

Mode of Transposition and Expression of 16S rRNA Methyltransferase Gene *rmtC* Accompanied by *ISEcp1*

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A newly identified 16S rRNA methyltransferase gene, *rmtC*, was accompanied by an *ISEcp1* element at its 5' end. This *ISEcp1* element, which contained a transposase gene, *tnpA*, provided a promoter activity for expression of the adjacent *rmtC*; and this structure enabled the *rmtC* gene to be transposed onto another plasmid in *Escherichia coli*.

Four types of plasmid-mediated 16S rRNA methyltransferase genes, *rmtA*, *rmtB*, *rmtC*, and *armA*, which confer high levels of resistance to various aminoglycosides, have been found worldwide among a number of pathogenic gram-negative rods (3, 4, 8, 14, 17–19). The distribution of these plasmid-mediated 16S rRNA methyltransferase genes among pathogenic bacteria seems attributable to the fact that these genes are associated with some bacterium-specific DNA recombination systems, such as a transposon (3, 5, 7, 16). In fact, it was recently reported that transposition of *armA* was mediated by a composite transposon, Tn1548 (5). However, little is known about the transposition system of the other three plasmid-mediated 16S rRNA methyltransferase genes, *rmtA*, *rmtB*, and *rmtC*. Therefore, in the present study we characterized in detail the transposition system of *rmtC*, which was located on a plasmid (pARS68) found in a clinical *Proteus mirabilis* strain, ARS68 (14).

A *SacI*-digested 11-kb fragment carrying *rmtC* was cloned from pARS68, and both strands were entirely sequenced. By using the bacterial genetic code, ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) was used to search for open reading frames (ORFs). A schematic representation of the cloned fragment is shown in Fig. 1A. *ISEcp1*, which contained a transposase gene, *tnpA*, was located just upstream of *rmtC*. Although there were several ORFs (*orf1* to *orf8*) around *ISEcp1* and *rmtC*, their functions remained unknown, even though their sequences were compared with the sequences in the public databases of GenBank and EMBL by using the BLAST and FASTA search tools.

It is well known that *ISEcp1* is often located at the 5' ends of several β -lactamase genes, such as *bla*_{CTX-M} and *bla*_{CMY} (1, 2, 6, 9, 11, 12, 15), and enables these genes to be transposed to other DNA target sites (2, 10, 13). Moreover, *ISEcp1* provides promoter activity for expression of a downstream CTX-M-type β -lactamase gene (2, 12). These findings strongly suggested

that the transposition and expression of *rmtC*, as seen in pARS68, were also regulated by *ISEcp1*. *ISEcp1* was bracketed with two imperfect 14-bp inverted repeat (IR) sequences (the left IR [IRL] and the right IR [IRR]) (Fig. 1A). A putative 5-bp target site (TTCAA) was located in the immediate vicinity of IRL (Fig. 1A). This 5-bp target site might be duplicated, most likely after the insertion of a DNA fragment mediated by a transposon, and is subsequently located on both faces of a transposed DNA fragment. The presence of duplicated 5-bp target sites can be a trace that the insertion of a DNA fragment by transposon occurred at that position. Considering the flanking genetic organizations of *rmtC*, it is speculated that a DNA fragment containing *ISEcp1* and *rmtC* bracketed on one side by IRL and on the other by a putative second IRR, IRR', constituting a potential transposon on pARS68. To determine if *rmtC* could transpose with *ISEcp1* and to determine the structural limits of the transposable unit, we tried to identify a potential transposon carrying *ISEcp1* and *rmtC* bracketed with IRL and putative IRR' by an in vitro transposition experiment. For this purpose, the transposition of *rmtC* carried by several donor recombinant plasmids based on two different backbones (Fig. 1A) to the recipient plasmid R388 (TMP^r) was investigated with a standard mating assay. *Escherichia coli* DH5 α harboring R388 together with various recombinant plasmids and *E. coli* HB101 (STR^r) were used as the donor and recipient strains, respectively. Transconjugants were selected on Luria-Bertani agar plates supplemented with gentamicin (10 μ g/ml), trimethoprim (50 μ g/ml), and streptomycin (100 μ g/ml). Transconjugants were obtained when *E. coli* DH5 α carrying recombinant plasmids (pBCS68, pBCHS68, pMCLS68, and pMCLHS68) (Fig. 1A) was used as a donor strain at a frequency of ca. 10^{-7} to 10^{-6} per recipient. On the other hand, transconjugants could not be obtained (frequency, $< 10^{-9}$ per recipient) when *E. coli* DH5 α carrying plasmids (pBCES68 and pMCLES68) lacking a part of *ISEcp1* was used (FIG. 1A). These findings strongly suggested that *ISEcp1* plays an essential role in the transposition of *rmtC*.

Twenty recombinant plasmids carrying *rmtC* with the backbone of plasmid R388 obtained when pBCS68 was used as a donor plasmid were extracted from the transconjugants. The sequences of both terminal ends of the transposed fragments

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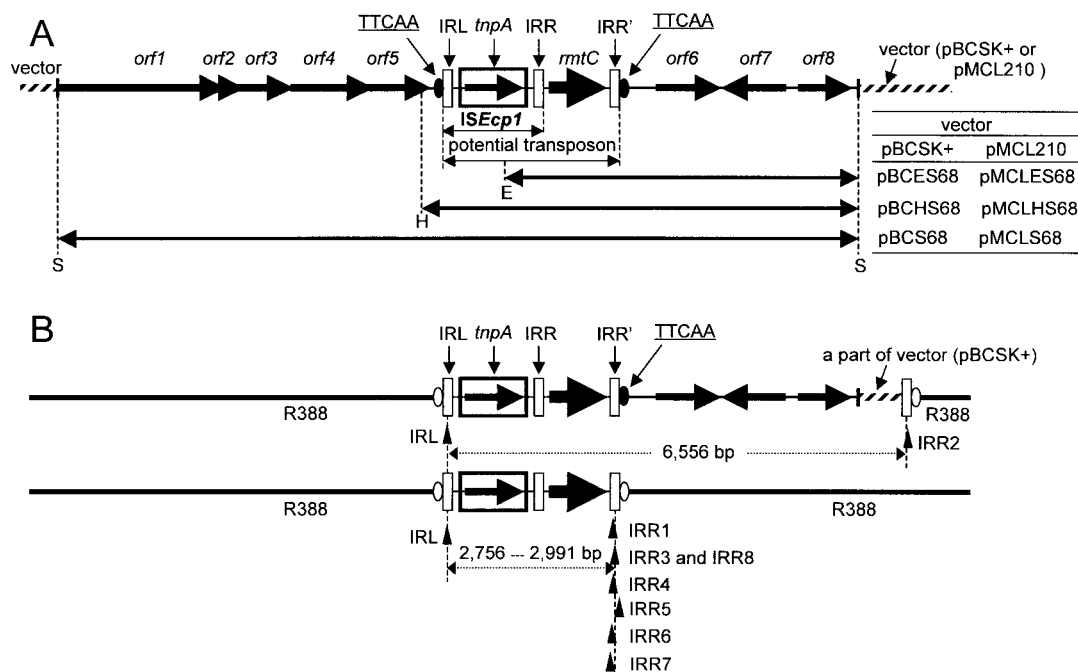


FIG. 1. (A) Schematic organization of genes in the 11-kb *SacI* fragment of pBCS68. ORFs and genes are shown as arrows indicating transcription orientation. The IRL, IRR, and IRR' motifs are indicated by open rectangles. Possible 5-bp target sites (TTCAA) are shown as a closed ellipse. DNA fragments carried by recombinant plasmids used in the transposition experiments are indicated by double-headed arrows with the corresponding recombinant plasmid names. Fragments on pBCHS68 and pMCLHS68 were generated by ligating the fragment on pBCES68 or pMCLES68, and the PCR fragments were amplified by using primers supplemented with restriction sites (*HindIII* or *EcoRI*). Restriction sites: S, *SacI*; H, *HindIII*; E, *EcoRI*. (B) Transposition of the *ISEcp1-rmtC* element onto R388 from pBCS68. The positions of the IRRs (IRR1 to IRR8) were confirmed by transposition experiments with the *ISEcp1-rmtC* element in *E. coli* cells by using R388 as the recipient plasmid. Positions of the 5-bp duplicated target sites are shown as open ellipses. Upward-pointing arrowheads indicate the positions of the IRL and the IRR' extremities of each probable transposed fragment (horizontal broken lines). This result reveals that the IRR' elements of *ISEcp1* are not so rigorous but are comparatively multifarious or flexible and that the 5-bp target sites or recombination junctions at the IRR' side are also diverse.

were determined in detail by direct sequencing of these recombinant plasmids with customized primers. As a result, eight types of transposed fragments, which were structurally different from each other, were obtained. All transposed fragments analyzed contained both *ISEcp1* and *rmtC*, and each left end (IRL) of those fragments was perfectly identical (Fig. 1B). However, the right end of each fragment (IRR1 to IRR8) varied (Fig. 1B and Table 1). IRR was within 459 bases of the

end of *rmtC* in seven of eight cases, although only IRR2 belonged to the cloning vector region (Fig. 1B). The locations of IRR3 and IRR8 were adjacent to the typical 5-bp nucleotide sequence, TTCAA, which seemed to be an innate target site on pARS68. Therefore, it is probable that the 2,973-bp fragment bracketed on the left side by IRL and on the right end by IRR3 or IRR8 constituted a potential transposon on plasmid pARS68.

TABLE 1. Characteristics of inverted repeats and target sites for *ISEcp1*-mediated transposition

Description of sequence	Nucleotide sequence of IRL and IRRs (14 bp) ^a	5-bp duplicated target site sequence	Size of transposed fragment (bp)
IRL of <i>ISEcp1</i>	5'-CCTAGATTCTACGT-3'	TTCAA	
Expected perfect IRR of <i>ISEcp1</i> (complementary sequence of IRL)	5'-ACGTAGAATCTAGG-3'		
IRR of <i>ISEcp1</i>	5'-ACGTGGAATTTAGG-3'		
IRR'	5'-CCTAGGAACTCGGC-3'	TTCAA	2,973
IRR1	5'-GCCTGGGATTTCGA-3'	TTCTT	2,943
IRR2	5'-GAACAGTATTTGGT-3'	TATGT	6,556
IRR3	5'-CCTAGGAACTCGGC-3'	ACGCA	2,973
IRR4	5'-TCCTAGGAACTCGG-3'	GCCAA	2,972
IRR5	5'-ATATGGTGTTCCT-3'	ATGAA	2,991
IRR6	5'-AATCTTTTTTCGGA-3'	AATTT	2,774
IRR7	5'-ACGCCGTAACCTCGG-3'	GTGAC	2,756
IRR8	5'-CCTAGGAACTCGGC-3'	GAAAT	2,973

^a Underlining of IRRs indicates nucleotide residues identical to the corresponding complementary nucleotide residues of the IRL sequence. IRR', IRR3, and IRR8 showed identical nucleotide sequences.

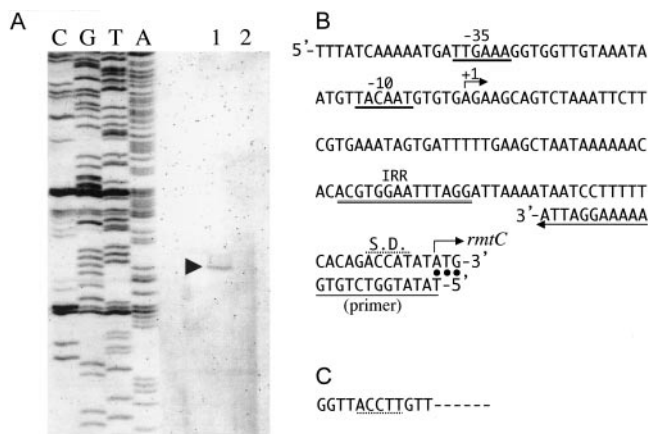


FIG. 2. Identification of transcriptional start site for *rmtC* in *E. coli* DH5 α (pBC-KB1) by primer extension analysis (14). (A) Lanes C, G, T, and A, results of sequencing reactions performed with DNA prepared by PCR as a template and the primer; lane 1, primer elongation product obtained with total RNA of *E. coli* DH5 α (pBC-KB1); lane 2, primer elongation product obtained with total RNA of *E. coli* DH5 α (pBCSK+). (B) Promoter region of *rmtC*. -35 and -10 promoter sequences upstream of transcriptional start site are underlined with a thin line; dots indicate the ATG start codon of *rmtC*; a probable Shine-Dalgarno (S.D.) sequence is marked with a dotted line; the IRR of *ISEcp1* is underlined with double thin lines; the primer used in primer extension analysis shown with an arrow at bottom. (C) Complementary sequence of the 3' terminus of the 16S rRNA of *Proteus mirabilis*. The portion of the nucleotide residues that coincide with the upstream region of ATG start codon of *rmtC* is marked with a dashed line. The sequence was referred to the EMBL/GenBank database and can be found under accession no. AJ301682.

The numbers of base pairs in IRR, which is identical to those in IRL, ranged from three to nine (Table 1). The 3' ends of the IRRs identified varied (GA, GT, GC, GG, or CT), although it was reported that *ISEcp1B* needs a guanosine (G) residue at the 3' ends of the IRRs when it transposed the adjacent genes *bla*_{KLU-A} and *bla*_{CTX-M-19} (10, 13). In any event, it was commonly observed that *ISEcp1* and *ISEcp1B* were able to transpose adjacent antibiotic resistance genes by using IRRs composed of a wide variety of nucleotide sequences.

Primer extension analysis of RNA from RmtC-producing *E. coli* transformant, *E. coli* DH5 α (pBC-KB1) (14), revealed the start residue of the mRNA transcription of *rmtC* (Fig. 2A and 2B). Transcription was initiated at an A (adenine) residue, located 99 nucleotides upstream of an AUG translation initiation codon of *rmtC*. This position was located within *ISEcp1* near its IRR. Although diversity in the start residue of transcription was observed among *ISEcp1*-bearing antimicrobial resistance genes, including *rmtC*, *bla*_{CTX-M}, and *bla*_{CMY}, *ISEcp1* commonly provides promoter sequences within the right-end region near its IRR for expression of downstream antibiotic resistance genes (2, 6, 12).

Recently, it was experimentally confirmed that *ISEcp1B* could transpose upstream of chromosomally located *bla*_{KLU-A} of *Kluyvera ascorbata*, which is thought to be a progenitor of CTX-M type β -lactamases, and, consequently, could also transpose *bla*_{KLU-A} to other target sites in *E. coli* (10). This hybrid structure of *ISEcp1B* and *bla*_{KLU-A} seems to be the origin of that of *ISEcp1* and *bla*_{CTX-M}, which is widely distrib-

uted among members of the family *Enterobacteriaceae* worldwide. Although the overall schemes for the development of the hybrid structure of *ISEcp1* and *rmtC* have not been elucidated, it is probable that *ISEcp1* first transposed into the 5' end of chromosome-carrying *rmtC* in unknown aminoglycoside-producing bacteria and that subsequently the *ISEcp1-rmtC* element transposed to other DNA target sites on a residential plasmid of the *Enterobacteriaceae*. To understand the development of the hybrid structure of *ISEcp1* and *rmtC*, it would be necessary to identify natural reservoirs of *rmtC*. In conclusion, we report here that *ISEcp1* plays an essential role in the transposition and expression of a 16S rRNA methyltransferase gene, *rmtC*.

Nucleotide sequence accession number. The nucleotide sequence of the 11-kbp *SacI* fragment shown in Fig. 1A was submitted to the EMBL/GenBank database through the DNA Data Bank of Japan (DDBJ) and can be found under accession no. AB194779.

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