Virology 404 (2010) 240–245

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Identification of bases required for P2 integrase core binding and recombination

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ARTICLE INFO

Article history:
Received 12 April 2010
Returned to author for revision 9 May 2010
Accepted 12 May 2010
Available online 3 June 2010

Keywords:
Bacteriophage
Integrase
Site-specific recombination

ABSTRACT

Temperate coliphage P2 integrates its genome into the host chromosome upon lysogenization via a site-specific recombination event mediated by an integrase belonging to the complex family of tyrosine recombinases. The host integration site *attB* (BOB′) is localized in the end of the *cyaR* gene and shares 27 nucleotides with the core of *attP* (COC′). In the present study we determine the minimal *attB* site using an *in vivo* recombination assay. Ten nt on the left side (B) are found to be nonessential for recombination. We show that the integrase has higher affinity for the right side (B′) compared to B and that artificial B′OB′ and an *attP* site with a matching core (C′OC′) are efficient substrates for recombination *in vitro*. We have analyzed single nucleotides in *attB* and find that sequence homology within a non-centrally located quadruplet in the hypothetical overlap region is essential for efficient recombination *in vivo*.

Introduction

Bacteriophage P2 is a temperate phage that integrates into the *Escherichia coli* chromosome upon lysogenization. Integration is promoted by the phage-encoded integrase (Int) that mediates recombination between the phage *attP* site and the bacterial *attB* site, generating the phage–host junctions *attL* and *attR*. The integration requires also the host encoded architectural protein IHF, and the reverse reaction, excision, requires in addition to Int and IHF, the phage-encoded Cox protein (Yu and Haggård-Ljungquist, 1993a). The P2 integrase belongs to the tyrosine family of integrases and like the prototype lambda integrase, it has two DNA binding domains, the large C-terminal domain that contains the core recognition and the catalytic site, and the N-terminal domain that recognizes the arm-sites present only in *attP* (Frumerie et al., 2005a). The *attP* core region contains an imperfect inverted repeat surrounding the hypothetical crossover region (COC′) located within a 27 nucleotides long sequence that is identical to the *attB* site (BOB′) (Yu et al., 1989), and the arm-sites that constitute two directly repeated sequences on either side of the core (P1, P2 and P′1 and P′2, respectively) (Yu and Haggård-Ljungquist, 1993b). The IHF binding site is located between the P-arm and the core, and the Cox binding sites are located between the core and the P′-arm (and consequently in *attL*) (Yu and Haggård-Ljungquist, 1993a). The heterobivalent integrase works as a dimer and is believed to bridge the arm and core-binding sites with the help of IHF or Cox, which bind cooperatively with the integrase (Frumerie et al., 2005a; Frumerie et al., 2005b). P2 integrase binds with high affinity to the core, but with low affinity to the arm-sites (Frumerie et al., 2005a), which is in contrast to the well-studied lambda integrase that binds with a low affinity to the core and with a high affinity to the arms. The lambda integrase has been shown to regulate the cleavage activity by the formation of the intasome containing four molecules of the integrase where only one pair of Int molecules are active at a time ensuring an ordered pairwise exchange of DNA strands, where the arm-sites play an important role in directing which strand in the core is cleaved first (Azaro and Landy, 2002; Radman-Livaja et al., 2006).

The P2 bacterial integration site, *attB*, is located in a hypothetical small RNA gene, *cyaR* (*ryeE*), where the C-terminal end of CyaR is restored by the prophage sequence after recombination (the nt corresponding to the end of the CyaR RNA are underlined in Fig. 1) (Wassarman et al., 2001). CyaR is an 85 nt long RNA that is activated by the global regulator Crp during conditions where cAMP levels are high. The activated CyaR regulates the expression of a number of genes negatively by a direct binding near the ribosome binding sites of its target genes (De Lay and Gottesman, 2009).

In this work we have addressed the importance of the nucleotides in the *attB* for integrase binding and recombination, by defining a minimal recombinogenic *attB* site and also by analyzing the effects of nucleotide substitutions within the right half of *attB*. Attempts to locate the Int cleavage site have so far failed, and since the presumed Int recognition sites, i.e. the inverted repeat, contain several mismatches we have used the information obtained by the mutation studies to propose a cleavage site for the P2 integrase.

Results

Construction of a reporter system to analyze the efficiency of recombination

A plasmid reporter system (pEE2035) was constructed, containing the *GFPuv* gene under the control of the *lac* promoter, with the 27 nt

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doi:10.1016/j.virol.2010.05.009

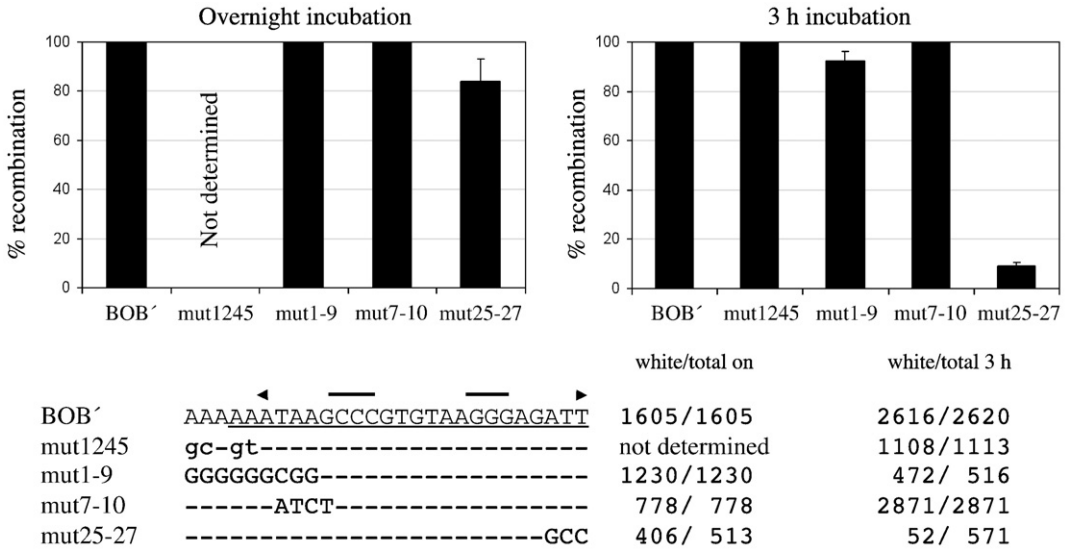

Fig. 1. Recombination efficiencies of the wild type and deleted/mutated *attB* sites using the plasmid assay system. The percentage of white colonies over total number of transformed colonies after retransformation of the extracted plasmids is shown. The plasmids were extracted 3 h after transformation of the recombination assay plasmid, or after overnight incubation. The sequences of the *attB* sites are shown below the graphs. Identical nt are indicated with a dash and the potential imperfect inverted repeat is indicated as arrows above the sequence. The sequence corresponding to the CyaR RNA is underlined. Mut1245 indicates that the *attB* site has a deletion of the first 5 nt, and the bases shown below the figure in non-capitalized letters originate from the vector. The sum of white colonies out of total number of colonies screened in all the experiments are given as white/total on the right side of the sequences and the standard deviations are based on the percentage obtained in independent experiments.

attB site inserted in frame inside the *GFPuv* gene and the *attP* site inserted downstream of the *GFPuv* gene. The *GFPuv* gene is expressed from the *lac* promoter. Cells containing the reporter plasmid show a high fluorescent signal under UV light. Transformation with a compatible plasmid containing the wild type *int* gene leads to excision of the *GFPuv* gene and after overnight growth all colonies will have lost their fluorescent signal (Fig. 1). The reporter plasmid is a high copy plasmid and the Int protein is supplied in trans from a low copy plasmid expressing the Int protein. The *int* gene in this plasmid is under control of the T7 promoter, but since there is no T7 polymerase present in the bacteria used, *int* expression is caused by a low background level. To be able to quantitate the fraction of reporter plasmid that had recombined 3 h after transformation, the plasmid was extracted and retransformed into cells lacking the *int*-expressing plasmid. 100% of the colonies were white, demonstrating that 100% of the plasmids had recombined already 3 h after transformation. Plasmids from a few colonies were extracted and after PCR amplification and DNA sequencing it was confirmed that they only contained the *attL* junction (data not shown).

The first 10 nt of the common core sequence are not required for recombination

The identity between *attB* and *attP* in P2 is quite long compared to lambda, 27 nt versus 15 nt. One possible explanation for this long identical region could be to restore the *cyaR* sequence upon integration, rather than the identity being important for the recombination process as such. To investigate this 5 nt of the *attB* site were deleted, or purines were exchanged with the other purine and pyrimidines with the other pyrimidines at the respective end of *attB*. As can be seen in Fig. 1, the deletion of the first 5 nt which generated vector sequences at positions 1, 2, 4, and 5 that differed from the *attB* sequence did not reduce recombination efficiency. It is as high as with full length *attB* after only 3 h incubation. An exchange of the first 9 nt showed 100% recombination after overnight incubation, and 90% already after 3 h incubation. Thus, the first 9 nt have no major effect on recombination. Since the possible inverted repeat differs on the right and left side, the left side was modified to match the right side, i.e. nt 7–10 were changed from TAAG to ATCT. This modification had no negative effect on recombination, it had reached 100% already after 3 h incubation, indicating that an identity between the first 10 nt of *attB* and the core of *attP* is not required for efficient recombination. Exchange of nt 25–27 in the other extreme end, however, leads to a clear reduced recombination efficiency, 79% had recombined after overnight incubation and only about 9% after 3 h incubation (Fig. 1).

P2 Int has a higher affinity to the right half (B′) of attB compared to the left half (B)

The integrase is presumed to recognize the left (B) and the right (B′) sides of the intervening overlap sequence (O) in *attB*, but since the sequences B and B′ only show a weak palindromic symmetry, we wanted to investigate whether the integrase had different affinity to the repeats in *attB*. To clarify this, symmetric core sequences were constructed and analyzed for their capacities to be bound by Int, using an electromobility shift assay. It was found that the integrase protein binds to the palindromic B′OB′ sequence with a considerably higher affinity than to the wild type BOB′ sequence, while the BOB sequence was barely recognized by the integrase (Figs. 2a,b). Using an *in vitro* recombination assay, the BOB substrate, as opposed to the B′OB′ substrate, was unable to recombine under similar conditions (data not shown), but the recombination between *attP* and the B′OB′ substrate was reduced compared to the wild type BOB′ substrate. However, the B′OB′ substrate recombined more efficiently with a modified *attP* substrate (*attP* coreR), with a perfect homology to B′OB′, than with the wt *attP* substrate (Figs. 2b,c). The unannotated band is believed to correspond to a shift of the linear labeled *attB* substrate caused by Int binding.

A library screen for mutations in the right half (B′) of attB that affect integrase binding

To analyze the importance of the individual bases in the right half of *attB* a screening method was developed to identify mutants to which integrase has a low affinity. The *attB* sequence was inserted

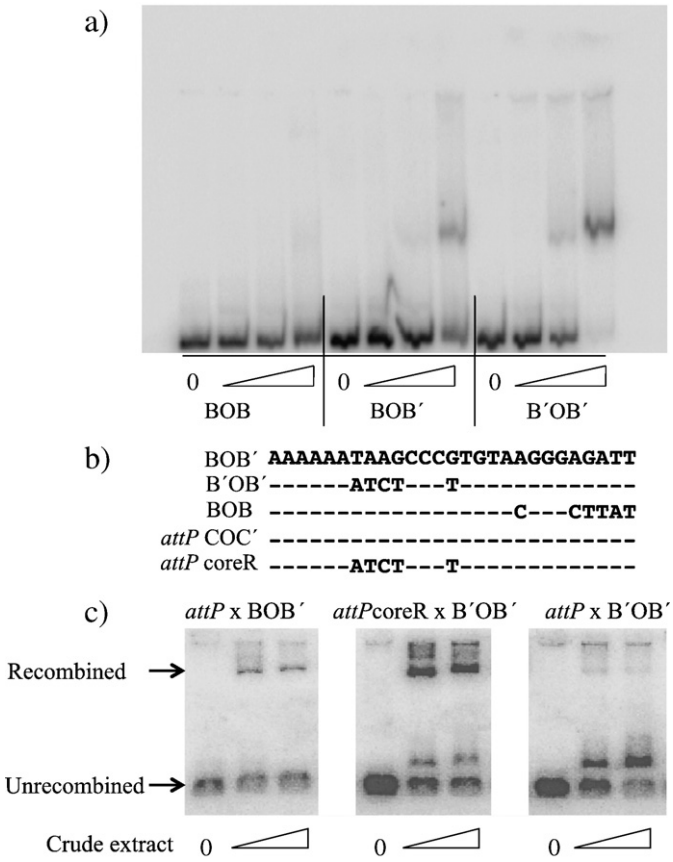

Fig. 2. a) Phosphorimager pictures of an EMSA showing the binding of Int to wild type or mutated *attB* sequences. About 4 ng of labeled DNA was incubated with an increasing amount of a crude extract of cells overexpressing the Int protein. The protein concentration of the crude extract was 13 μg μl^{-1}, where the over expressed Int protein was about 1/10 of the total amount of protein. 0.5 μl, 1 μl and 3 μl of crude extract were used for each DNA construction. b) The sequence of the respective *attB* region, wt BOB′, BOB and B′OB′ and the mutated *attP* coreR. Identical nt are indicated by a dash. c) *In vitro* recombination assay with *attP*/BOB′, *attP* coreR/B′OB′ and *attP*/B′OB′. In the assay 0.5 μl, 1 μl of crude extract (with 50% overexpressed IHF and 50% overexpressed Int extracts) was used (see Materials and methods).

between the *lac* promoter and the ribosomal binding site directing the *lacZ* gene in pUC18. The *attB* sequence was inserted in both orientations, generating plasmids pEE2032 and pEE2033. The insertion of the *attB* site does not affect the expression of the *lacZ* gene, and transformed bacteria give black colonies on S-gal plates, but when integrase is supplied *in trans* from a compatible plasmid it binds to the *attB* site and the colonies on S-gal are white (i.e. grayish).

A library of the right repeat of the *attB* sequence was generated in plasmid pEE2032 and pEE2033, using site-directed mutagenesis with a degenerate primer in positions 20–26. Black colonies were picked and sequenced. The total number of plasmids analyzed where the integrase was unable to block *lac* expression was 39, where most contained multiple mutations in the right half of *attB*. The mutations were analyzed further, and the minimal amount of mutations needed to disrupt integrase binding is shown in Fig. 3. The only single mutation that allows *lac* expression is the mutation G22C. Mutations in the other locations were only found in combination with one or more mutations. In Fig. 3, only the minimal combinations of mutations are shown. The double and triple mutations were found in combination with other mutations that were not found in combination with other mutations. The same mutations were obtained in both plasmid libraries. This indicates that a combination of nt are involved in Int binding and that mutations in only one of them, with the exception of the G22C mutation, will not abolish binding.

```
attB        AAAAAATAAGCCCGTGTAAGGGAGATT
Primer      ----------SSSHKWW-

Minimal mutants obtained
Single      ----------C-----
Double      ----------C--C----
Triple      ----------C-CT---
Quadruple   ----------CC-T-T--

S=(GC); H=(ACT); K=(GT); W=(AT)
```

Fig. 3. The DNA sequence of the pertinent region of the primer used to construct the library of the right half of *attB*, and the minimal mutants obtained that prevented Int binding using the *lac* gene as a reporter.

Effects of point mutations in attB on recombination in vivo

To determine the nucleotides in *attB* that are essential for recombination, the recombination assay reporter plasmid system was used (pEE2035) as described above. Single nucleotide exchanges, purines with purines and pyrimidine with pyrimidines, within the essential part of *attB* was inserted into the assay plasmid, and the effect of GFPuv expression was analyzed after transformation and incubation for 3 h with the Int-expressing plasmid, as described above. As can be seen in Fig. 4, the two single substitutions C13T and G14A on the left side of the presumed overlap region had no major effect on recombination after 3 h of incubation, 99 and 73% respectively. Surprisingly, the T15C exchange, located in the hypothetical overlap region (G14–A19) did not affect recombination. The G16A, T17C and G24A substitutions resulted in a reduced frequency of recombination, 57, 14 and 18% respectively, but after incubation overnight the recombination frequencies increased to 89, 66 and 88%, respectively (data not shown). Only four exchanges strongly affected the recombination frequency, namely A18G, A19G, G20A and G22A. The frequencies increased slightly after overnight incubation for A18G to 5% and G22A to 29% (data not shown), whereas the recombination frequencies for A19G and G20A remained very low of 0.5 and 1.8% respectively (of totally 3100 and 3233 colonies analyzed respectively).

The attB mutations T17C, A18G, A19G and G20A, but not G16A and G22A, are rescued by complementary mutations in attP

Int is expected to recognize the bases on either side of the overlap region, and a requirement for complementary sequences within the overlap region in *attB* and *attP* is expected for the isomerization step and the second cleavage-joining reaction in the recombination pathway. Thus, mutations restoring complementarity were introduced in the *attP* region of the recombination assay plasmid, for the mutations affecting the recombination frequency. As can be seen in Fig. 5, all mutations in *attB* analyzed, except for G16A and G22A, were rescued by the complementary mutation in *attP*. The recombination efficiency after overnight incubation with G22A *attB* mutation and the complementary mutation in *attP* remained low 3%. Thus it seems as if the four bases TAAG constitute the overlap region. The fact that G22A is not rescued by a complementary mutation in *attP*, supports the finding of the *attB* binding assay where Int was unable to bind to the *attB* site when the G22 nucleotide was changed to C.

Discussion

P2 integrase belongs to the complex family of tyrosine recombinases and it catalyzes the integration of the phage genome into the host genome by a mechanism similar to the well-characterized lambda system. In this work we show that the 10 first nucleotides of the 27 nt common sequence between the core of *attP* and *attB* are not required for efficient recombination. More likely, they are maintained

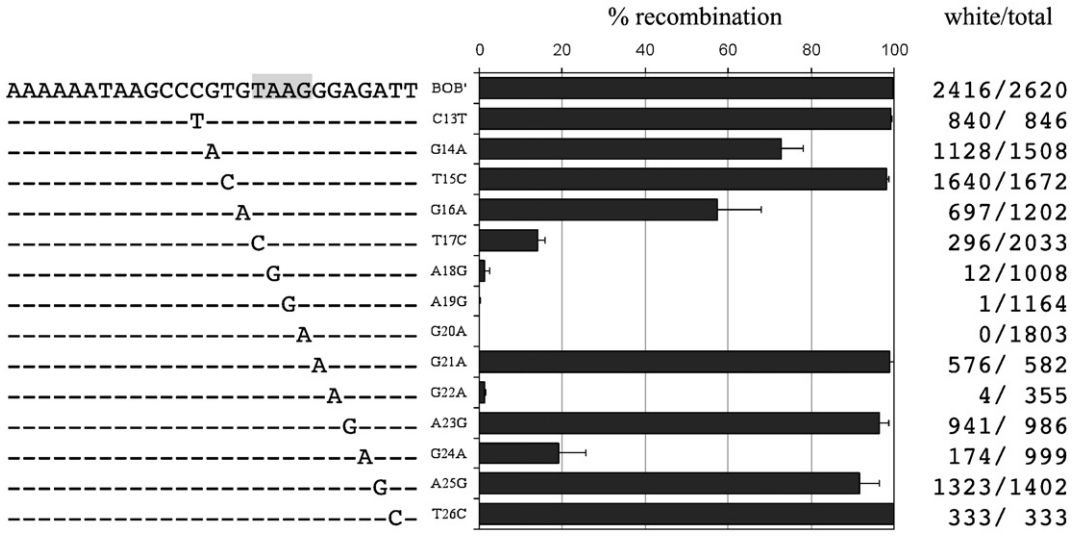

Fig. 4. An analysis of nucleotides in *attB* affecting recombination using the *in vivo* plasmid reporter system. The percent of white colonies over total number of transformed colonies after retransformation of the extracted plasmids are shown in the graph. The plasmids were extracted 3 h after transformation of the recombination assay plasmid. The sum of white colonies out of total number of colonies screened in all the experiments is given as white/total on the right side of the sequences and the standard deviations are based on the percentage obtained in independent experiments.

so as not to inactivate the *cyaR* gene upon integration. The core region contains an imperfect inverted repeat believed to be the recognition sequence for the C-terminal domain of the Int protein, and our finding that it binds very poorly to the left side but well to the right side, indicates that the right side is the primary recognition sequence. Only six single nt substitutions within the right half of the *attB* site have significant effect on recombination and out of these, four are rescued by a complementary mutation in the core of *attP*. Hence it seems as if the crossover region consists of the TAAG quadruplet, even though the T17C *attB* mutation shows a rather high frequency of recombination with wt *attP* after overnight incubation (66%). Plasmids from two white colonies of each single mutation were sequenced, and the mutations at positions 13–7 had maintained the mutation in *attL*, while the mutations 21–26 all had wild type *attL* sites supporting that this short sequence belongs to the crossover region (data not shown). The plasmids extracted from two white colonies after recombination between the A18G mutant and wt *attP*, generated wt *attL*. Plasmids from white colonies obtained after overnight incubation of the A19G and G20A mutations, also with wt *attP*, showed in the case of A19G one that had maintained the mutation and two others that showed illegitimate recombination while the G20A only showed illegitimate recombination. Probably, the Int promoted cleavage reaction leads to mutations and aberrant recombination since the heterologous intermediate is unable to resolve.

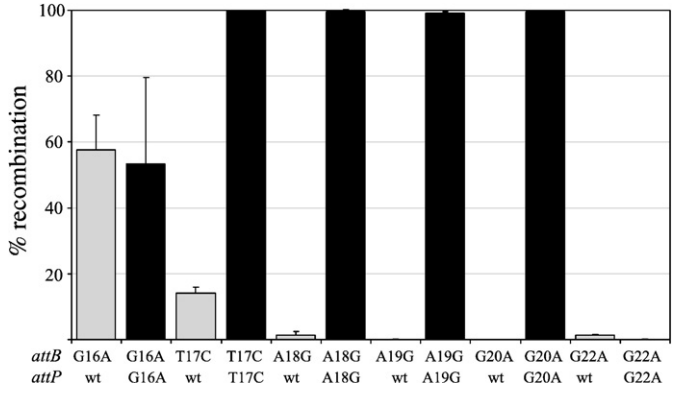

Fig. 5. The effect on recombination of *attB* mutants defective in recombination when introducing a complementary mutation in the core sequence of *attP*. The percentage of white colonies over total number of transformed colonies after retransformation of the extracted plasmids is shown. The plasmids were extracted 3 h after transformation of the recombination assay plasmid. The combination of *attB* and *attP* sites in the recombination reporter plasmids are given below the graph.

P2 has a preferred integration site in *E. coli* C, *locI*, but three secondary integration sites have been sequenced, *locII*, *locIII* and *locH* (Barreiro and Haggård-Ljungquist, 1992), and a point mutation, P2 *saf*, in the core of *attP* has been shown to give P2 a preference for *locII* instead of *locI* (Six, 1966; Yu et al., 1989). An alignment of the *attB* sites is shown in Fig. 6. There is an extensive variation in the sequence of the first 9 nt which fits with our finding that they are not required for efficient recombination. Furthermore, the alignment supports our finding that the TAAG quadruplet (nt 17–20) constitutes the overlap region since it is maintained in *locIII* and *H*. The fact that the *saf* mutation has a T17G mutation and that it favors *locII* instead of *locI*, and that we find a reduced frequency of recombination of T17C with the wt *attP*, site supports the importance of T17 for recombination. Furthermore nt G22, identified as essential for Int recognition is also conserved among the secondary integration sites.

Assuming that homology is required in the sequence separating the 1st and 2nd cleavage reaction, P2 has a short overlap region of 4 nt, and it is not centrally located relative to the previously identified imperfect repeats. However, the requirement for homology for the cleavage-joining reactions among the tyrosine recombinases has been challenged since some conjugative transposons have been shown not to require homology between the two cleavage sites (Rajeev et al., 2009). In the case of the IntDOT recombinase it has been shown that initial strand exchange is sequence identity-dependent, but the isomerization and second cleavage-joining reaction is sequence identity-independent (Laprise et al., 2010). Thus, it is possible that the P2 Int initiates recombination by a first cleavage between nt G20 and G21 and that Watson–Crick base pairing where the conserved TAAG quadruplet is located is needed for the first strand exchange.

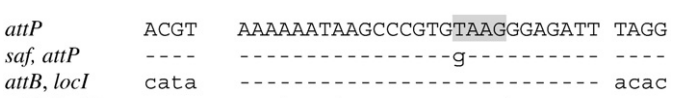

Fig. 6. An alignment of the *attB* sites of the preferred host integration site and secondary sites. Identical nucleotides are indicated by a dash.

This would be followed by an isomerization step and the second cleavage 6–7 nt to the left where sequence identity could be less crucial. Indeed, this would make the P2 site-specific recombination system more related to the conjugative transposons than to the lambda system, which will be analyzed in the future.

Materials and methods

Biological materials

E. coli strains C-1a (Sasaki and Bertani, 1965) or DH5α (Woodcock et al., 1989) were used for transformations. Plasmids pEE856, a pACYC177 derivative containing P2 *attB* site (Frumerie et al., 2008); pEE2007 a pET8c derivative containing the P2 *attP* region (Yu and Haggård-Ljungquist, 1993b); and pEE2026, a pACYC177 derivative containing the *int* gene and part of the *attP* site flanked by the T7 promoter and terminator (Frumerie et al., 2008) were used.

All oligonucleotides used in cloning were purchased from DNA Technology A/S (Denmark) or from Thermo Fisher Scientific (Germany). Restriction enzymes were purchased from Fermentas (Germany) and PfuTurbo and PfuUltra II Fusion HS polymerases used in site-directed mutagenesis (SDM) from Stratagene (Agilent Technologies, USA). Oligonucleotide sequences used for site-directed mutagenesis are available upon request.

Plasmid constructions

All constructs were performed according to standard procedures (Sambrook et al., 1989). All constructs were sequenced before use at Macrogen Inc. (Korea) or Eurofins MWG Operon (Germany).

pEE2027

A derivative of pEE2026 where *attP* was removed by amplification of the plasmid using primers on either side of *attP*, directed away from *attP* (KpnI-72.8R: ATC GGT ACC GCC CTT AAA CGT TAC TCT ACT GTG G and KpnI-pET8c541rev ATC GGT ACC CAT GAC TGG TGG ACA GCA AAT GG). The amplified region was ligated and transformed into DH5α.

pEE2029

Derivative of plasmid pEE856 where the right half was modified so that the inverted repeat corresponds to the left side denoted BOB (see Fig. 2a).

pEE2030

Derivative of plasmid pEE856 where the left half was modified so that the inverted repeat corresponds to the left side denoted B′OB′ (see Fig. 2a).

pEE2032 and 2033

Integrase binding assay plasmid. An AgeI restriction site was constructed in pUC18 by SDM, (at position 495–500). The restriction site is located upstream of the *lacZ* gene, between the RBS and promoter sequences. Oligonucleotides containing the core sequence were hybridized and inserted into the AgeI site. The right repeat (C′) of the core is next to P*lac* in pEE2032 and in pEE2033 the direction is inversed.

pEE2035

Recombination assay plasmid. The P2 *attP* region was amplified by PCR from pEE2007 and inserted between the EcoRI and ApaI sites of the pGFPuv vector (Clontech, USA) located downstream of the *GFPuv* gene. Oligos containing *attB* and derivatives were hybridized and inserted in frame within the *GFPuv* gene between the PstI and XbaI sites. All constructs are able to express the modified *GFPuv* gene.

In vivo recombination assay

The *in vivo* reporter plasmid pEE2035 and its derivatives were transformed into C-1a competent cells containing the Int-expressing plasmid pEE2027. Cells were allowed to recover for 1 h at 37 °C shaking before ampicillin (100 μg/ml) and kanamycin (50 μg/ml) were added. Incubation was continued for 3 h or overnight. The reactions were stopped by extraction of plasmids using the E.Z.N.A. Plasmid Mini Kit I (Omega Bio-Tek Inc., USA). Approximately 70 ng of purified plasmid was either directly transformed into C1-a or first cleaved with BglII (Fermentas) to remove pEE2027 and grown on LA plates containing ampicillin. The number of white colonies over the total number of transformants was determined in UV light. White colonies were randomly picked and analyzed by PCR using primers located in the vector outside the *attB* and *attP* insertions to confirm correct recombination, and at least one clone from each experiment was sequenced. The results are presented as the average percentage of white colonies of total number of transformants in all experiments, and the standard deviations are based on the average percentage of recombination in independent experiments (between 2 and 5 experiments).

In vitro recombination assay

The *in vitro* recombination assay was performed as described (Frumerie et al., 2008). The crude extract used was a 50/50 mix of a crude extract overexpressing the Int protein and the other one overexpressing the IHF protein. The protein concentration of the crude extracts was 13 μg/μl and was estimated to contain about 10% Int and about 1% IHF. The linear *attB* fragments were labeled in the 5′ ends using γ̄ [^{32}P]-ATP and polynucleotide kinase. Approximately 0.1 μl labeled linear fragment and approximately 1 μg supercoiled *attP* plasmid, 5 μl crude extract, 2.5 μl buffer (150 mM Tris–HCl, 25 mM NaCl, 450 mM KCl, 20 mM spermidine, 25 mM EDTA, 5% glycerol) and water to a final volume of 25 μl, was incubated for 2 h at 37 °C. 5 μl of Maxan dye solution (xylenecyanol 0.1%, bromophenol blue 0.1%, NaOH 10 mM, EDTA 1 mM, formamide 80%). Twelve μl of the sample was loaded on a 1% agarose gel. The gel was dried prior to phosphorimage analysis.

Site-directed mutagenesis (SDM)

Mutations in the *attB* and *attP* sites in various plasmids for the EMSA, the *in vitro* and *in vivo* recombination experiments were generated by site-directed mutagenesis using PfuTurbo or PfuUltra II Fusion HS polymerase. The mutations in *attB* had no effect on the capacity to express the *GFPuv* gene.

Electromobility shift assay

The wild type or mutated *attB* sites were amplified from plasmids pEE856, pEE2029, or pEE2030 using primers located on either side of the *attB* site generating 210 nt long DNA fragments. The fragments were labeled, incubated with crude extracts from cells overexpressing the *int* gene, run on a non-denaturing polyacrylamide gel and autoradiographed as described in Frumerie et al. (2005a).

Construction of library of substitutions in the right repeat of the core sequence

Plasmids pEE2032 and pEE2033 were used as substrates for SDM using the QuikChange® Multi Site-Directed Mutagenesis Kit (Stratagene) using the degenerate primer GTA AAA AAT AAG CCC GTG TAA SSS HKW WTA CCG GTC ACA ATT CCA C, where H = A/C/T, K = G/T, S = G/C, W = A/T. After the mutagenic PCR, the plasmids were first transformed into the competent cells supplied by the manufacturer,

and after growth overnight in LB supplemented with ampicillin (100 μg/ml) they were transformed into strain DH5α containing the compatible plasmid pEE2026 expressing P2 Int, whereafter black and white colonies on LA plates containing S-gal were picked for further analysis (sequencing).

Acknowledgment

This work was supported by the Swedish Research Council.

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