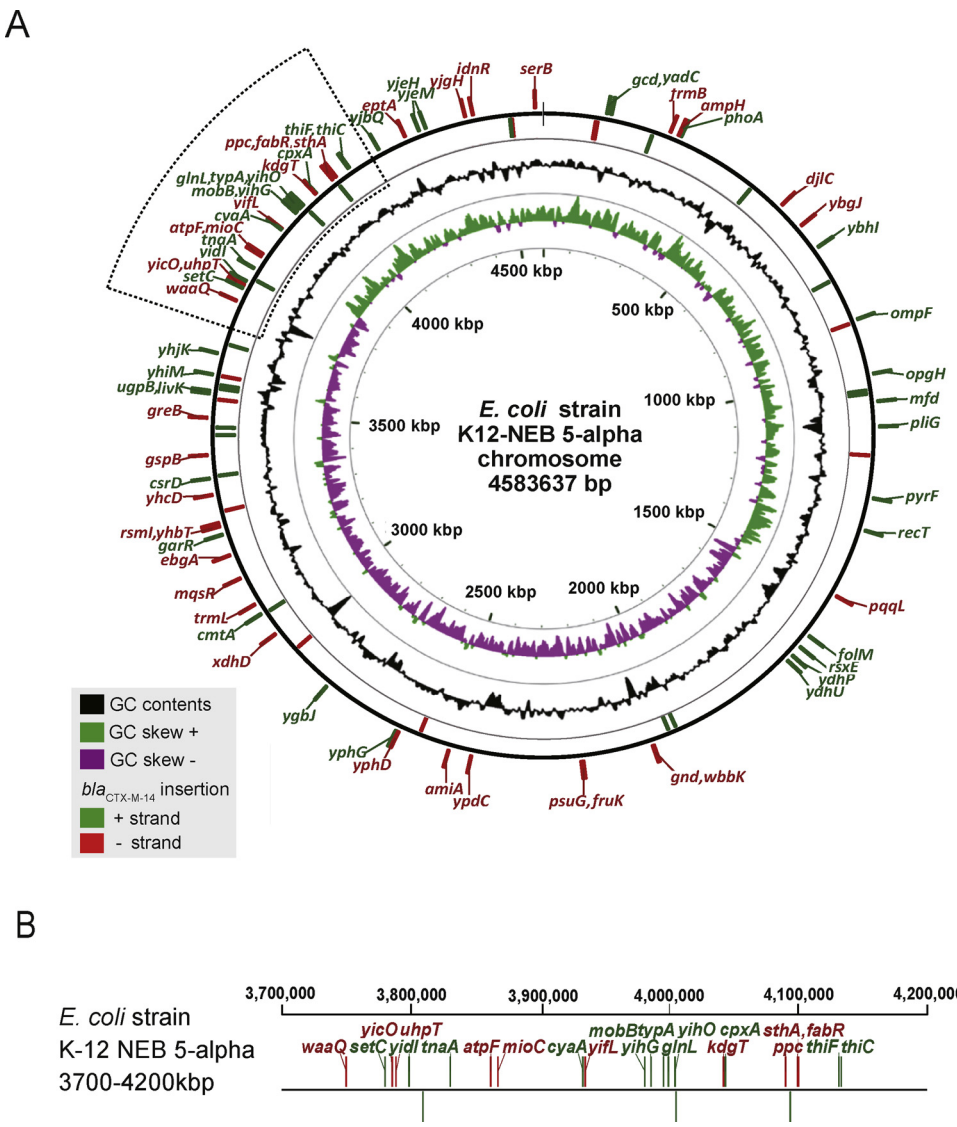

Fig. 2. Graphical map of $bla_{CTX\text{-}M\text{-}14}$ insertion sites plotted on the circular whole genome of the *E. coli* NEB 5-alpha laboratory strain. A The insertion sites of chromosomally located $bla_{CTX\text{-}M\text{-}14}$ transposition units determined in this study are visualized by plotting these sites on a circular whole-genome map of the *E. coli* NEB 5-alpha laboratory strain (CP017100) using the CGView Server. Insertion sites of the $bla_{CTX\text{-}M\text{-}14}$ transposition units on each positive strand and negative strand are shown by green and red lines, respectively. Insertions on each coding region and non-coding region are indicated in the outer and inner circle, respectively. B. The insertion sites of the $bla_{CTX\text{-}M\text{-}14}$ transposition units on the chromosomal region engaged with broken line are shown. Insertions on each coding region and non-coding region are indicated in above and below the lines, respectively (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

E. coli experimental strain because CRISPR/Cas9 activity might not perfectly remove the $bla_{CTX\text{-}M\text{-}14}$ plasmid. In addition, during our preliminary consideration, we could not exclude a possibility that KAN selection cassette might be transferred as a component of $bla_{CTX\text{-}M\text{-}14}$ transposition. Therefore, we employed ligation-mediated PCR to characterize downstream region of $bla_{CTX\text{-}M\text{-}14}$ transposition unit transferred from plasmid to chromosome by IS*Ecp1* mediated transposition. However, KAN selection cassette was not found as a component of $bla_{CTX\text{-}M\text{-}14}$ transposition unit (Fig. 3B). Therefore, in our study, we evaluated the location of the $bla_{CTX\text{-}M\text{-}14}$ transposition units by S1-PFGE, Southern blotting, and sequencing analysis.

It is known that IS*Ecp1* mobilizes ESBL genes by recognizing IRL and IRRalts (Lartigue et al., 2006; Partridge, 2011). Consistently, we observed 31 kinds of IRRalts (Fig. S1). One IRRalt (5′-GCGTTGATTCC TGG-3′), which was found in chromosomally located $bla_{CTX\text{-}M\text{-}14}$ transposition units and had a nucleotide length of 4798 bp, was the most frequently observed IRRalt (9 different chromosomal locations; Figs. 3B and S1). This IRRalt (5′-GCGTTGATTCCTGG-3′) has also been found in case of IS*Ecp1B*-mediated transposition events, as demonstrated in a previous study (Poirel et al., 2005). The GenBank searches indicated that this IRRalt (5′-GCGTTGATTCCTGG-3′) was found in the chromosomes of the *E. coli* strain 2452 (CP031833; nucleotide positions 4238501–4243298) and *Klebsiella pneumoniae* strain SCKP020046 (CP028783; nucleotide positions 4706164–4710961). In addition, the third most used IRR (5′-ATGTGGAACCTCGA-3′; 6 different chromosomal locations; Figs. 3B and S1) was found in the chromosomes of the *E. coli* Ecol_224 strain (CP018948; nucleotide positions 2041644–2045461) and *Proteus mirabilis* strain GN2 (CP026581; nucleotide positions 1191886–1195703). These data suggested that IS*Ecp1*'s ambiguous recognition of IRR sequences results in a variety of transpositions of the $bla_{CTX\text{-}M\text{-}14}$ transposition unit, even though there might be a preference with regards to nucleotide sequences during IRR recognition.

In this study, the frequency of the transfer of $bla_{CTX\text{-}M\text{-}14}$ from a plasmid to a chromosome via IS*Ecp1*-mediated transposition was determined. Compared with the frequency of the IS*Ecp1*-mediated transposition of $bla_{CTX\text{-}M\text{-}2}$ from the chromosome of *K. ascorbata* to a transferable plasmid (determined to be $(6.4 \pm 0.5) \times 10^{-7}$) (Lartigue et al., 2006), the frequency of the transfer of $bla_{CTX\text{-}M\text{-}14}$ by IS*Ecp1*-mediated transposition observed in this study was remarkably high (mean = 0.51%, SD = 0.37). In addition, the enrichment of $bla_{CTX\text{-}M\text{-}14}$ insertion sites was observed especially in case of positions between the 3700000th and 4200000th nucleotide, as shown in Fig. 2A and B. In a previous report, a high-density mini-Tn*5* transposon insertion library has been used to identify the essential genes of the *E. coli* K-12 strain BW25113 (**CP009273**) (Goodall et al., 2018). We compared the whole-genome sequences of the *E. coli* NEB 5-alpha laboratory strain (**CP017100**) and *E. coli* K-12 strain BW25113 (**CP009273**) using

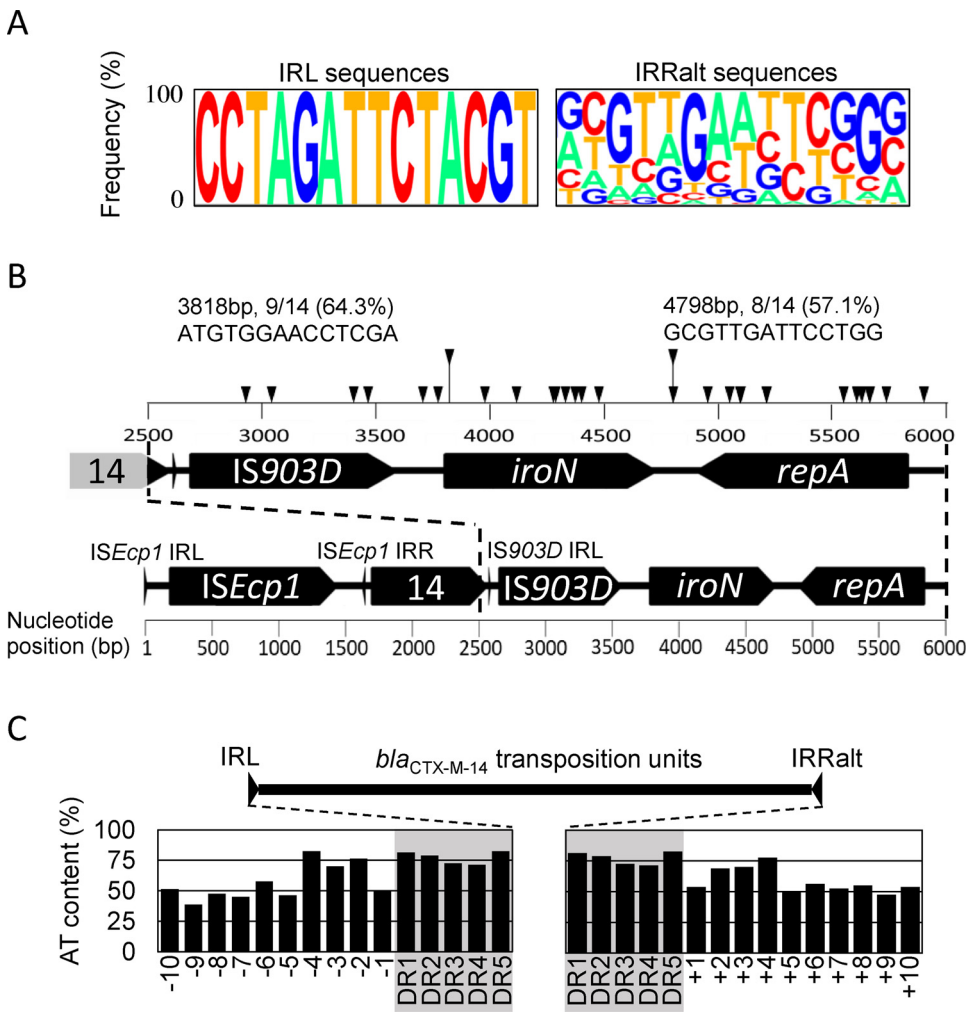

Fig. 3. The genetic structures of the IRL and IRRalt of the $bla_{CTX\text{-}M\text{-}14}$ transposition units and their surrounding sequences. A Pictogram of 14-bp nucleotide sequences of the IRL and IRRalt are depicted by using the Pictogram web tool (http://genes.mit.edu/pictogram.html). B. The locations of the IRRalts observed in 80 insertion sites of chromosomally located $bla_{CTX\text{-}M\text{-}14}$ are shown. The locations of 31 types of confirmed IRRalts are indicated by inverted triangles. Two IRRalts observed in the $bla_{CTX\text{-}M\text{-}14}$ transposition units, with nucleotide lengths of 3818 bp and 4798 bp, are shown, along with their similarity to IS*Ecp1*-specific IRRs. C. AT contents (%) at each position located 15 nucleotides upstream and downstream of the IRR and IRRalt, including direct repeat (DR) sequences, are shown. IRL, Left terminal inverted repeat sequence; IRR, Right terminal inverted repeat sequence; IRRalt, alternative IRR.

MAUVE (Darling et al., 2010). As a result, the chromosomal regions, i.e., the regions between the 3700000th and 4200000th nucleotides, for which enrichment of the $bla_{CTX\text{-}M\text{-}14}$ transposition unit was observed in this study corresponded to the regions showing frequent Tn5 insertions in the *E. coli* K-12 strain BW25113 (data not shown). This indicated that IS*Ecp1*-mediated $bla_{CTX\text{-}M\text{-}14}$ transposition occurred randomly, and consequently, transformants possessing the $bla_{CTX\text{-}M\text{-}14}$ transposition unit in non-essential genes were selected, as mentioned in a previous studies (Partridge, 2011; Poirel et al., 2005).

Other than $bla_{CTX\text{-}M}$, several IS*Ecp1*-associated antimicrobial resistance (AMR) genes such as the carbapenemase gene $bla_{OXA\text{-}181}$ (Sonnevend et al., 2017; Zowawi et al., 2015) and AmpC-type β-lactamases such as $bla_{CMY\text{-}2}$ and $bla_{CMY\text{-}42}$ (Fang et al., 2018; Feng et al., 2015) have been reported. Considering the high frequency of the transfer of $bla_{CTX\text{-}M\text{-}14}$ via IS*Ecp1*-mediated transposition observed in this study, mobile genetic elements of IS*Ecp1*-associated AMR genes could possibly be spread rapidly via the capture of distal genes such as insertion sequences and AMR genes.

For formulating effective countermeasures against bacteria showing AMR, how bacterial strains possessing AMR genes are distributed among humans, animals, and the environment is one of the fundamental questions that should be addressed. Probably, the molecular analysis of IS*Ecp1*-associated AMR genes and their surrounding sequences may be helpful in tracing the distribution of bacteria showing AMR that possess chromosomally located AMR genes.

5. Conclusion

In this study, we determined the frequency of the transfer of $bla_{CTX\text{-}M\text{-}14}$ from a plasmid to a chromosome by IS*Ecp1*-mediated transposition (mean = 0.51%, SD = 0.37). Meanwhile, IS*Ecp1*'s ambiguous recognition of IRRalts, in which there may be a preference for nucleotide sequences similar to the IRR unique to IS*Ecp1*, was related to the transposition event of $bla_{CTX\text{-}M\text{-}14}$. Considering the high detection rates of bacteria from the family Enterobacteriaceae that possess chromosomally located $bla_{CTX\text{-}Ms}$ transferred by IS*Ecp1*-mediated transposition (Coelho et al., 2010; Hamamoto and Hirai, 2018; Hamamoto et al., 2016; Harada et al., 2012; Hirai et al., 2013), these results suggest that the molecular nature of IS*Ecp1* observed in this study could plausibly be one of the factors contributing to the high detection rates of *E. coli* possessing chromosomally located $bla_{CTX\text{-}M\text{-}14}$ in both clinical and community settings.

Declaration of Competing Interest

Not applicable.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2020.151395.

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