Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 368 (2008) 192–198
BBRC
www.elsevier.com/locate/ybbrc

A novel site-specific recombination system derived from bacteriophage ϕMR11

Mohammad Rashel [a], Jumpei Uchiyama [a,b], Takako Ujihara [c], Iyo Takemura [a,d], Hiroshi Hoshiba [a], Shigenobu Matsuzaki [a,*]

[a] Department of Microbiology and Infection, Kochi Medical School, Kohasu Oko-cho, Nankoku, Kochi 783-8505, Japan
[b] Department of Pediatrics, Kochi Medical School, Kohasu Oko-cho, Nankoku, Kochi 783-8505, Japan
[c] Science Research Center of Kochi University, Kohasu Oko-cho, Nankoku, Kochi 783-8505, Japan
[d] Department of Biochemistry, Kochi Medical School, Kohasu Oko-cho, Nankoku, Kochi 783-8505, Japan

Received 2 January 2008
Available online 22 January 2008

Abstract

We report identification of a novel site-specific DNA recombination system that functions in both *in vivo* and *in vitro*, derived from lysogenic *Staphylococcus aureus* phage ϕMR11. *In silico* analysis of the ϕMR11 genome indicated *orf1* as a putative integrase gene. Phage and bacterial attachment sites (*attP* and *attB*, respectively) and attachment junctions were determined and their nucleotide sequences decoded. Sequences of *attP* and *attB* were mostly different to each other except for a two bp common core that was the cross-over point. We found several inverted repeats adjacent to the core sequence of *attP* as potential protein binding sites. The precise and efficient integration properties of ϕMR11 integrase were shown on *attP* and *attB* in *Escherichia coli* and the minimum size of *attP* was found to be 34 bp. In *in vitro* assays using crude or purified integrase, only buffer and substrate DNAs were required for the recombination reaction, indicating that other bacterially encoded factors are not essential for activity.

Keywords: Site-specific recombination; Serine recombinase; Integrase; Bacteriophage ϕMR11; *S. aureus*

Site-specific recombination involves rearrangement or exchange of segments of DNA within or between DNA molecules at a particular DNA sequence. Enzymes that catalyze site-specific recombination rearrange DNA to integrate, excise or invert the DNA segments for a variety of biological purposes [1] such as genome replication, differentiation, pathogenesis, and movement of some mobile elements (transposons, plasmids, bacteriophages, and integrons) [2]. In the field of molecular biology, the molecular mechanisms of site-specific DNA recombination have long been a major focus of interest [3,4] that may lead to many applications of integrases as molecular tools for DNA manipulation [5].

Site-specific recombinases can be classified into two major families: the tyrosine recombinase (or λ-integrase) family and the serine recombinase (or resolvase/invertase) family [2]. Tyrosine recombinases break and rejoin single strands in pairs to form a Holiday junction intermediate, while the serine recombinases cut all strands in advance of strand exchange and religation [4]. Based on the available information, λ integrases have been efficiently utilized to develop the high throughput Gateway® Technology for DNA cloning [6]. Both Cre recombinase of phage P1 and FLP invertase from yeast have been effectively used to create gene deletions, insertions, inversions and exchanges in flies, mammalian cell cultures and mice [4].

Serine recombinases (e.g., γδ resolvases, Mu Gin invertase, ϕC31, R4 and TP901-1 integrase) possess a catalytic serine residue [2]. Both γδ-resolvase and ϕC31 integrase have been extensively studied in order to understand their

* Corresponding author. Fax: +81 88 880 2324.
E-mail address: matuzaki@kochi-u.ac.jp (S. Matsuzaki).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.01.045

molecular mechanism of action [4,7]. Exploiting its efficient activity in *Escherichia coli* and mammalian cells [8], ϕC31 integrase has been used to develop molecular tools and has been tested in a variety of genetic disease settings [9]. New members of the serine recombinase family are rapidly being added to databases, and detailed functional analyses of large serine recombinases from mycobacteriophage have been reported [10].

Most strains of *Staphylococcus aureus* are lysogenized by temperate phages in nature [11], but surprisingly studies of recombination systems in phages of *S. aureus* origin are extremely rare. We have isolated *S. aureus* phage ϕMR11 by mitomycin C induction of a clinical *S. aureus* strain MR11 [12]. We also produced artificially a ϕMR11 lysogen of *S. aureus* strain SA37 (SA37/ϕMR11), which indicated that the phage must have machinery to integrate into the bacterial chromosome. Here we describe identification and activity of the site-specific recombination system of phage ϕMR11. To our knowledge, this is the first site-specific DNA recombination system that has been shown to be functional in both *in vivo* and *in vitro* in *S. aureus* phages.

Materials and methods

Primers and plasmids. A list of primers and description of plasmids are provided as supplementary materials.

Identification of integrase gene and attachment sites. We have sequenced the whole genome of ϕMR11 [12] and identified a possible 67 ORFs using GENETYX-MAC Version 11.2.7 software. To identify the possible integrase gene, the individual ORFs were analyzed by using the NCBI resident BLAST program [13]. The sequence of the putative integrase gene (*orf1*) was deposited into the DNA Data Bank of Japan (DDBJ) under the Accession No. AB367799.

Identification of attachment junctions between phage ϕMR11 and the genome of its natural lysogenic host *S. aureus* MR11 was performed using the LA-PCR™ In-vitro Cloning Kit (Takara Shuzo, Kyoto, Japan) according to the manufacturer's instructions. To identify the *attL* region (the left end of the integration junction), *Hind*III digested *S. aureus* MR11 genomic DNA was ligated with *Hind*III cassettes and amplified by cassette primers C1 and L4-RP-0.01 (first step). This product was again amplified with primers C2 and L4-RP-0.02 (second step). Primers L4-RP-0.01 and L4-RP-0.02 were synthesized based on the sequence of *orf1*. The PCR products were sequenced and the junction was determined by comparing the genomic sequence of phage ϕMR11 and *S. aureus* N315 (Accession No. NC_002745).

We also constructed primer ATT-P1 (from phage) and ATT-B1 (from bacteria) based on the sequence as determined above, and amplified MR11 genomic DNA by PCR to reconfirm the *attL* region by sequencing of the PCR product. In contrast, the *attR* (right site of integration junction) region was amplified using MR11 genomic DNA as template with the primers L4-MP-0.1 (from phage) and ATT-B2 (from bacteria) that were synthesized based on the sequence around *attL*. The PCR product was purified from the gel and sequenced. The 150 bp sequence of left or right side around the *attB* on MR11 genome was deposited into DDBJ under the Accession No. AB373133 or AB373134, respectively.

Identification of connection sites between phage ϕMR11 and the host genome in artificially lysogenized *S. aureus* SA37 (SA37/ϕMR11) was performed by PCR amplification of the junctions based on the sequence information obtained from the natural lysogen MR11. By using SA37/ϕMR11 genomic DNA, *attL* was amplified by primers ATT-B1 and ATT-P1 and *attR* was amplified by primers L4-MP-0.1 and ATT-B2. PCR amplified products were confirmed by sequencing. The bacterial attachment site (*attB*) in the SA37 genome was determined by PCR amplification using primers ATT-B1/ATT-B2 and the SA37 genome as template DNA. The PCR product was confirmed by sequencing. The sequence around the *attB* of SA37 genome was deposited into DDBJ under Accession No. AB373135.

Intermolecular integration assay in E. coli. The pCold-Int-attP (expresses the integrase gene in *E. coli*) (Supplementary material) plasmid was transformed into *E. coli* DH5α and was selected on an Luria–Bertani (LB)-ampicillin plate. The resulting transformant was retransformed with different pACYCattB plasmids (Supplementary material) and the transformants were selected on LB plates containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol and grown at 37 °C. Colonies were picked and grown overnight in liquid terrific broth (TB) medium containing ampicillin and chloramphenicol, and plasmids were purified using Amersham FlexiPrep™ (Piscataway, NJ). The recombination event was checked by running the purified plasmid on agarose gel before and after restriction digestion with *Xba*I, PCR analysis and confirmed by sequencing.

Preparation of crude integrase for assay of recombination in vitro. *E. coli* BL21 transformed with pCold-Int-His was grown overnight in TB/ampicillin medium, diluted 50× with fresh TB/ampicillin medium and grown until OD_{600} reached 0.7. The culture was transferred to 15 °C and kept there for 30 min. Expression of the integrase was induced by 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 24 h at 15 °C, and the bacterial cells were pelleted at 6000*g* for 5 min and washed with phosphate buffered saline (PBS). A crude extract was prepared by suspending the cells in 10 mM Tris/100 mM NaCl, pH 8, and sonication using an ultrasonic disintegrator. A clear supernatant was collected by centrifuging at 14,000*g* for 20 min and was used for assay. One microgram of substrate DNAs (pUC18attP48 and pACYCattB40) were mixed with approximately 20 μl of crude integrase in 10 mM Tris–HCl, pH 8/100 mM NaCl/1% glycerol/2.5 mM EDTA, and incubated at 37 °C. The reactions were stopped by phenol extraction at different time points, and after ethanol precipitation pellets were suspended in 30 μl water and were amplified by PCR with primer pair 184BamP1/M4 to amplify *attL* and 184BamP2/RV to amplify *attR*. The recombination reaction was then confirmed by sequencing of PCR products.

Purification of integrase and assay of recombination in cell-free system. *E. coli* BL21 transformed with pCold-Int-Gst (expresses the integrase fused with GST in *E. coli*) (Supplementary material) was grown overnight with TB/amp, diluted 50× with fresh TB/amp medium and grown until OD_{600} reached 1.2. Culture was transferred to 15 °C and kept there for 30 min. The integrase was induced with 1 mM IPTG for 24 h; the bacterial cells were pelleted at 6000*g* for 5 min, washed with PBS and stored at −70 °C until protein purification. Protein was purified according to the manufacturer's instructions with some simple modifications. The cell pellets were resuspended in 50 mM Tris–HCl, pH 8, 100 mM NaCl, and 2.5 mM EDTA supplemented with 0.05 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) (Roche Diagnostics Penzberg, Germany) and 0.1 mM phenylmethanesulfonyl fluoride (PMSF), sonicated with an ultrasonic disintegrator then bound to prewashed Glutathione Sepharose 4B resin (Amersham). After extensive washing, the protein was eluted with 50 mM Tris–HCl, pH 8, 10 mM glutathione and 100 mM NaCl. The purified protein was dialyzed against 10 mM Tris–HCl, pH 8, 100 mM NaCl, and 10 mM $MgCl_2$ and stored at −145 °C until use. One microgram of each substrate DNA was mixed with approximately 1 μg purified recombinant integrase in 10 mM Tris–HCl, pH 8, 100 mM NaCl, 1% glycerol, 2.5 mM EDTA, 5 μg/μl BSA, 10 mM $MgCl_2$ and incubated at 37 °C. The reactions were stopped by phenol extraction, and after ethanol precipitation the pellets were suspended in 30 μl water. Recombination reactions were checked by PCR amplification of the reaction products with primer pair 184 BamP1/M4 to amplify *attL* and with primer pair RV/ACYC184-SalL3 to amplify *attR*. A second round of PCR was performed to obtain a sufficient amount of purified DNA from the initial PCR product for sequencing.

Phylogenetic analysis. Homologues of the integrase gene were identified by searching the NCBI database with the resident Protein BLAST program [13]. Multi-alignment was performed by the GENETYX-MAC Version 13.0.14. A phylogenetic tree was constructed using the Treeview program [14] from multiple protein sequence alignments generated by Clustal W [15].