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Characterization of Site-Specific Recombination by the Integrase MJ1 from Enterococcal Bacteriophage ΦFC1

PARK, MI-OK[1], KI-HONG LIM[1], TAE-HYUNG KIM[2], AND HYO-IHL CHANG[1]*

[1]*School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea*
[2]*Department of Biochemistry, Chosun University School of Medicine, Gwangju, Korea*

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Abstract Bacteriophage ΦFC1 integrase (MJ1) was previously shown to perform a site-specific recombination between a phage attachment site (*attP*) and a host attachment site (*attB*) in its host, *Enterococcus faecalis*, and also in a non-host bacterium, *Escherichia coli*. Here, we investigated biochemical features of MJ1 integrase. First, MJ1 integrase could perform *in vitro* recombination between *attP* and *attB* in the absence of additional factors. Second, MJ1 integrase interacted with *att* sites. Electrophoretic mobility shift assays and DNase I footprinting revealed that MJ1 integrase could efficiently bind to all the *att* sites and that MJ1 integrase recognized relatively short sequences (~50 bp) containing an overlapping region within *attB* and *attP*. These results demonstrate that MJ1 integrase indeed catalyzes an integrative recombination between *attP* and *attB*, the mechanism of which might be simple and unidirectional, as found in serine integrases.

Key words: *attB*, *attP*, *Enterococcus faecalis*, ΦFC1, integrase

The genome of bacteriophages, in the lysogenic state, is inserted into the host chromosome through integration, a process of site-specific recombination driven by a phage-encoded protein, so-called integrase. Integrases are categorized into two families, tyrosine and serine integrases, according to catalytic amino acid residues used for breakage of DNA [reviewed in 8]. The site-specific integration is mediated by phage integrases between two defined sites, an attachment site in bacteriophage DNA (*attP*) and an attachment site in host genome (*attB*), resulting in two new hybrid sites, *attL* and *attR*. The *att* sites usually comprise a core sequence, where crossing-over occurs, and flanking regions that integrases or accessory factors recognize and bind to [reviewed in 12]. The core sequence is short (ranging from 2 bp to >10 bp) and identical among all the *att* sites in the same phage system [9, 18]. In a family of tyrosine integrases, flanking regions of *attP* are composed of multiple regulatory elements; however, those of *attP* of serine integrases are shorter and simpler [4, 20, 21]. For recombination reactions, integrases of the tyrosine family require host factors, but serine integrases act autonomously [20, 21]. The reverse reaction of integration, so-called excision, is an event of phage DNA escaping from host chromosome, which is also mediated by integrases, but requires another phage-encoded protein, excisionase [4, 15].

ΦFC1 temperate bacteriophage was isolated from *Enterococcus faecalis* by ultraviolet irradiation [11]. The integration of ΦFC1 into the host chromosome is an event of site-specific recombination, which is mediated by a ΦFC1-encoded integrase, MJ1 [22]. MJ1 is an integrase of 464 amino acid residues, contains a resolvase catalytic domain in its N-terminus, and is homologous with previously characterized serine integrases (Fig. 1). MJ1 has significant homology with the serine integrases such as U153 from *Listeria monocytogenes* (52.2% identity over the whole amino acid sequence and 74.8% over N-terminal 150 amino acid sequences) [13], TP901-1 from *Lactococcus lactis* (44.3% identity over the whole amino acid sequence, 67.8% over N-terminal 150 amino acid sequences) [7], R4 from *Streptomyces parvulus* (32% identity over the whole amino acid sequence, 39.2% over N-terminal 150 amino acid sequences) [16], and ΦRv1 from *Mycobacterium tuberculosis* (17% identity over the whole amino acid sequence, 24.2% over N-terminal 150 amino acid sequences) [2]. Site-specific integration, which is now considered as a promising tool for gene therapy by virtue of its specificity, has been intensively studied on tyrosine integrases such as λ integrase, but the molecular mechanisms on integrations by serine integrases are still scarce. In this study, we investigated MJ1-mediated recombination *in vitro* and molecular interactions with *att* sites.

*Corresponding author
Phone: 82-2-3290-3421; Fax: 82-2-923-9923;
E-mail: hichang@korea.ac.kr

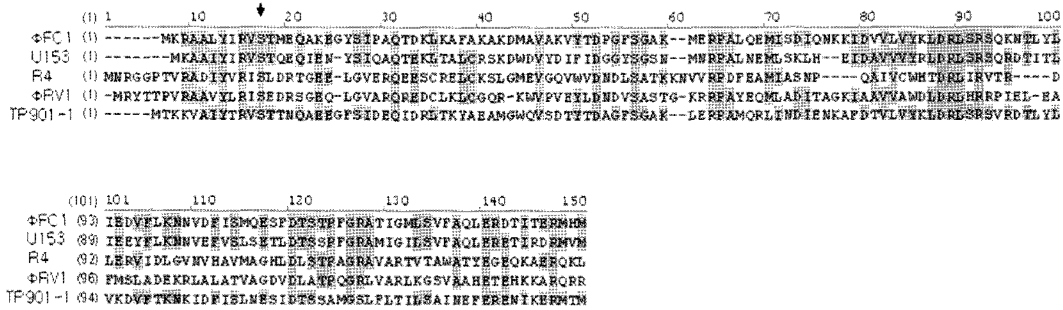

Fig. 1. Alignment of N-terminal 150 amino acid sequences of several serine integrases.
ΦFC1 from *Enterococcus faecalis*, U153 from *Listeria monocytogenes*, R4 from *Streptomyces parvulus*, ΦRv1 from *Mycobacterium tuberculosis*, and TP901-1 from *Lactococcus lactis* were aligned. Amino acid residues identical among at least three integrases are shown against a dark background. The presumptive catalytic serine residue is indicated by an arrow head.

MATERIALS AND METHODS

Plasmid Constructions

To construct N-terminally thioredoxin (Trx)- and hexahistidine (His)-tagged MJ1-expression vector (pET32a-MJ1), an MJ1-encoding DNA fragment was PCR-amplified from ΦFC1 DNA with primers, (mj1 forward) 5'-CGGGATCCATGA-AACGTGCAGCATTG-3' and (mj1 reverse) 5'-CGGGA-ATTCACCGAATGCATGTTCGTA-3', and inserted between the BamHI and EcoRI sites of pET32a(+) (Novagen, Madison, WI, U.S.A.). To construct pATTP, pATTB, pATTL, and pATTR for *in vitro* recombination assays, which carry *attP*, *attB*, *attL*, and *attR*, respectively, *attP*-, *attB*-, *attL*-, and *attR*-containing DNA fragments were PCR-amplified using primer sets as follows: phy2 and phy3 from ΦFC1 DNA for *attP* (738 bp), ON-1 and ON-2 from *E. faecalis* 707 (a non-lysogenic strain) chromosome for *attB* (290 bp), ON-1 and phy3 from *E. faecalis* 703 chromosome (a lysogenic strain) for *attL* (404 bp), and phy2 and ON-2 from *E. faecalis* 703 chromosome for *attR* (624 bp), which were inserted into pT7Blue T vector (Novagen). The primer sequences have been previously described [22].

Recombinant MJ1 Integrase from *Escherichia coli*

Trx-MJ1 or Trx were expressed in *E. coli* BL21(DE3), purified using nickel-chelating resin, and monitored by SDS-PAGE and Coomassie blue staining.

In Vitro Recombination Assays

Supercoiled or linearized substrate DNAs (1 μg) were incubated with the indicated amounts of Trx-MJ1 or Trx in 50 μl of buffer M (30 mM Tris-Cl, pH 7.6, 15 mM NaCl, 80 mM KCl, 0.7 mM EDTA, 4 mM spermidine, 7% glycerol) for 1 h at 37°C, followed by phenol extraction and ethanol precipitation. The resultant DNAs were digested with HindIII or EcoRI and electrophoresed on 1–2% agarose gel.

Electrophoretic Mobility Shift Assay (EMSA)

AttP (284 bp using mj1 reverse and phy-3 primers from ΦFC1), *attB* (290 bp in pATTB), *attL* (404 bp in pATTL) and *attR* (172 bp using mj1 reverse and ON-2 primers from *E. faecalis* 703 chromosome) were produced using PCR with ^{32}P-labeled primers. The *att* fragments were incubated with the indicated amounts of Trx-MJ1 or Trx in 50 μl of buffer E (30 mM Tris-Cl, pH 7.6, 15 mM NaCl, 80 mM KCl, 0.1 mM EDTA, 0.3 mg/ml calf thymus DNA, 0.3 mg/ml bovine serum albumin, 5% glycerol) for 20 min at room temperature. For competition assays, 20-fold molar excess of unlabeled *att* fragments were included in the reaction mixtures. At the end of the incubation, the reaction mixtures were electrophoresed on 5% polyacrylamide gel in 0.5× TBE, followed by autoradiography.

DNase I Footprinting

The *attB* and *attP* fragments used in EMSA (~50,000 cpm) were incubated with the indicated amounts of Trx-MJ1 or Trx in 50 μl of buffer E supplemented with 5 mM $MgCl_2$ and 1 mM $CaCl_2$ for 15 min at room temperature, followed by incubation with 2 μg of DNase I for 1 min at room temperature. The reactions were discontinued by the addition of 75 μl of DNase I stop solution (20 mM EDTA, pH 8.0, 1% SDS, 200 mM NaCl, 125 μg/ml yeast tRNA), followed by phenol extraction and ethanol precipitation. The products were electrophoresed on 7 M urea-containing 8% polyacrylamide gel, followed by autoradiography. The protected areas were determined by the positions of G bases by Maxam-Gilbert chemical sequencing.

RESULTS AND DISCUSSION

In Vitro Recombination Between *attP* and *attB* by MJ1 Integrase

MJ1 integrase of ΦFC1 bacteriophage from *E. faecalis* performs a site-specific integration between the *attP* and *attB* site in *E. faecalis* and a non-host, *E. coli* [22]. To further confirm that MJ1 integrase indeed mediates the site-specific recombination between *attP* and *attB* in a defined *in vitro* condition, we performed *in vitro* recombination

Fig. 2. *In vitro* recombination between *attP* and *attB* by MJ1 integrase.

A. Trx-fused MJ1 (Trx-MJ1) and Trx were produced from *Escherichia coli* and analyzed by 12% SDS-PAGE and Coomassie blue staining. **B.** One μg of supercoiled plasmids, pATTP containing *attP* and pATTB containing *attB*, were incubated with Trx (lane 2) or with increasing amounts of Trx-MJ1 (0.05, 0.1, 0.25, 0.5, 1 μg in lanes 3–7). The products were linearized by HindIII and electrophoresed on 1.2% agarose gel. An arrowhead indicates *attR*-containing DNA resulting from recombination (upper). Schematic diagram of pATTP, pATTB and a chimeric plasmid (lower). **C.** Trx-MJ1 (0.5 μg) was incubated with different combinations of supercoiled (S) or linearized (L) pATTB and pATTP and analyzed as in **B**. Arrowheads indicate resulting chimeric plasmids. **D.** *In vitro* recombination was performed with 0.5 μg of Trx-MJ1 and 1 μg of linearized pATTP and pATTB in the presence of 1 mM ATP or 1 mM $MgCl_2$.

assays using purified integrase. Increasing amounts of thioredoxin-fused MJ1 (Trx-MJ1), which was produced from *E. coli* (Fig. 2A), were incubated with two supercoiled plasmids, pATTP and pATTB, containing *attP* or *attB*, respectively. The formation of a larger recombinated plasmid containing *attL* and *attR* was expected if *in vitro* recombination by Trx-MJ1 occurred (Fig. 2B, lower). Thus, the resultant plasmids from recombination reactions were analyzed using agarose gel electrophoresis after linearization by HindIII. As shown in Fig. 2B, two new plasmid bands of 6.8 kbp and 0.68 kbp, supposed to contain *attR* and *attL*, respectively, were observed and their formations were enhanced as the amount of Trx-MJ1 was increased (the DNA band of 0.68 kbp was hardly detectable on a gel because of its small size). The identical result was also obtained when linearized using EcoRI, and the presence of *attL* and *attR* were confirmed by PCR (data not shown). MJ1 could also carry out recombinations between substrate DNAs of different conformations such as between supercoiled and linearized DNAs or between linearized and linearized DNAs (Fig. 2C). However, ATP or $MgCl_2$ did not affect MJ1-mediated recombination reactions (Fig. 2D). Excisive

recombination between *attL* and *attR* is also known to be mediated by integrases with the help of additional proteins, *e.g.*, phage-encoded excisionase [4, 15]. Thus, we examined if MJ1 could catalyze a recombination between *attL* and *attR* like *attP* and *attB*. However, any new DNA fragments by an excision event was not produced, implying that MJ1 alone was not capable of carrying out the excisive recombination in a high efficiency. These results indicate that MJ1 is able to catalyze the integrative recombination between *attP* and *attB*, irrespective of the conformations of substrate DNA fragments, in the absence of additional factors.

Binding of MJ1 Integrase on *att* Sites

To know if MJ1 indeed bound to *att* sites, we performed electrophoretic mobility shift assays. The *attP* (284 bp) and *attB* (290 bp) were radiolabeled and incubated with increasing amounts of Trx-MJ1 or Trx. As shown in Fig. 3, MJ1 incubation with *attP* or *attB* produced shifted bands whereas Trx did not. The formation of *att*-MJ1 complexes was elevated as the amounts of MJ1 increased and inhibited by unlabeled *att* fragments, indicating that MJ1 specifically bound to *attP* and *attB*. Additionally, we found that MJ1 also bound to *attL* and *attR*, suggestive of a possible involvement of MJ1 integrase in an excisive recombination. To precisely localize MJ1-recognizing sequences, we performed DNase I footprinting with *attP* and *attB* segments used in EMSAs. As shown in Fig. 4, MJ1 incubation with *att* fragments prior to DNase I addition generated a series of protected regions. Protected regions by MJ1 contain a common core sequence (5'-AGT-3') in their centers and extend ~50 bp. The *attP* sequence has an imperfect inverted repeat, but *attB* does not have any noticeable sequence element.

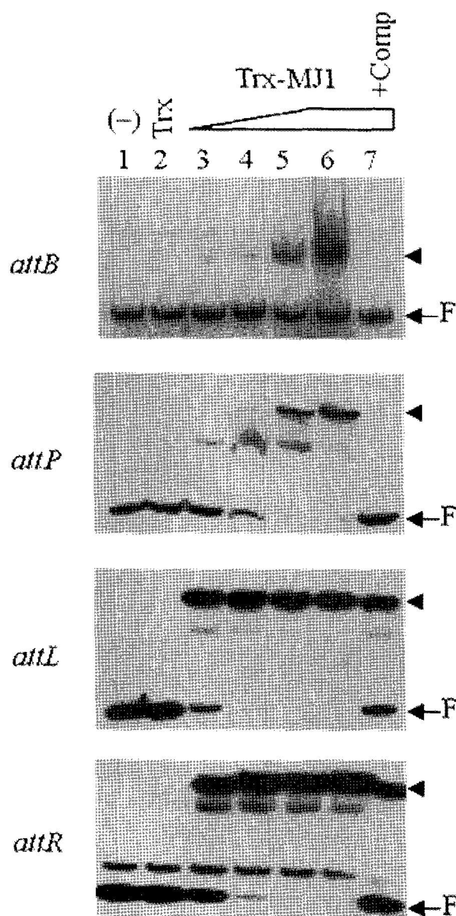

Fig. 3. Electrophoretic mobility shift assays (EMSAs).
Ten ng of *attP* (287 bp), *attB* (290 bp), *attL* (404 bp), or *attR* (172 bp) was incubated with 0.1 μg of Trx (lane 2) or with increasing amounts of Trx-MJ1 (0.01, 0.02, 0.05, 0.1, 0.1 μg [lanes 3–6] in the case of *attP* and *attB*, 0.05, 0.1, 0.2, 0.5, 0.5 μg [lanes 3–6] in the case of *attL* and *attR*). Lane 1 indicates probes only and lane 7 includes 20-fold molar excessive cold-specific competitors. Arrowheads indicate shifted bands and F indicates free probes.

In this study, we investigated some molecular mechanisms of MJ1-mediated recombination and found that they were similar to those of the serine integrases studied to date, such as TP901-1, ΦC31, R4, or ΦRv1. First, MJ1-mediated integration requires neither high energy cofactor nor DNA supercoiling. Second, MJ1-recognizing *att* sites were short and simple, which is a typical feature common in serine integrase-mediated recombination systems. In the case of tyrosine integrases, *attP* extends long and contains multiple elements [18], whereas the minimal regions of *attB* and *attP* of serine integrases were determined to be as short as 50 bp, as elucidated in ΦC31 [20], TP901-1 [4], R4 [16], or ΦRv1 [2] integrases. Third, MJ1 integrase alone could perform a recombination reaction between *attP* and *attB* in a defined reconstituted system. In contrast, most tyrosine integrases require additional host cofactors such as IHF for λ integrase [14], and it has been known that serine integrases do not require accessory factors [20]. Fourth, MJ1 is highly homologous with previously characterized serine integrases (Fig. 1). All of these integrases have a resolvase domain and a conserved catalytic serine residue around their N-termini. In these respects, MJ1 is considered to belong to the serine integrase family and likely to mediate the site-specific integration in a similar mechanism. It is also known that the reverse reaction of integration, excision, could not be mediated by serine integrases alone and MJ1 integrase seems to be also the case. TP901-1 and ΦRv1 are, among the serine integrases, the only cases of which excisionases have been identified and characterized [3, 4]. Our results suggest, like TP901-1 and ΦRv1, that MJ1 might also be involved in the excisive recombination, but a certain additional factor, probably a phage-encoded excisionase, may still be required for efficient excision. It will be necessary to identify the excisionase to better understand the integration-excision mechanisms of ΦFC1, in a future study.

Efficient and controlled gene manipulation of cells or organisms is a major concern in modern biology [10, 19]. Recently, trials for introducing a desired gene into

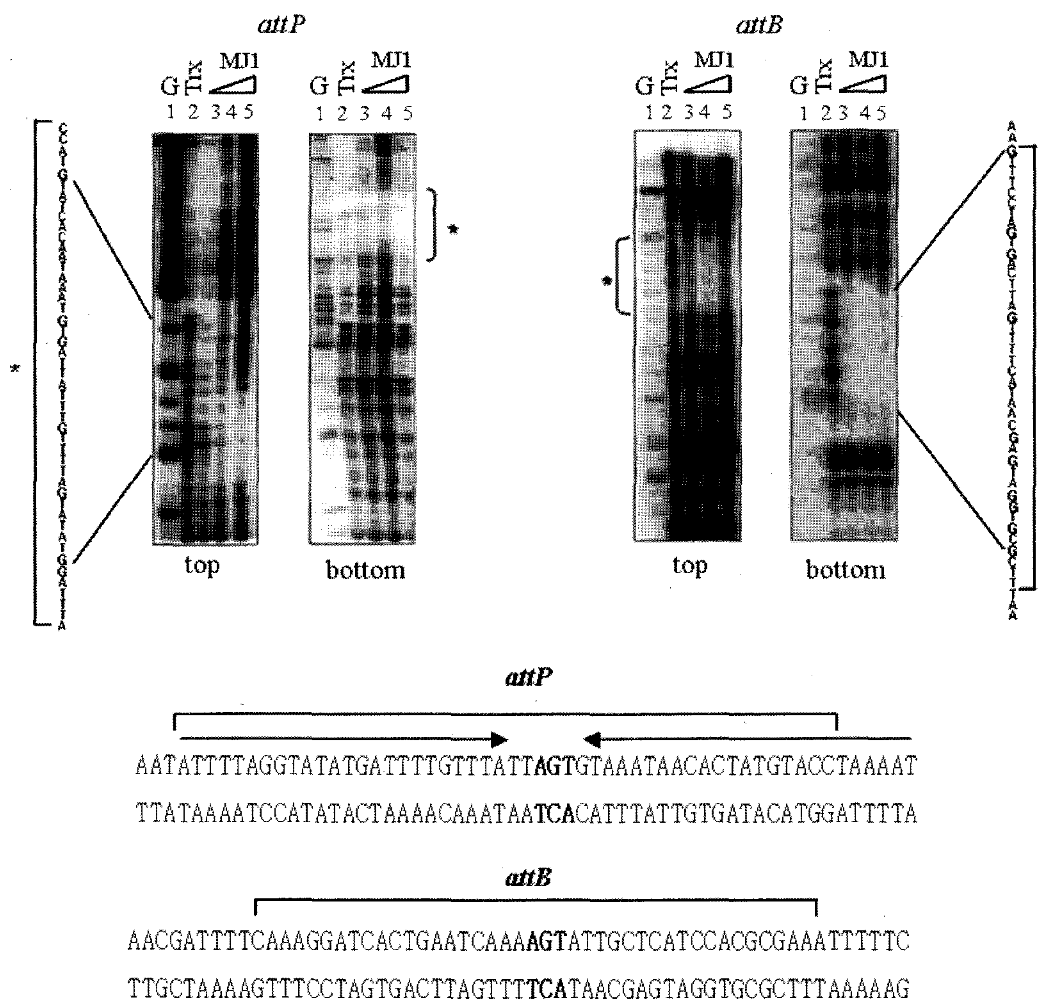

Fig. 4. DNase I footprinting.

One pmole of labeled DNA was incubated with 1 μg of Trx or with increasing amounts of Trx-MJ1 (1, 2.5, 5 μg in lanes 3–5) and DNase I treatment followed. G (lane 1) indicates G bases by chemical sequencing reaction, and protected areas are indicated by brackets (upper). DNA sequences protected by MJ1 are shown (lower). Brackets indicate protected areas and arrows indicate an imperfect inverted repeat.

mammalian chromosome in a controlled way using the integration system of bacteriophages are ongoing. It has been proven that the integration reaction by serine integrases in mammalian cells is efficient, and the stable insertion of a foreign gene into pseudo *att* sites present in mammalian chromosome is possible [1, 6]. These simple and unidirectional characteristics of serine integrases render them to be a potentially useful tool for genetic engineering of bacterial and mammalian cells. We believe that MJ1 integrase of ΦFC1 is probably the case, and we are proceeding to utilize this system for genetic manipulation of mammalian cells.

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