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Title:

New *Bacillus subtilis* vector, pSSβ, as genetic tool for site-specific integration and excision of cloned DNA, and prophage elimination

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Running head: *B.subtilis* integration/excision vector

Summary

Site-specific recombination (SSR) systems are employed in many genetic mobile elements, including temperate phages, for their integration and excision. Recently, they have also been used as tools for applications in fields ranging from basic to synthetic biology. SPβ is a temperate phage of the Siphoviridae family found in the laboratory standard *Bacillus subtilis* strain 168. SPβ encodes a serine-type recombinase, SprA, and recombination directionality factor (RDF), SprB. SprA catalyzes recombination between the attachment site of the phage, *attP,* and that of the host, *attB,* to integrate phage genome into the *attB* site of the host genome and generate *attL* and *attR* at both ends of the prophage genome. SprB works in conjunction with SprA and switches from *attB*/*attP* to *attL/R* recombination, which leads to excision of the prophage. In the present study, we took advantage of this highly efficient recombination system to develop a site-specific integration and excision plasmid vector, named pSSβ. It was constructed using pUC plasmid and the SSR system components, *attP*, *sprA* and *sprB* of SPβ. pSSβ was integrated into the *attB* site with a significantly high efficiency, and the resulting pSSβ-integrated strain also easily eliminated pSSβ itself from the host genome by the induction of SprB expression with xylose. This report presents two applications using pSSβ that are particularly suitable for gene complementation experiments and for a curing system of SPβ prophage, that may serve as a model system for the removal of prophages in other bacteria.

Key Words:

site-specific integration vector, site-specific recombination, SPβ, SSR, *Bacillus subtilis,* integrase, RDF

Introduction

Bacillus subtilis is a Gram-positive model organism which has genetic competence to uptake external DNA. In particular, *B. subtilis* 168 is a well studied strain in terms of its natural competence. Exogenous DNA can be integrated into the *B. subtilis* chromosome by RecA-driven homologous recombination (HR). This property has classically been used not only in gene disruption, but also in gene complementation experiments, which examine the regeneration of wild-type phenotypes through the insertion of an intact gene at a specific position of the chromosome in the mutant strain (**Harwood et al., 2013**). In complementation experiments, the double-crossover HR event is employed for the introduction of a single copy of native target gene using linearized plasmids or linear DNA fragments. However, to introduce the target gene into the chromosome by double-crossing over HR event, it is necessary to add 1-kb (or longer) of homologous DNA fragments at both sides of the corresponding region (e.g. *amyE* locus) on the host chromosome. Therefore, preparation of donor DNA for HR integration appears to be complicated. On the other hand, integration vectors have been employed to introduce exogenous target genes, which are not homologous to any host genes, into the host genome by single crossover HR. The integration vector harbors a host gene, such as *amyE*, to induce the occurrence of single crossover HR to the locus in the chromosome (**Harwood et al., 2013**). However, it is sometimes difficult to use for complementation experiments, because HR may occur between the target gene and its mutant locus in the chromosome.

Site-specific recombination (SSR) systems are employed for the integration of lysogenic phages and integrative conjugative elements (ICEs) into host genomes **(Groth and Calos, 2014; Johnson and Grossman 2015; Suzuki et al., 2020)**. In contrast to HR, SSR does not require such a long DNA region for the integration process; the recombination simply occurs between short sequences—*attP* in the phage genome and *attB* in the host genome—and is specifically catalyzed by a phage-encoded integrase. The SSR enzymes are broadly classified into two types of integrases: the serine-type and tyrosine-type. Although the former recognize specific nucleotide sequence with lengths of approximately 50 bp for each *attP* and *attB, attP,* the sequences of tyrosine-type integrases are relatively large (i.e., 240-bp for phage λ), and their SSR also requires host factors (i.e., IHF and Fis for phage λ) **(Laxmikanthan et al., 2016; Singh et al., 2014**).

Thus, the techniques for introducing exogeneous DNA into the host genome has been developed by utilizing the SSR system, especially with serine-type integrases. The advantage of utilizing a SSR harboring integrase gene (*int*) and *attP* in vectors is that they can integrate at a specific position (*attB*) in the host genome and can be stably retained. As a phage integrase induces recombination between *attP* and *attB*, transformation by vectors containing *attP* and the integrase gene (*int*) results in the integration of the vector into the bacterial genome at *attB*.

Serine-type integrases derived from phages φC31, φBT1, Bxb1 and R4 have been shown to be capable of promoting site-specific integration of DNA into their original, or other, host chromosome (**Fogg et al., 2014**). To date, various approaches using serine integrases have been developed as tools in synthetic biology **(Colloms et al., 2014; Merrick et al., 2018)**. In *B. subtilis,* site-specific tyrosine recombination systems, such as Cre/*loxP* from bacteriophage P1 and Xer/*dif,* have been reported (**Dong and Zhang, 2014; Pohl et al., 2013; Yan et al., 2008**). However, the construction of site-specific integration vectors harboring serine-type integrase genes in *B. subtilis* have rarely been reported.

During phage lysogeny, integrases lead to the formation of the hybrid attachment sites *attL*/*attR* at the junctions between *attP* and *attB*. A phage genome integrated into the host genome's *attB*, which is called prophage, contains *attL* and *attR* at both ends of the prophage genome. To excise the phage DNA from its host genome, recombination between *attL*/*attR* sites is performed by the integrase with a phage-encoded recombination directionality factor (RDF) or Xis. RDF accesses its cognate integrase and changes the direction of DNA recombination from integration to excision (**Fogg et al., 2014; Merrick et al., 2018**). So far, almost no attempt has been made to adapt an excision system by SSR into an integration vector and to use it for gene manipulation.

In this study, we developed a vector system based on the site-specific recombination apparatus of SPβ. The *attP* site, *sprA* (serine-type integrase), and *sprB* (RDF) were cloned into a plasmid to generate the site-specific integration plasmid vector pSSβ. It was delivered successfully into the chromosomal DNA of a SPβ-free *B. subtilis* strain. The integrated pSSβ was easily excised from the host genome and placed back into the parental genotype again, and consequently it was then available for the gene

complementation experiment. Furthermore, taking advantage of the characteristics of pSSβ, an efficient method for curing the SPβ prophage from its lysogen using pSSβ was developed. As some lysogenic phages convert non-pathogenic bacteria into pathogenic bacteria by lysogenization (**Asadulghani et al., 2009; Kim et al., 2017**), the system for prophage removal reported in this study is expected to be a practical approach to overcome prophage-based bacterial infectious diseases.

Materials and Methods

Bacterial strains, plasmids, and general methods

Table S1 lists the strains and plasmids used in this study. The oligonucleotide primers used for PCR amplification are listed in Table S2. Bacterial cultures were grown in liquid or solid Luria-Bertani (LB) (**Sambrook and Russell, 2001**), or CI medium (**Dubnau and Davidoff-Abelson, 1971**). Solid media were fortified with 1.5% agar (Wako). The Min-CH medium (**Rutberg, 1969**) was Spizizen's minimal glucose medium supplemented with 0.05% amicase (Sigma). Antibiotics for the selection of various *B. subtilis* strains were used at the following concentrations: chloramphenicol, 5 μg/ml; ampicillin, 100 μg/ml added, 1% (w/v) xylose added to the medium if indicated. Plasmids were constructed in *E. coli* strain DH5α.

Table S1

Table S2

Plasmid construction

pSSβ is a derivative of the vector, pCA191 and pMF20 (**Murakami et al., 2002**). A 1,977-bp *sprA* fragment, amplified by **P1** and **P2,** was ligated with the inverse PCR fragment of pCA191 amplified by **P3** and **P4** by Gibson assembly reaction, designated as pCAsprA. The *Hin*dIII/*Bam*HI-digested *sprB* fragment (741-bp), amplified by **P5** and **P6,** was ligated with the *Hin*dIII/*Bam*HI digested pMF20, designated as pMFsprB. To remove multiple cloning sites, **P7** and **P8** were used to amplify pMF20sprB, and the fragment was self-assembled by Gibson assembly reaction, designated as pMFsprBΔ35. The xylose-inducible promoter, *xylR*, and *sprB* region of pMF20sprBΔ35 were amplified by **P9** and **P10**. The resulting pMF20 fragment and pCAsprA, amplified with **P11** and **P12,** were ligated by Gibson assembly reaction, designated as pSSβpro. To remove multiple *Bam*HI sites, pSSβpro was amplified with primers **P13** and **P14,** and self-assembled by Gibson assembly reaction. The resulting plasmid was designated as pSSβ (DDBJ accession number LC648923).

To construct pCAamyE, a 537-bp internal segment of the *amyE* gene, amplified by **P15** and **P16,** was digested by *Bam*HI/*Hin*dIII and ligated with the *Bam*HI/*Hin*dIII-digested pCA191.

To construct pSSβ-trpC, primers **P17** and **P18,** and **P19** and **P20** were used for the

amplification of the promoter region of the tryptophan operon and *trpC* gene of NCIB3610, respectively, using NCIB3610 genome DNA as template. The amplified fragments were ligated by overlap PCR using primers **P17** and **P20**. The resulting fragment was digested with *Eco*RI and *Xba*I, and then ligated with *Eco*RI/*Xba*I digested pSSβ.

The constructed strains and primers used in this study are shown in Table S1 and Table S2, respectively.

Measurement of *B. subtilis* transformation efficiency

Competent cells were prepared following a protocol previously described in **Dubnau and Davidoff-Abelson (1971**) with some modifications. A single colony of the *B. subtilis* strain to be transformed was inoculated into LB medium and incubated for 16 hours at 37°C while being subjected to shaking. It was then diluted to an OD_{600} of 0.04 in 3 ml of CI medium and grown at 37°C until the early-stationary phase, with agitation, to induce the competent state. An aliquot of 0.2 ml of CI competent cells was mixed with 200 ng of DNA (final concentration, 1 ng/μl) and incubated at 37°C for 1 hour with agitation, and cells were plated into LB plates containing appropriate antibiotics to determine colony forming units. These plates were incubated at 37°C for overnight and colonies were counted. The integration of plasmids was verified through colony PCR amplification (**Suzuki et al., 2020**) using primers of **P21**/ **P22** for pSSβ and pSSβ-trpC, **P23**/ **P24** for pCAamyE.

pSSβ excision and elimination assay on plates

SPless-pSSβ cells were inoculated into LB, LB+Cm, and LB+1% xylose medium and grown at 37°C for 16 hours with shaking. These cells were serially diluted by 10-fold and spotted onto LB or LB supplemented with chloramphenicol plates, and were then incubated at 30°C for 16 hours.

pSSβ excision and elimination assay in liquid medium

SPless-pSSβ cells were grown in LB supplemented with chloramphenicol medium at 37°C for 16 hours with shaking. The cell culture was diluted into fresh LB medium at

1:100, and grown to OD600 ≈ 0.2 at 37°C with shaking. Then 1% xylose was added into the cell culture and was plated on to the LB and LB supplemented with chloramphenicol plates. The frequency of pSSβ-eliminated cells was calculated by dividing the number of chloramphenicol resistant cells by the number of total cells.

SPβ prophage curing using pSSβ

Preparation of competent cells of *B. subtilis* 168, SPβ lysogen, was performed as described above; 200 ng of pSSβ was added to 0.2 ml of competent cells (final concentration, 1 ng/μl) and incubated at 37°C for 15 min with shaking. Subsequently, 1% xylose was added into the cells and these were further incubated for 1 hour and 30 min. The xylose-treated cells were centrifuged at 8000 ×*g* for 3 min, and the cell pellets were washed using 0.2 ml of fresh LB, twice. The washed cells were plated on the LB supplemented with chloramphenicol plate and incubated at 37°C. The obtained chloramphenicol resistant cells were selected and subjected to single colony isolation on a LB plate containing 1% xylose. The chloramphenicol sensitivity of these single colonies was confirmed by placing them on chloramphenicol containing plates. The generated $attB_{SP\beta}$ and presence of SPβ prophage sequences were verified using colony PCR amplification, as described in **Suzuki et al. (2020),** using primers **P22**/ **P25** for $attB_{SP\beta}$, and **P26**/ **P27** for curing the SPβ prophage. The sequence of the generated $attB_{SP\beta}$ was also confirmed by Sanger-sequence analysis using primers **P22**/ **P25**.

Results

Construction and characterization of the site-specific integration and excision vector, pSSβ

Integration and excision of SPβ was regulated by the serine-type integrase, SprA, and its cognate RDF, SprB (**Fig. S1**; **Abe et al., 2014**). SprA catalyzed integration between the *attP* (52-bp) of the SPβ genome and the *attB* (44-bp) of the *B. subtilis* genome which was located within the *spsM* gene **(Fig. 1A and S1; Abe et al., 2017)**. The integration reaction generated the prophage genome with new attachment sites, *attL* (48-bp) and *attR* (48-bp), which were hybrids of *attP* and *attB*. The *sprA* and *sprB* genes were located proximal to *attP* site in SPβ genome **(Fig. S1)**. As SprA is constitutively transcribed by a promoter for the major σ factor, σ^A, the SPβ prophage genome is stable in the host genome. SprB is expressed only when the SPβ prophage is induced by stress such as UV radiation, and it works in conjunction with SprA to switch the recombination direction from *attB-attP* to *attL-attR,* to excise the prophage genome. In the pSSβ vector, *sprA* was cloned with its native σ^A promoter and $attP_{SP\beta}$ region adjacent to *sprA*, and *sprB* was located downstream of the xylose-inducible promoter P_{xylA} to regulate its transcription and control the excision reaction **(Fig. 1B)**. pSSβ also contains ampicillin (*bla*) and chloramphenicol (*cat*) resistant genes as selection marker for the transformation of the SPβ-cured strain (SPless). Because pSSβ carries only the pUC-derived origin of replication, it can be replicated in *E. coli* but not in *B. subtilis*. Fig.1

To examine the transformation efficiency of pSSβ, SPless was transformed with pSSβ and selected chloramphenicol resistant cells. As a transformation control, pCAamyE was used, which harbors 537-bp of the internal segment of the *amyE* gene to be integrated into the *amyE* locus by single crossover recombination event. The transformation efficiency of pSSβ was approximately 500-fold higher than that of pCAamyE **(Table 1)**. The integration of plasmids into the target site in these transformants was confirmed by PCR, and both pSSβ and pCAamyE were specifically integrated into the target sites, *attB* and *amyE* locus, respectively. These results indicate that pSSβ is a promising integration cloning vector to achieve a considerably higher integration efficiency than that obtained by single crossover HR event using pCAamyE.

We next confirmed the excision of pSSβ from its host genome by induction of *sprB* expression with xylose. The pSSβ containing cells were grown in LB medium, with or without 1% xylose, at 37°C for 16 hours. These cultures were serially diluted and spotted on LB or chloramphenicol (Cm) containing LB agar plates. On Cm plates, the number of Cm resistant cells were substantially decreased in xylose-treated cells, and in this cell population the loss of pSSβ was approximately 1000-fold higher than in non-treated cells **(Fig. 2A)**. We next examined the timing of pSSβ excision by induction of *sprB* expression. The pSSβ integrated strain was grown in LB medium, and xylose was added at $OD_{600} \approx 0.2$. The cells were harvested at indicated time points, and then plated on LB and LB+Cm plates. The frequency of pSSβ-eliminated cells was calculated as described in the Materials and Methods section. As shown in **Fig. 2B**, approximately 80% of pSSβ was eliminated two hours after the induction, and 99.6% was eliminated from host cells after 24 hours **(Fig. 2B)**. A decrease in the number of pSSβ-carrying cells grown for 24 hours in the absence of xylose was also observed, which is probably due to leaky expression of *sprB* in the absence of xylose-inducer. These results indicate that pSSβ can be rapidly excised from the genome by the induction of *sprB* expression and the host genome reverts to the parental strain. Fig. 2

Complementation analysis of *B. subtilis* 168 tryptophan auxotroph using pSSβ

B. subtilis 168 (*trpC2*) is tryptophan auxotrophic because of a mutation of the *trpC* gene coding the indole-3-glycerol-phosphate synthase enzyme **(Albertini and Galizzi, 1999)**. In contrast to 168, the ancestral strain of *Bacillus subtilis* NCIB3610 (3610) exhibits tryptophan prototrophy. A 99.6% homology exists between the 3610 *trpC* and 168 *trpC* genes. We next attempted the cloning of 3610 *trpC* into pSSβ and examined whether this gene was able to complement the tryptophan auxotrophy of 168 or not. The promoter region of the 3610 tryptophan operon (P_{trp}) homologous to that of 168 (**Shimotsu and Henner, 1984**) was amplified and connected with the *trpC* gene by overlap PCR, and the connected unit was cloned into the MCS of pSSβ, designated as pSSβ-trpC (**Fig. 3A**). The pSSβ-trpC was used to transform the SPless strain, and chloramphenicol (Cm) resistant cells were obtained. The integration of pSSβ-trpC into the $attB_{SP\beta}$ site of the chromosome was confirmed and it showed that pSSβ-trpC was Fig. 3

correctly integrated into the target region (**Table 1**). This result indicates that even if pSSβ contains a gene (*trpC*), which is almost identical to the gene (*trpC2*) in the host chromosome, the integration position of pSSβ-trpC in the host chromosome is in preference to *attB* than the gene (*trpC*) where HR may occur. All Cm^r transformants streaked on minimal medium (Min-CH) agar plates could grow normally. **Fig. 3B** (-xylose) shows the cell growth of one of the Cm^r transformants containing pSSβ-trpC on Min-CH, and it indicates that pSSβ is an effective vector for complementation experiments. If it were shown, not only that the phenotype was restored to the wild-type strain by the introduction of *trpC,* but also that the resulted strain was reverted to tryptophan auxotrophy (*trpC2*) by the elimination of *trpC*, then the complementarity would be confidently confirmed. **Fig. 3B** (+xylose) showed that the cells were reverted to tryptophan auxotrophy by the elimination of pSSβ from the host genome through induction of SprB expression with xylose. Together, these results suggest that the integration and excision of additional genes at the ectopic region could be performed by using pSSβ, even if the genes were highly homologous, to induce HR with the gene in the host genome. Therefore, pSSβ is also utilized for complementation and excision assays using homologous sequence genes; in other words, pSSβ can reversibly change the phenotype of the strain using homologous genes.

Curing the SPβ prophage using pSSβ

It has been reported that the induction of *sprB* in SPβ lysogen leads to excision of the prophage genome from the host genome without cell-lysis **(Abe et al., 2014)**. Thus, it should be possible to cure the SPβ prophage by using pSSβ. To examine this possibility, we developed the following strategy. Competent cells of *B. subtilis* SPβ lysogen were transformed with pSSβ and were incubated for 15 min to uptake pSSβ. The competent cells were then treated with xylose (final concertation 1%, vol/vol) for 1 hour and 30 min at 37ºC. These cells were centrifuged and washed with fresh LB medium twice to remove xylose, and plated on LB+Cm agar plates **(Fig. 4A)**. Approximately 6×10^3 /ml of Cm resistant colonies was obtained. The cells were transferred on to the LB+Cm+xylose plate, and 81.6±11.8% of them showed severe growth inhibition. Subsequently, the Cm resistant colonies were selected and streaked on LB+xylose, and Cm sensitive colonies were

Fig. 4

obtained. The absence of SPβ in the Cm sensitive cells was examined by PCR amplification of the internal region of SPβ and $attB_{SP\beta}$ site (**Fig. 4B**). The sequence of generated $attB_{SP\beta}$ was analyzed by Sanger sequencing method, and the construction of the intact $attB_{SP\beta}$ site was confirmed (**Fig. 4C**). Therefore, these results indicate that pSSβ is a useful tool for SPβ prophage-curing vector.

Discussion

In this study, we utilized the SSR of a SPβ phage to develop a genome integration and excision vector, referred to as pSSβ, which had the following advantageous features: (1) highly efficient integration, (2) excision from the genome by xylose treatment, and (3) curing of its cognate phage, SPβ.

The first notable point about pSSβ is its high integration efficiency. As shown in Table 1, pSSβ and its derivatives integrated completely at the *attB* site. This result indicates that *sprA* in pSSβ functionally expressed and accurately catalyzed DNA recombination between *attP* and *attB*. The integration frequency of pCAamyE, which codes 537-bp of internal *amyE* region, was very low, whereas the integration efficiency of pSSβ was 500-fold higher than that of pCAamyE. In this experiment, pSSβ and pCAamyE were uptaken at an equivalent level by competent *B. subtilis* cells, indicating that SSR using pSSβ is considerably more efficient than the single crossover recombination event using pCAamyE. We also confirmed that pSSβ can be used as complementation assay. The ancestral *B. subtilis* strain NCIB3610 showed homology of the *trp* operon of *B. subtilis* 168 with *trpC2* mutation (**Albertini and Galizzi, 1999**). pSSβ-trpC was specifically integrated into *attB* and it complemented the auxotrophy of the *B. subtilis* phenotype, indicating that pSSβ can be used for this type of complementation assay. However, it is necessary to be careful that, due to an integration of pSSβ within sporulation-related gene *spsM* which is polysaccharide synthesis gene, pSSβ-carrying cells will form spore without polysaccharide layer, and sporulation frequency might be slightly decreased compared with a wild type strain. (**Abe et al., 2014**).

The most unique feature of pSSβ is that it can be removed from the host genome by induction of SprB expression with xylose. To date, some reports are available on the construction and analysis of this eliminable vector. For example, replication temperature-sensitive (Ts) multicopy plasmid vectors can replicate only at low temperatures, so the plasmid is eliminable from the host if temperature is shifted during incubation of the host cell (**Maguin et al., 1992**). The Ts plasmid vector is also used for temporal gene induction, similarly to transposase-encoded plasmids pIC333 (**Steinmetz and Richter, 1994**) and pMarA/B (**Le Breton et al., 2006),** to generate random mutagenesis. The difference

between the removal methods using the Ts plasmid or pSSβ is that the former represents an elimination system for replicable multicopy plasmids in the cytoplasm, while the latter is an elimination system for single copy plasmids integrated in the host genome. This study showed that the excision of pSSβ occurred rapidly after xylose treatment, and about 80% of the plasmids were removed within two hours. Efficient excision was also confirmed using pSSβ-*trpC*. Therefore, pSSβ can provide unique methods to analyze the real-time phenotype after the removal of genes while incubating in liquid medium.

Moreover, pSSβ was used as a phage curing tool for the SPβ prophage. This curing method consists of two processes, (1) by induction of the *sprB* gene, the SPβ prophage is removed from *attB,* and subsequently pSSβ is integrated into *attB* to select SPβ-lost cells, and (2) pSSβ is excised and antibiotic-sensitive cells are selected (**Fig. 4A**). After these processes, the SPβ-cured strain can be used as a recipient cell for pSSβ. To obtain the prophage-cured strain, a counter selection approach using *sacB* **(Euler et al., 2016; Shmidov et al., 2021),** and an approach based on the isolation of thermoinducible lysogen **(Shimizu-Kadota and Sakurai, 1982)** were previously reported. However, these approaches require a long time to select cells without the prophage. As shown in the present study, strains that were prophage-cured by pSSβ might be selected more accurately and smoothly than those obtained by using previously reported methods. It has been reported that some bacterial phages have pathogenic genes, and they can cause their host cells to become pathogenic through infection, e. g., the CTX prophage of *Vibrio cholerae* (**Kim et al., 2017**) and enterohemorrhagic *Escherichia coli* (EHEC) (**Asadulghani et al., 2009**). Therefore, curing phages from their host cells can avoid the risks of pathogen transfer, and the phage curing approach using integration and excision vectors—such as pSSβ—could be applied for the detoxification of these pathogens. While, to construct the integration and excision vectors, an integrase and its cognate RDF gene are needed to be identified. RDFs are typically small and have diverse amino acid sequences, therefore it might need time and some efforts to develop of pSSβ-like vectors for curing uncharacterized prophages. In other words, if the genes encoding integrase and RDF in the prophage genome were identified, it is possible to create pSSβ-like vectors for curing the prophage.

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Figure legends

Fig. 1. Integration of pSSβ into *attB*$_{SPβ}$. A: Minimal attachment site sequence of *attP*$_{SPβ}$ and *attB*$_{SPβ}$ required for recognition and recombination of SprA. The core sequence is shown in bold. Arrows denote inverted repeat sequences. Underlines indicate the central nucleotides of *attP*$_{SPβ}$ and *attB*$_{SPβ}$. B: pSSβ contains *sprA*, *sprB*, and *attP*$_{SPβ}$ derived from the SPβ phage, *sprA* is a serine recombinase regulated by a constitutive expression promoter (P$_{sprA}$), *sprB* is a recombination directionality factor (RDF) controlled by a xylose-inducible promoter (P$_{xylA}$). Multicloning site (MCS) is located within *lacZα* gene. Cloning of a gene of interest can use the MCS and the positive colonies can be selected by blue/white selection method in *E. coli*. Abbreviations: *cat*; chloramphenicol resistant gene, *bla*; ampicillin resistant gene, *xylR*; xylose repressor.

Fig. 2. pSSβ excision and elimination by xylose treatment. A: pSSβ-eliminated cells after xylose treatment; pSSβ harboring cells were grown in LB medium, with or without 1.0% xylose, at 37°C for 16 hours; 5 ul of serially diluted cells were spotted on LB or chloramphenicol plates. B: Frequency of pSSβ-carrying cells after xylose treatment; pSSβ-carrying cells were grown in LB medium and added with 1.0% xylose at $OD_{600} \approx$ 0.2. Cells were plated on LB or chloramphenicol plates at indicated time points. The frequency of the Cm resistant colonies after xylose-treated cells and -untreated cells are indicated white and black circles, respectively. The bottom panel shows the movement of excised plasmids in growing cells and their resistance to chloramphenicol. Abbreviations: Cm^r; resistant cell, Cm^s; sensitive cell.

Fig. 3. Complementation analysis of *B. subtilis* 168 tryptophan auxotroph using pSSβ. A: Schematics of pSSβ-trpC; the putative promoter of the tryptophan operon (P$_{trp}$) and *trpC* derived from NCIB3610 were cloned into pSSβ. B: Excision and elimination assay of pSSβ-trpC by xylose treatment on Min-CH agar plate.

Fig. 4. Curing of the SPβ prophage using pSSβ. A: Schematic representation of the SPβ prophage curing process using pSSβ. B: Detection of SPβ genome DNA and *attB*$_{SPβ}$ site;

PCR amplified fragments were analyzed by 0.5% agarose electrophoresis. The positions of used primers, P22/P25 for *attB*$_{SP\beta}$ and P26/P27 for internal SPβ, are indicated in A with gray arrow heads. C: Sequence analysis of generated *attB*$_{SP\beta}$ by curing SPβ prophage; the top sequence shows the native *attB*$_{SP\beta}$, and the bottom sequence shows the generated *attB*$_{SP\beta}$. The bottom panel shows sequencing chromatograms of the generated *attB*$_{SP\beta}$ sequence. Bold characters indicate the core sequence that the site of strand exchanges. Underlines indicate the central nucleotides of *attB*$_{SP\beta}$.

Fig. S1. Site-specific recombination of SPβ

Schematic representation of the SPβ integration/excision reaction with the host genome. *sprA* is a serine recombinase regulated by a constitutive σ^A dependent promoter (P_{sprA}), *sprB* is a recombination directionality factor (RDF) controlled by a mother cell-specific $\sigma^{E/K}$-dependent sporulation promoter ($P_{E/K}$) and stress inducible σ^A dependent promoter (P_{St}).

A


attP SPβ
gtagtaagtatcttaaaaaACAGATAAAGCTGTATattaagatacttactac
attB SPβ
GTCATTAATATCAGTACAGATAAAGCTGTATCTCCTGTGAACAC

B

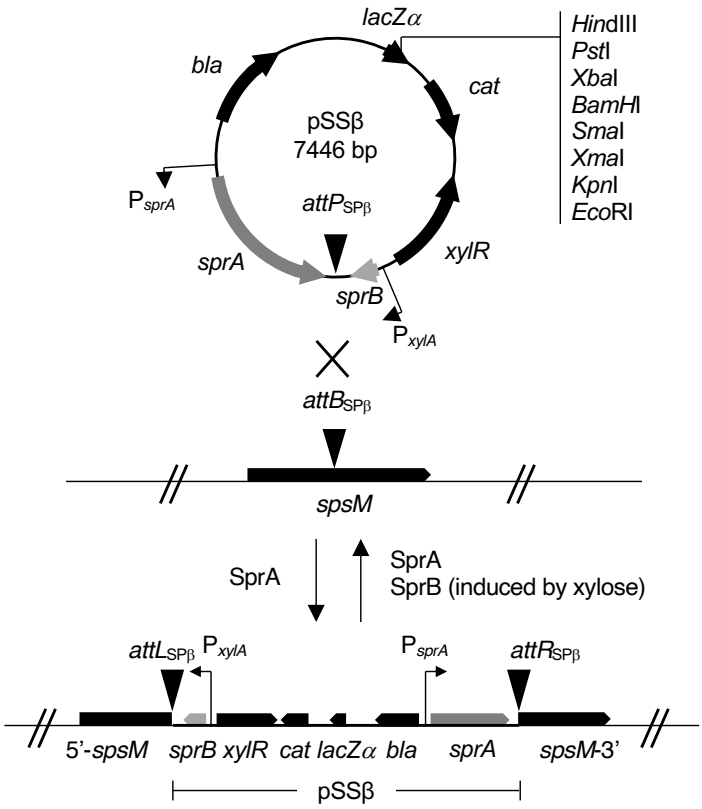
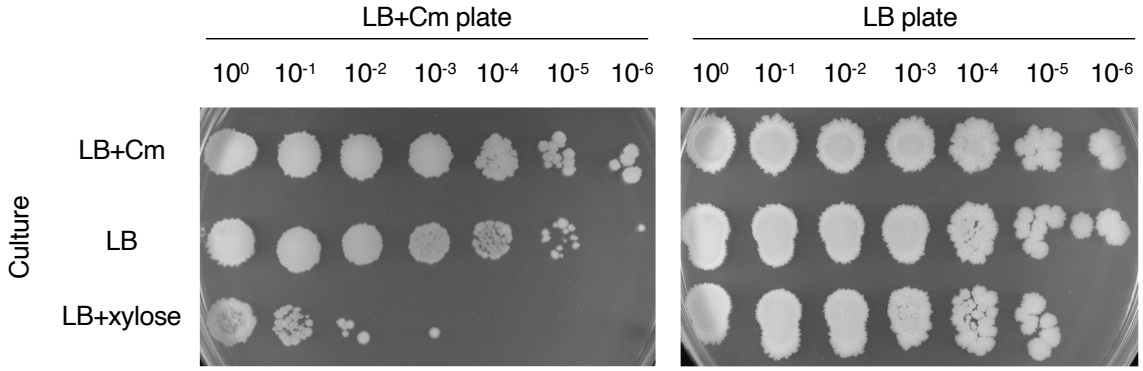
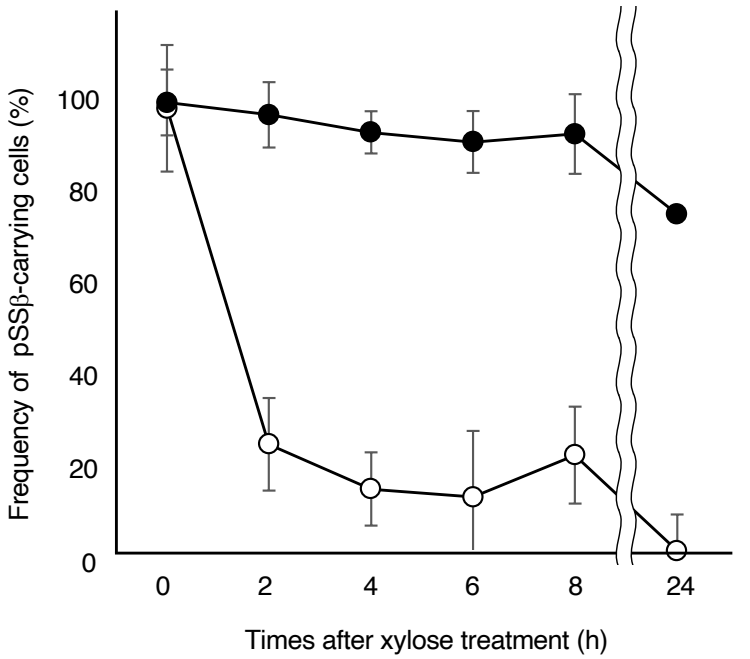

lacZα
HindIII
PstI
XbaI
BamHI
SmaI
XmaI
KpnI
EcoRI
bla
cat
pSSβ
7446 bp
P sprA
attP SPβ
sprA
xylR
sprB
P xylA
attB SPβ
spsM
SprA
SprA
SprB (induced by xylose)
attL SPβ
P xylA
P sprA
attR SPβ
5'-spsM sprB xylR cat lacZα bla sprA spsM-3'
pSSβ

FIG. 1

A


LB+Cm plate
LB plate
10^0 10^-1 10^-2 10^-3 10^-4 10^-5 10^-6
10^0 10^-1 10^-2 10^-3 10^-4 10^-5 10^-6
Culture
LB+Cm
LB
LB+xylose

B


Frequency of pSSβ-carrying cells (%)
100
80
60
40
20
0
0
2
4
6
8
24
Times after xylose treatment (h)

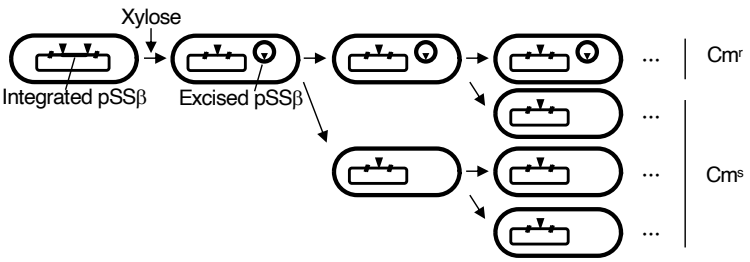
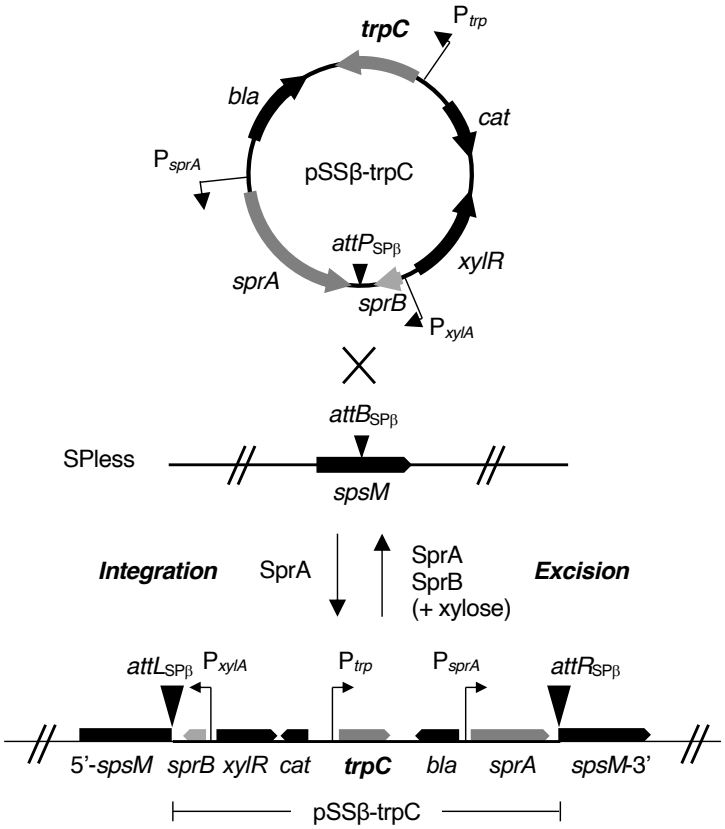

Xylose
Integrated pSSβ
Excised pSSβ
Cm^r
Cm^s

FIG. 2

A


trpC
P trp
bla
cat
P sprA
pSSβ-trpC
attP SPβ
xylR
sprA
sprB
P xylA
attB SPβ
SPless
spsM
Integration
SprA
SprA
SprB
(+ xylose)
Excision
attL SPβ
P xylA
P trp
P sprA
attR SPβ
5'-spsM sprB xylR cat trpC bla sprA spsM-3'
pSSβ-trpC

B

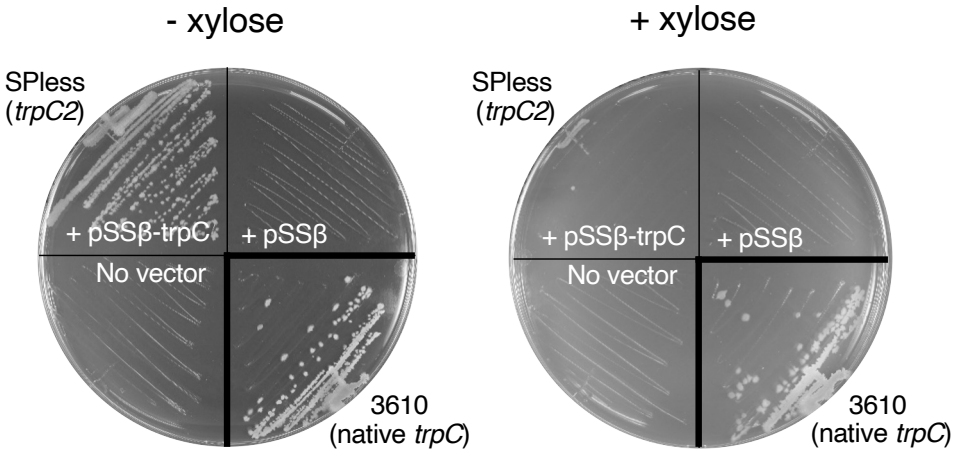
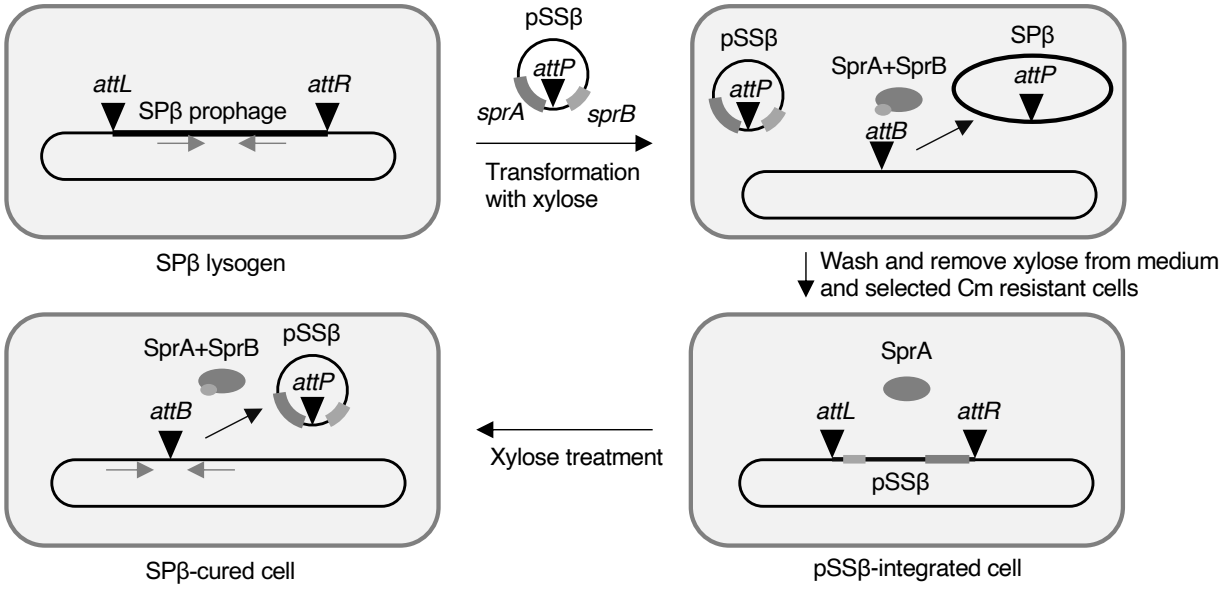
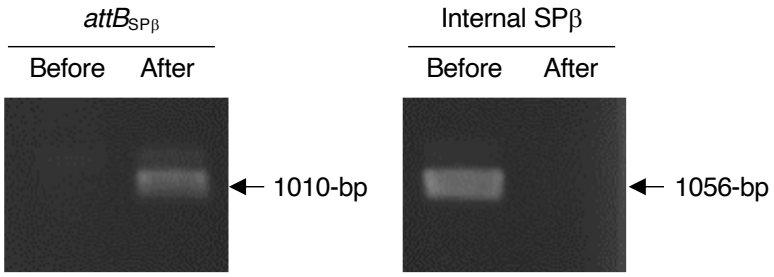

- xylose
+ xylose
SPless
(trpC2)
+ pSSβ-trpC
+ pSSβ
No vector
3610
(native trpC)
SPless
(trpC2)
+ pSSβ-trpC
+ pSSβ
No vector
3610
(native trpC)

FIG. 3

A



B



C

attB$_{SPβ}$ GTCATTAATATCAGT**ACAGATAAAGCTGTAT**CTCCTGTGAACAC

SPβ-cured strain GTCATTAATATCAGT**ACAGATAAAGCTGTAT**CTCCTGTGAACAC

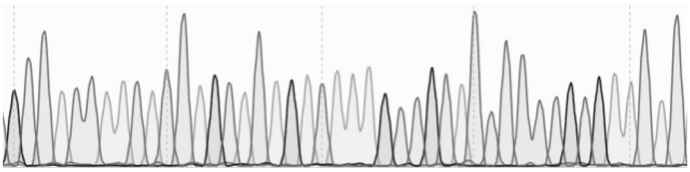


FIG. 4

Table 1. Transformation efficiency of the site-specific integration vectors.

Plasmid	Recipient strain	Relevant features in *B. subtilis*	Transformation efficiencies (CFU /ml)[a]	Integration efficiencies at a*myE* or *attB* site in s*psM*[b]
pSSβ	SPless	*cat*	9.7 (± 5.8) × 10^3	100% at $attB_{SP\beta}$
pCAamyE	SPless	*cat amyE* (537 bp)	1.9 (± 1.2) × 10	100% at *amyE*
pSSβ-trpC	SPless	*cat trpC*	4.9 (± 3.4) × 10^3	100% at $attB_{SP\beta}$
pCA191	SPless	*cat*	< 1.0 × 10	ND[c]
pSSβ	168	*cat*	5.7 (± 4.8) × 10^3	NT[d]

[a]The data shown are the average of three independent experiments ±SD.

[b]Ten transformants were investigated in three independent expreriments.

[c]ND, not detected.

[d]NT, not tested.