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Site-Specific Integration by Temperate Phage Integrase for Application in Gene Therapy

Hyo-Ihl Chang

Laboratory of Biochemical Genetics, Graduate School of Biotechnology, Korea University, Seoul, Korea

Introduction

Enterococci are gram-positive anaerobic bacteria that normally occur in the intestines of most animals. Enterococci tolerate a variety of growth conditions, including hypotonic, hypertonic, acidic and alkaline environments and can survive at temperatures ranging from 10 to 45 °C.

Bacteriophage ΦFC1 was first isolated in our laboratory from a culture of the lysogenic strain of *Enterococcus faecalis* (KBL703) following induction by UV irradiation. The bacteriophage has a double-stranded DNA genome of approximately 40.5 kbp, an icosahedral head, and a sheath-less non-contractile tail. It has been classified into Bradley's group B or Siphoviridae according to the International Committee on Taxonomy of Viruses classification system.

Phage ΦFC1 integration into the host chromosome occurs by a site-specific mechanism. A gene that encodes a putative site-specific recombinase and that is upstream of the *att*P site has been identified. The gene, mj1, encodes a 465-amino-acid polypeptide with similarity in its N-terminal domain to site specific recombinases. The MJ1 integrase displays significant overall homology (57%) with the integrases of listerial phage A118 and lactococcal bacteriophage TP901-1. Analysis of the DNA sequences around the *att*P region identified two predicted bacterium-phage junction regions (*att*L and *att*R).The corresponding bacterial attachment site (*att*B) was deduced from the sequences of these regions.

Materials and methods

Bacterial strains, bacteriophage, and plasmids.

E. faecalis strains were propagated at 37°C in Todd-Hewitt broth (THB; Difco Laboratories, Detroit, Mich.) without shaking. *E. coli* was grown with agitation at 37°C in Luria-Bertani broth.

Temperate phage ΦFC1 was purified after induction by UV irradiation from *E. faecalis* KBL 703, and its DNA was extracted in accordance with standard procedures.

UV inducibility of bacteriophage ΦFC1

Bacteriophage ΦFC1 was induced from the *E. faecalis* lysogenic strain by UV irradiation. Active strains were incubated at 37°C in THB until the optical density at 600 nm reached approximately 0.4. Cells were

then harvested and resuspended in 50 ml of sterile 0.1 M $MgSO_4$, and, after UV irradiation with a 15W germicidal lamp emitting 16 ergs/mm2 for 15 s, they were transferred to double-strength THB and incubated at 37°C again. Changes in the turbidity of the cultures were recorded every 20 min.

Amplification of the attB, attL, attR, and attP regions.

The putative integrase gene and the mj1 and attP sites were amplified from phage ΦFC1 DNA with primers PHY-1 (5'-AAC TGC AGG GCG CAA GAA ACA ACT GCT T-3') and PHY-2 (5'-GAA GAT CTT GTT CTC GAG CAT AGT CTC C-3').

The attL region was amplified from the genomic DNA of *E. faecalis* KBL 707 transformants with primers ON-2 (5'-CGG ATT GCC AGA TGG ATG AT-3') and PHY-2. The attR region was amplified with primers ON-1 (5'-CGG CCA TTG AAT TAG GGT GT-3') and PHY-1. A 290-bp fragment containing attB was amplified from *E. faecalis* KBL 703 genomic DNA with intermolecular integration assay primers PATB-1 (5'-CCC TCG GGC GGA TTG CCA GAT GGA TGA T-3') and PATB-2 (5'-CCC CCG AGC GGC CAT TGA ATT AGG GTG T-3'). A junction region of pREC1 was amplified from plasmid DNA, which was extracted from *E. coli* cotransformed with pATTB1 and pETMJ1, with primer set PATB-1 and PHY-2. PCR was conducted as follows: 94°C for 5 min, and then 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 to 3 min. This was followed by a final extension period of 72°C for 10 min. PCR products were analyzed on 0.8 to 1.2% agarose gels in Tris-acetate-EDTA buffer.

For transformation, 20 μl of frozen cells was thawed on ice, mixed with plasmid DNA, and transferred to the electrode pin. A single electric pulse (PG 240 bacterial electrode and Progenitor II; Hoefer Co.) with the following parameters was applied: 12,000 V/cm peak voltage, 4.7 ms, and 100 μF. The cell suspension was mixed with ice-cold SGM17MC (4) and left on ice for 5 min.

After incubation at 37°C for 2 h, transformed cells were spread on a streptococcal regeneration plate containing 5 μg of chloramphenicol per ml. Southern blotting. DNA restriction fragments separated on an agarose gel were transferred to a Hybond-N membrane (Amersham) by the capillary method(32). Appropriate probes were labeled with digoxigenin-11-dUTP, and prehybridizations and hybridizations were performed as recommended by the supplier (Boehringer-Mannheim). Digoxigenin-11-dUTP-labeled λ DNA digested with DraI was used as a molecular marker for the hybridization.

DNA sequencing

DNA cycle sequencing was done using the ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin-Elmer Co.). Double-stranded template DNA (0.5 μg) and 3.5 pmol of primer were mixed with 8 μl of terminator ready reaction mixture, and cycle sequencing was carried out with a GeneAmp PCR system 9600 (Perkin-Elmer Co.). The amplified DNA was purified with Centri-Sep spin columns (Applied Biosystems), and the nucleotide sequence was analyzed with an ABI PRISM 310 genetic analyzer. The left junction region, *att*L, which lies between the *att*B site and pEMJ1-1 on a 617-bp DNA fragment, was amplified from the genomic DNA of the *E. faecalis* KBL 707 transformant by PCR using primers ON-2 and PHY-2 and sequenced. *att*R, the other junction region of the 400-bp fragment, was amplified from the genomic DNA of the *E. faecalis* KBL 707 transformant by PCR using primers ON-1 and PHY-3 (5'-GCG TTA ACT GCC AAT ATA GC-3') and then sequenced. The nucleotide sequence data have been deposited

in GenBank under accession no. AY026043 and AY026044.

Purification of MJ1 integrase

Purification of MJ1 from *E. coli* strain BL21 (DE3) pLysS (Novagen Co., U.S.A) carrying plasmid pETMJ1, induced by addition of 0.5mM IPTG (isopropyl-D-thiogalactoside) at 15°C, was accomplished as follows. Cells from a 500ml culture were pelleted and frozen; thawed pellets were resuspended in 10ml of cold Lysis buffer (50mM NaH_2PO_4 (pH8.0)/300mM NaCl/ 10mM imidazole/ 1mM PMSF), sonicated, and clarified by centrifugation. The supernatant was extracted in batch by addition of Ni-NTA resin, which was then washed with Wash buffer (20mM Tris-HCl/ 0.5M NaCl/ 40mM of imidazole) and then extracted with elution buffer (20mM Tris-HCl/ 0.5M NaCl/ 1M imidazole)

***in vitro* recombination assays**

Approximately 1μg of substrate DNAs were mixed with integrase in a volume of 50μl. Final reaction conditions were 30mM Tris-HCl, pH7.6/ 15mM NaCl/ 80mM KCl / 0.7mM EDTA/ 4mM spermidine/7% glycerol, incubated at 37°C. The reactions were terminated by phenol extraction, and then the product DNAs were collected by ethanol precipitation, and the pellets were resuspended in 20μl of D.W. After restriction, the reaction products were separated by electrophoresis on 1.0-2.0% agarose gel. DNA fragments in the gel were stained with Ethidium bromide and visualized under UV light. DNAs for use in the recombination reactions were purified by alkaline lysis method. To prepare linearized substrates, plasmids were cleaved with EcoRI.

Results

In this study, a vector system based on the site-specific recombination apparatus of temperate bacteriophage ΦFC1 of *E. faecalis* KBL 703 was constructed. We wanted to determine whether putative integrase gene mj1 could serve as an efficient mediator of integration and if the mj1 and *att*P sites were sufficient for site-specific integration into the *att*B site. The vector we constructed, which included the *att*P site and mj1, could integrate into the *att*B-like site on chromosomal DNA of *E. faecalis* strain KBL 707, which contains no bacteriophage. This strain also was easily lysogenized by phage ΦFC1, produced from *E. faecalis* KBL 703 after UV irradiation. In addition, a 290-bp fragment containing the *att*B site was cloned into vector pACYC184 for use in intermolecular integration assays to determine the minimum size of the *att*B site and the eventual necessity for other host factors required for site-specific recombination.

And bacteriophage-T7 derived expression vector pET32a(+) was chosen to ensure an efficient expression of the recombinant protein in soluble form. The pET32a(+) is a gene fusion expression system that uses thioredoxin, the product of the *E. coli trx*A gene, as the fusion partner. This recombinase has been partially purified and used in DNA binding experiments using the four recombination sites, *att*P, *att*B, *att*L and *att*R. These characteristics of binding by ΦFC1 recombinase are significantly different from those of lambda integrase binding to its sites, which uses multiple binding sites for the recombinase and its accessory proteins.

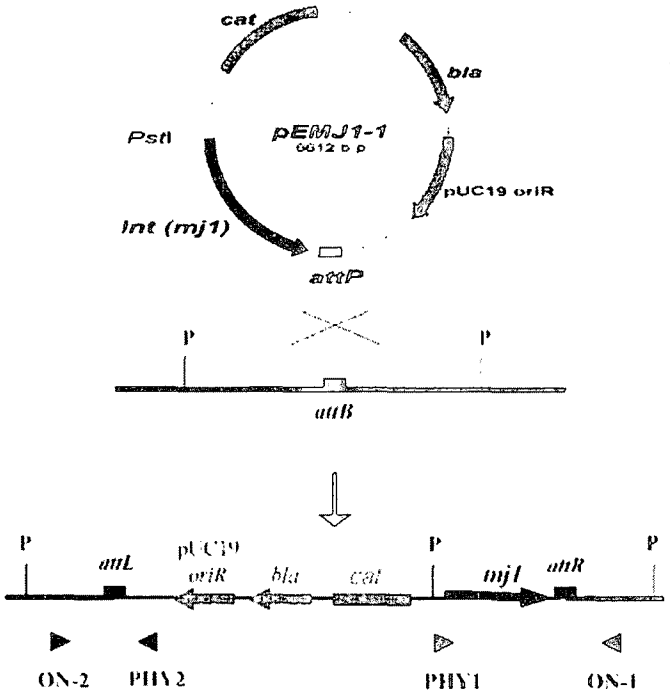

Fig. 1. Schematic reprentation of the site-specific integration of pEMJ-1 into the attB locus of the *E. faecalis* KBL 707 chromosome. P, PstI aite; arrowheads, primers.

AttB- ATATTTAACCGCTTCCCGAAAAATTTCGCGTGGATGAGCAAT ACT TTGATTCAGTGAACCTTTGAAAATCGTTTTCTGTTGGATAA

r1 r2 r2 r1

AttP- aaactacagaaaataaaatccttatcatcaaagagtttgttgaacgtatagaactattcgatgatgaggtaatt

r3 r3

attaaatataaattttagtacatagtgttatatac act aataaacaaaatcatatacctaaaatattacatt

AttL- ATATTTAACCGCTTCCCGAAAAATTTCGCGTGGATGAGCAAT ACT aataaacaaaatcatatacctaaaatattacatt

AttR- attaaatataaattttagtacatagtgttatatac ACT TTGATTCAGTGAACCTTTGAAAATCGTTTTCTGTTGGATAA

Fig. 2. Nucleotide sequences of the arrB, *att*P, *att*L, and *att*R regions. *att*L and *att*R were sequences after pEMJ1-1 integrated into the *E. faecalis* KBL 707 chromosome, Uppercase, *E. faecalis* KBL 707 chromosomal DNA sequences; lowercase, phage ΦFC1 DNA sequences; boldface, 3-bp core. In the *att*B sequence, the single nucleotide difference in the real *att*B nucleotide sequence of *E. faecalis* KBL 703 in underlined (T in place of A in *E. faecalis* KBL 703). Arrows (*att*P sequence), inverted repeats r1, r2, and r3.

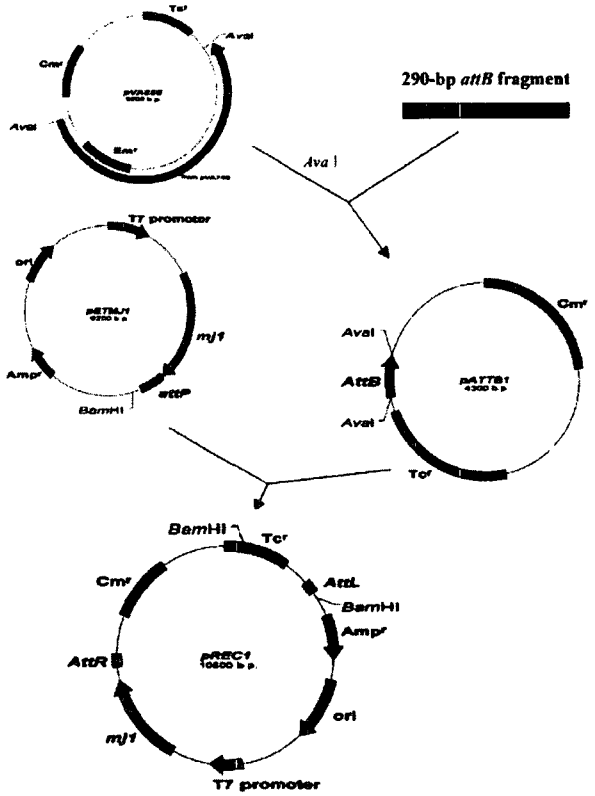

Fig. 3. Schematic representation of the construction of *att*B-containing plasmid pATTB1 and chimeric plasmid pREC1. pVA856 is a shuttle vector constructed by joining pACYC184 and pVA749 at the AvaI site. The pACYC184 fragment from pVA856 was used for construction of pATTB1. A 290-bp *att*B fragment was amplified from the *E. faecalis* KBL 703 chromosome by PCR. pATTB1 and pETMJ1 were cotransformed into *E. coli* JM 109 for the intermolecular integration assay.

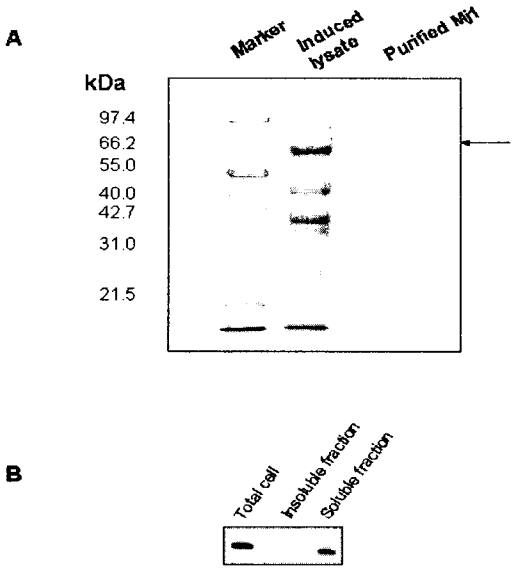

Fig. 4. Overexpression and purification of the MJ1 protein (A) SDS-PAGE. MJ1 integrase has a predicted molecular size of 70KDa (B) Western blot. Line 1, induced total cell; Line 2 and 3, insoluble and soluble proteins of IPTG-induced cells harboring pETMJ1-1

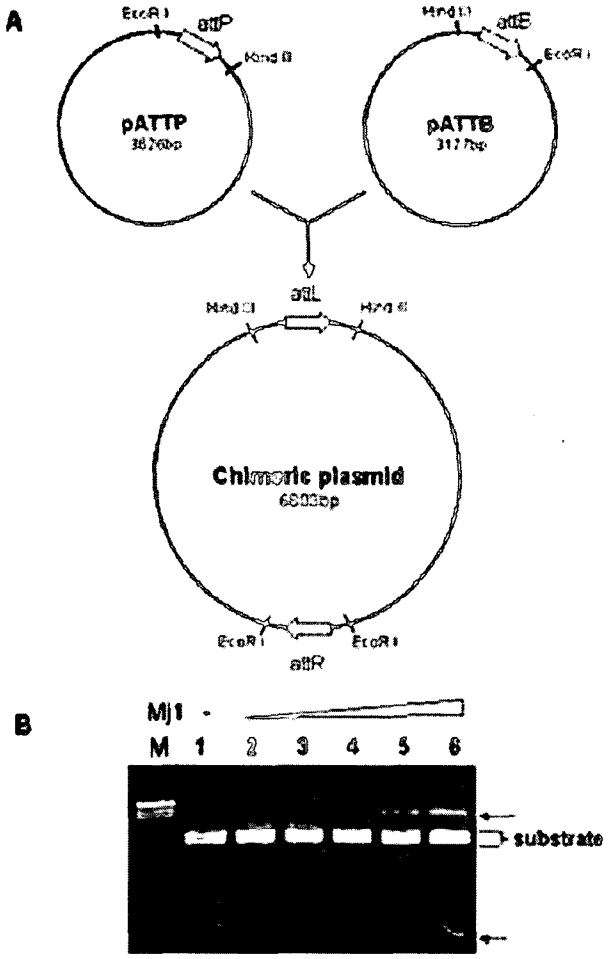

Fig. 5. in vitro recombination by ΦFC1 recombinase MJ1 between *att*P and *att*B site (A) Circular maps of the plasmids pATTP and pATTB and the chimeric plasmid resulting from MJ1-mediated recombination between these two plasmids. The revelant genetic markers, restriction sites are shown. The arrows on the attachment sites (*att*P, *att*B, *att*L, *att*R) indicate the direction of the core site. (B) Restriction analysis by EcoR I of parental and recombinant products. Molecular weight markers (M) are provided by digesting lambda DNA with Dra I.

References

Rice, E. W., J. W. Messer, C. H. Johnson, and D. J. Reasoner. 1995. Occurrence of high-level aminoglycoside resistance in environmental isolates of enterococci. Appl. Environ. Microbiol. 61:374–376.

Jett, B. D., and M. S. Gilmore. 1994. Virulence of enterococci. Clin. Microbiol. Rev. 7:462–478.

Landy, A. 1989 Annu. Rev. Biochem. 58, 913-949

HY Yang, YW Kim, and HI Chang, 2002. Construction of an Integration-proficient Vector Based on the Site-Specific Recombination Mechanism of Enterococcal Temperate Phage ΦFC1. J. Bac. 184(7): 1859-64

Kim, M.-J., J. Y. Lee, Y. W. Kim, and H. I. Chang. 1996. Molecular characterization of the region encoding integrative functions from enterococcal temperate phage ΦFC1. J. Biochem. Mol. Biol. 29:448–454.

Kim, Y. W. 1999. Genetic studies of bacteriophage ΦFC1. from Enterococcus faecalis. Ph.D. thesis. Korea University, Seoul, Korea.

Kim, Y. W., and H. I. Chang. 1994. Isolation and molecular characterization of ΦFC1, a new temperate phage from Enterococcus faecalis. Mol. Cell 4:155-158.

Thrope HM, Smith MC. 1998 *In vitro* site-specific integration of bacteriophage DNA catalyzed by a recombinase of the resolvase/invertase family. Proc. Natl. Acad. Sci. USA 95: 5505-10

Thrope HM, Wilson SE, Smith MC. 2000. Control of directionality in the site-specific recombination system of the Streptomyces phage ΦC31. Mol. Microbiol. 38:232-41

Thyagarajan B, Guimaraes MJ, Groth AC, Calos MP. 2000 Mammalian genomes contain active recombinase recognition sites. Gene 244: 47-54