Compatibility of Site-Specific Recombination Units between Mobile Genetic Elements

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HIGHLIGHTS
Lysogenic phage-derived SSR unit is sufficient to drive SSR of ICE and vice versa
Defective prophage-derived SSR unit can drive the excision of the active lysogenic phage
Closely related prophages with distinct SSR units control each gene rearrangements
Correspondence between MGEs and their cognate SSR units is not absolute

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Article

Compatibility of Site-Specific Recombination Units between Mobile Genetic Elements

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SUMMARY

Site-specific recombination (SSR) systems are employed for transfer of mobile genetic elements (MGEs), such as lysogenic phages and integrative conjugative elements (ICEs). SSR between attP/I and attB sites is mediated by an integrase (Int) and a recombination directionality factor (RDF). The genome of Bacillus subtilis 168 contains SPb, an active prophage, skin, a defective prophage, and ICEBs1, an integrative conjugative element. Each of these MGEs harbors the classic SSR unit attL-int-rdf-attR. Here, we demonstrate that these SSR units are all compatible and can substitute for one another. Specifically, when SPb is turned into a defective prophage by deletion of its SSR unit, introduction of the SSR unit of skin or ICE converts it back to an active prophage. We also identified closely related prophages with distinct SSR units that control developmentally regulated gene rearrangements of kamA (L-lysine 2,3-aminomutase). These results suggest that SSR units are interchangeable components of MGEs.

INTRODUCTION

Bacterial viruses (bacteriophages or phages) infect bacterial host cells by injecting their genetic material. Phage virions consist of a protein coat that protects a DNA or RNA genome. Lytic (virulent) phages cause host cells to lyse after production of viral particles. Lysogenic (temperate) phages can switch between the dormant state (prophage), where the phage genome is integrated into the host chromosome, and the productive state, following excision of the phage genome from the chromosome. The loci where lysogenic phages integrate into the host genome are known as attachment (att) sites. For example, the bacteriophage λ genome, which carries an attP site, integrates in the genome of its host Escherichia coli at the attB site located between the bio and gal operons (Campbell, 1962; Landy and Ross, 1977). Similarly, SPb from Bacillus subtilis has an attachment site within spsM, a gene required for spore polysaccharide synthesis (Abe et al., 2014, 2017b).

The basic genetic unit of site-specific recombination (SSR) systems in the genome of an integrated mobile genetic element (MGE) is attL-int-rdf-attR (SSR unit) (Groth and Calos, 2004). The phage-bacteria junctions, attL (left) and attR (right), are hybrids of the attP and attB sites. Attachment sites are recognized by an integrase, which either catalyzes phage integration (by recombination of attP and attB) or, conversely, excision of the DNA comprised between attL and attR. In addition, excision reactions require phage-encoded small proteins, known as recombination directionality factors (RDFs) (Ghosh et al., 2006; Fogg et al., 2014; Merrick et al., 2018). Integrases can be categorized into Ser (Large Ser-type Recombinase; LSR)- or Tyr-type families. Each type, however, uses common features to promote recombination between specific attP and attB sites. The bacteriophage λ integrase, Int, belongs to the Tyr-type family, whereas SprA (the SPb integrase) belongs to the LSR-type family. Each excision reaction requires an RDF, known as Xis for λ and SprB for SPb (Groth and Calos, 2004; Laxmikanthan et al., 2016; Abe et al., 2014; Abe et al., 2017b; Olorunniyi et al., 2017). Integrative conjugative elements (ICEs) are another type of bacterial MGEs that can be transferred between cells by conjugation and subsequent integration via the SSR mechanism (Johnson and Grossman, 2015). In the B. subtilis ICE, ICEBs1, integration and excision reactions are catalyzed by a Tyr-type integrase (IntICEBs1) and an RDF (XisICEBs1), respectively (Lee et al., 2007).

Yet, in bacterial genomes like B. subtilis strain 168, these SSR units can be carried simultaneously by active prophages, ICEs, defective prophages, or non-prophage-like elements. Inactive prophages are often

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observed in genomes of spore-forming bacteria. Many of these are found in sporulation-specific genes (Stragier et al., 1989; Sato et al., 1990; Serrano et al., 2016). A classic example is the inactive prophage skin, which interrupts sigK, a gene encoding a mother cell-specific σ factor. The mother cell is one of the two cell types (the other being the forespore) generated after asymmetric division of the sporulating cell. Importantly, both cell types receive identical copies of the bacterial genome after division, but because the mother cell eventually lyces and the forespore matures into a spore, the spore genome can be viewed as the germ cell genome that will retain the inactive prophage. Several other genes specifically expressed in the mother cell of spore forming bacteria are known to be interrupted by MGEs. In *B. weihenstephanensis*, these include spoVF(b) (dipicolinate synthase subunit B gene) and spoVR (involved in spore cortex formation) (Abe et al., 2013). Similarly, gerE (encoding a mother cell-specific transcription factor) is interrupted in *B. cereus* (Abe et al., 2017a).

Spores of *Bacillus* species are usually produced in response to nutrient deprivation. Their dormant state is sustained until environmental conditions become favorable again for growth. Sporulation is an elaborate developmental process with well-defined temporal stages in the differentiation of the mother cell and forespore. Each cell engages in specific gene expression programs governed by a cascade of cell-specific sigma factors (σ^m and σ^n in the forespore and σ^n and σ^k in the mother cell) that control the expression of sporulation genes temporally and spatially (Bassler and Losick, 2006; Bate et al., 2014; McKenney et al., 2013; Shapiro and Losick, 2000). The sigma factor σ^n is the last sigma factor to be expressed in the sporulation cascade. As mentioned above, it is encoded by the composite gene sigK (S^2-sigK and sigK-3) interrupted by the defective prophage skin. To reconstitute a functional sigK, excision of skin is necessary (Stragier et al., 1989; Takemaru et al., 1995). This defective prophage contains its own SSR unit recognized by an Int_int (SpoIVCA), which promotes excision (Sato et al., 1990; Kunkel et al., 1991). This process also requires an RDF (Skr, this work), whose expression levels are controlled by the mother cell-specific sigma factor ρ^EF (Sato et al., 1994). Mutations in spoIVCA cause sporulation defects because sigK remains interrupted by skin and the σ^n-dependent genes fail to be expressed. Since the excision event is limited to the mother cell genome, skin is transferred vertically to the progeny through the spore (Sato et al., 1990). Similarly, many mother cell-specific genes in various strains and species (sigK, spoIVFB, spoVR, spsM, and gerE) are split by an element that carry their cognate SSR unit. Although some rdf genes have not yet been identified, excision processes are likely mediated by an individual integrase and its cognate RDF.

It was recently discovered that the sporulation gene spsM was interrupted by an active prophage, SPβ, carrying the SSR unit attL-sprB (rfl-sprA (int)-attR). The timing of phage excision is controlled by sprB, whose expression is dependent on stress-inducible and mother cell-specific promoters (Abe et al., 2014). Thus, the SSR unit responds to two pathways (i.e., stress and sporulation) that can trigger SPβ excision. Promotion of gene reconstitution by SSR units is, however, not limited to sporulation genes. For instance, these units have been described in MGEs that interrupt nitrogen fixation genes (*nifD, fdxN, and hupL* (Golden et al., 1985, 1987; Carrasco et al., 1995, 2005). There are interesting parallels with the SSR units that are active during sporulation, considering that these elements were also excised in a developmental process, and heterocyst formation in the cyanobacterium *Anabaena* sp., where reconstitution of nitrogen fixation genes is similarly limited to one terminal cell type. Recently, Rabinovich et al. (2012) showed that the comK gene of *Listeria monocytogenes* was also interrupted by a prophage and that the prophage was excised during invasion into mammalian cells, thus allowing the bacteria to escape from phagosomes and colonize the host cell cytoplasm. With these examples in mind, we hypothesize that, because SSR units operate via a common recombination mechanism, they are independent of the MGE they reside in and could promote recombination in a variety of related elements.

Accordingly, SSR units could be transferred as independent units between lysogenic phages and ICEs. Here, we demonstrate that *B. subtilis* SSR units are compatible among lysogenic phages and ICEs. Our experiments also show that SSR units from a defective prophage and from an ICE can rescue a phage rendered inactive by deletion of its endogenous SSR unit. In addition, we found in other *B. subtilis* strains and related species closely related prophages with distinct SSR units, suggesting that transfer between MGEs of independent SSR units is possible.

**RESULTS**

**SSR Units Are Functionally Exchangeable between Lysogenic Phages and ICEs**

In the *B. subtilis* 168 genome, the 134-kb SPβ prophage (Lazarevic et al., 1999) integrates into attBspβ within the spsM gene at approximately 183.8°, whereas the 20-kb ICEB1 integrates at the attB(ICEB1) site within
Figure 1. Integration of Chimeric Phages and Chimeric Integrative Conjugative Elements (ICEs) into Distinct attB Sequences

(A) Integration sites of SPβ, ICEBs1, and skin in the *B. subtilis* 168 genome. SPβ, ICEBs1, and skin were cured from the *B. subtilis* 168 genome resulting in a strain (D3) that does not carry SPβ-, ICEBs1-, and skin.

(B) Integration of chimeric phages. The chimeric phages SPβ-ICEBs1 and SPβ-skin were constructed at attBSPβ (the native site of SPβ), generating the HSS001 and HSS004 strains, respectively. Chimeric-phage genomes were excised following mitomycin C (MMC) treatment and packaged into phage particles. cos refers to the cohesive end sites of phage genomes. To obtain lysogens, the D3 strain was infected with these phages and subjected to antibiotic selection. Genomes of SPβ-ICEBs1 and SPβ-skin were integrated into attB sites located within trnS-leu2 (attBICEBs1) and sigK (attBskin) genes, respectively. Red-shaded connectors represent SPβ-derived genomic regions. Horizontal black arrowheads indicate positions and directions of promoters as follows: PV, σA-dependent promoter; PE/K mother cell-specific σE/K-dependent sporulation promoter; PSt, stress inducible σA-dependent promoter; PimmR, σA-dependent promoter of ICEBs1; Pxis, stress or quorum sensing-controlled promoter of ICEBs1.
the tmS-leu2 gene at approximately 45.2° (Figures 1A, S1A, and S1B and Table S1) (Kunst et al., 1997). SPβ and SPβkan (i.e., a version of the prophage modified by introduction of a kanamycin resistance cassette) carry the SSR unit attLSPβ-sprB/SPβ-sprA-attRSPβ. Among the components of this unit, the integrase gene, sprA, encodes an LSR and has a σ^δ^N-dependent promoter (P_{σ^δ}^N), whereas its cognate RDF gene, sprB, has distinct promoters, i.e., a stress (mitomycin C, MMC)-inducible σ^δ^N-dependent promoter (P_{σ^δ}^N) (Figure 1B) (Abe et al., 2014). ICEBs1 and ICEBs1cat (a version carrying a chloramphenicol resistance cassette) harbor the SSR unit attLICEBs1-intICEBs1-xisICEBs1-attRICEBs1 (Tables S1 and S2) (Lee et al., 2007). The integrase gene intICEBs1 encodes a Tyr-type recombinase and is constitutively expressed with immA and immR from the P_{immR} promoter. In contrast, the cognate RDF gene xisICEBs1 is expressed from the P_{xis} promoter, which is regulated by SOS responses and quorum sensing signals mediated by ImmA (anti-repressor) and ImmR (immunity repressor) (Figure 1C) (Auchtung et al., 2005, 2007; Lee et al., 2007; Bose et al., 2008). We investigated whether the ICEBs1-derived SSR unit would function in the context of SPβ insertion and excision. First, we determined whether a chimeric SPβICEBs1 construct would integrate preferentially into the attICEBs1 site or the attSPβ site. The 137-kb chimeric SPβICEBs1 prophage was generated by replacing the SPβ-derived SSR unit (attLSPβ-sprB/SPβ-sprA-attRSPβ) with the ICEBs1-derived SSR unit (attLICEBs1-intICEBs1-xisICEBs1-attRICEBs1) (Figure 1B) and introduction of an erythromycin resistance cassette. In this construct, intICEBs1 and xisICEBs1 remained under the control of ICEBs1-derived P_{immR} and P_{xis} promoters, respectively, and, as expected, were expressed upon integration/excision of ICEBs1. The resulting SPβICEBs1 chimeric-phage lysogen (HS0001) was induced by MMC (0.5 μg/mL) and the phage lysate was used to infect a SPβ-, ICEBs1-, and skin-cured strain (Δ3) (Figure 1B). SPβICEBs1 lysogens were then selected for erythromycin resistance, and the sequences of the regions flanking attICEBs1 and attICEBs1 were determined. These analyses showed integration of SPβICEBs1 at attICEBs1 in the genome of the Δ3 strain (Figure S2A). Next, to detect phage excision and the regeneration of the attB and attF sites from the attL and attR junctions of the lysogen, we performed polymerase chain reaction- (PCR) and quantitative polymerase chain reaction (qPCR)-based analyses. These assays showed that, although the timing of SPβICEBs1 excision was slower than that of SPβkan because of transcriptional regulation of SSR unit in SPβICEBs1, excision rates were significantly increased in response to MMC-mediated induction for both the SPβkan and chimeric SPβICEBs1 lysogens (Figures 2A and 2B). SPβICEBs1 excision was also measured by counting plaque-forming units/mL (pfu/mL). Phage titers for SPβICEBs1 were found to be slightly lower than those of the positive controls, i.e., wild-type SPβ or SPβkan (Table 1). Even though the integration rate for SPβICEBs1 was approximately 30-fold lower than that of SPβkan (probably due to non-native SSR units), site-specific integration at attICEBs1 was 100% accurate in lysogens carrying SPβICEBs1 (i.e., there was no integration at the attBSPβ site). In total, these data indicate that the ICEBs1-derived SSR units are sufficient for integration/excision of SPβ at the attICEBs1 site. These results further imply that SSR units can be repurposed to control the life cycle of unrelated MGEs, including that of lysogenic phages.

Our next objective was to investigate whether the SPβ-derived SSR unit was similarly adaptable for use in ICEBs1. We engineered a 22-kb chimeric ICEBs1SPβ carrying a chloramphenicol resistance cassette and the SPβ-derived SSR unit instead of the ICEBs1-derived SSR unit (Figure 1C and Tables S1 and S2). In this construct, SPβ-derived sprA and sprB genes were initially placed under the control of P_{immR} and P_{xis} promoters, respectively. The resulting chimeric ICE was designated ICEBs1SPβ, yet excision of ICEBs1SPβ was detected even in the absence of MMC-mediated induction. Expression of sprB under these conditions could be due to the leakiness of the P_{xis} promoter combined with the strong Shine-Dalgarno (SD) sequence of xisICEBs1. To reduce sprB expression, we introduced a GGAGG to GcAGG mutation into the SD sequence, 11 bp upstream of the start codon (TTG) of xisICEBs1. This second chimeric ICE construct (designated as ICEBs1SPβ) was stably integrated into attBS18 under non-MMC treatment conditions and was excised following addition of MMC (0.5 μg/mL). We performed PCR and qPCR-based detection assays and showed that the excision rates of both ICEBs1SPβ and the parent ICEBs1cat were increased in response...
Figure 2. Excision of SP\textsubscript{kan} and ICE\textsubscript{Bs1}.

(A) Excision mechanisms for SP\textsubscript{kan} and ICE\textsubscript{Bs1}. SP\textsubscript{kan} and SP\textsubscript{ICE\textsubscript{Bs1}} phage excision was induced by MMC treatment. S'-spsM and spsM-3', as well as S'-trmS-leu2 and trmS-leu2-3', were combined to generate att\textsubscript{B} in host genomes, respectively. Horizontal black arrowheads indicate the positions of primers for PCR amplification.

(B) Analysis of SP\textsubscript{kan} and ICE\textsubscript{Bs1} genome excision. Total genomic DNA was extracted from MMC-treated cells. The presence of att\textsubscript{B} was confirmed by PCR (top panel) and quantitative PCR (qPCR) analysis (bottom panel). SP\textsubscript{kan} and
to MMC-mediated induction (Figures 2C and 2D). These observations indicate that the Spβ-derived SSR unit, attLspβ-sprB-sprA-attRspβ, is functional in the context of ICEBs1 integration and excision. Next, the strain harboring ICEBs1spβ was induced by addition of MMC and co-cultured with the Δ2CK strain. That strain had been cured of Spβ and ICEBs1, and natural transformation was prevented by disruption with a kanamycin resistance cassette of the major competence gene comK. We then selected for ICEBs1spβ Δ2CK strains based on acquisition of chloramphenicol resistance and maintenance of kanamycin resistance. ICEBs1spβ transconjugants were obtained with approximately the same frequency as parental-type ICEBs1spβ (Table S3). DNA sequencing of the flanking region of attLspβ and attRspβ confirmed that ICEBs1spβ had integrated at attLspβ in the Δ2CK strain, and site-specific integration of ICEBs1spβ at attLspβ had 100% accuracy (Figure S2B and Table S3). Hence, the Spβ-derived SSR unit is sufficient to drive SSR of ICEBs1. Taken together, these data indicate compatibility of SSR units between the prophage and the ICE.

The SSR Unit Derived from the Defective Prophage Skin Is Active when Inserted into a Modified Spβ Prophage

The sigK intervening skin element located at approximately 226.6 kb also carries an SSR unit (attLskin intskin = spoIVCA, attRskin). The integrase gene spoIVCA encodes an LSR that catalyzes the joining of the truncated 5’-sigK and sigK-3’ portions of the sigK gene (Sato et al., 1990; Kunkel et al., 1991) (Figures 1A and 3A). Although spoIVCA is well characterized, the cognate rdf gene remains unidentified. To identify rdf in the skin element, we performed deletion analyses in the region flanking spoIVCA, based on the observation that in other systems the rdf gene is usually located near the integrase gene (for instance, int-xis in phage λ). Because the promoter of spoIVCA (Pspβ) is located over 200 bp upstream of the translational start site, we hypothesized that an open reading frame (ORF) was present immediately upstream of the spoIVCA coding sequence. To confirm the presence of the ORF and its role as the rdf of skin, we introduced the IPTG-inducible Pspac promoter at positions +47, +18, and −17 nucleotides (nt) from the first nucleotide of the putative ORF (Figures S3A and S3B). We observed that the excision of skin was only induced following expression from the −17 nt position (Figures S3C and S3D). We named this small ORF skr. It encodes a 64-amino-acid (aa) protein and is required for the reconstitution of a full sigK gene from the 5’-sigK and sigK-3’ portions. Unexpectedly, the 3’-end of skr overlaps with the 5’-end of spoIVCA by 101 nt, owing to a +1 frameshift (Figure S3A).

Next, we constructed a 136-kb chimeric Spβskin prophage that carries the skin SSR unit attLskin-skr-spoIVCA-attRskin and a spectinomycin resistance cassette (Figure 1B and Tables S1 and S2). In this chimeric prophage, spoIVCA and skr transcription levels are controlled by Pspβ of sprA and both the Pspβ and PspR promoters of sprB, whereas the SSR unit of Spβ (attLspβ-sprB-sprA-attRspβ) was eliminated. The resulting lysogen contained the chimeric Spβskin prophage. Upon treatment with MMC, the phage lysate was used to infect the Δ3 host strain and the Spβskin lysogen was selected for spectinomycin resistance. DNA sequences of the flanking regions of attLskin and attRskin were then determined to confirm that Spβskin was integrated at attLskin in the Δ3 strain genome (Figure S2C). Phage titers were determined (pfu/mL) on a lawn of Δ3 strain cells (Table 1). Excision of Spβskin from attLskin after MMC addition was quantified by PCR and qPCR (Figure 3B). The integration frequency of Spβskin was comparable with that of Spβskin, and the accuracy of site-specific integration at attLskin was 100% (Table 1). As mentioned before, excision of skin and Spβ both occur during sporulation (Figure 3C), leading to reconstitution in the mother cell genome of the sigK and spoIVCA genes, respectively. Similarly, Spβskin excision was observed 3 h after initiation of sporulation (T3). Rearrangement of sigK was as accurate as skin (Figure 3C). These results indicate
that the integration system of SPβ can replace that of skin and that the skin-derived SSR unit can also drive the excision of the active lysogenic phage SPβ, even though it is derived from a defective prophage.

Diversity of Prophage Genomes Integrated at Specific attB Sites

Searching the microbial genomes database at NCBI (http://www.ncbi.nlm.nih.gov), we found that 16 B. subtilis strains had SPβ phage-like sequences inserted into the spsM gene. Each sequence was of similar size (131–134 kb) and gene organization was highly conserved (Figure S4), suggesting that they all function as lysogenic phages. In contrast, in the B. amyloliquefaciens group, the phage-like sequences inserted into spsM were much shorter (from 4 to 20 kb), indicating that they are unlikely to be active prophages (Abe et al., 2014) (Figure S5). Outside of the B. subtilis and B. amyloliquefaciens groups, no phage-like sequences were found inserted in spsM.

In B. subtilis strain D12-5, we noticed a prophage named φ12-5, whose genome organization closely resembled that of SPβ (Figure 4A). φ12-5 disrupted the kamA gene, which was previously reported to be a sporulation gene expressed in the mother cell under the control of σE (Eichenberger et al., 2003, 2004; Feucht et al., 2003). In B. subtilis 168, this gene is annotated as coding for an L-lysine 2,3-aminomutase. Interestingly, kamA (formerly yodO) is in the vicinity of spsM (formerly yodU), with just a few genes separating the two phage insertion sites (Figure 4B). Although the genome organization of φ12-5 is conserved in comparison with SPβ, its SSR unit is different. Homology searches (blastp) using the integrase (Intφ12-5) of strain D12-5 as query revealed that seven phage-like (128–136 kb) and one non-phage-like (11 kb) sequences are inserted into kamA in various Bacillus species (Figure 4A). Among these, φ3T of B. subtilis reportedly (Erez et al., 2017; Dou et al., 2018) functions as a vital prophage. Moreover, the integrase Intφ3T (displaying 18.9% identity with SprA of SPβ) and its cognate RDF, Rdfφ3T (no similarity with SprB of SPβ), were encoded by genes located in the flanking regions of the φ3T prophage (Figures 4A and 4B). A degenerate phage-sequencing (11 kb) disrupting kamA was found in only one B. subtilis strain, DKU_NT_02. Among the kamA-inserted prophages in other strains, diverse gene organizations were observed. Furthermore, genome organizations of φ3T and φ12-5 (both inserted into kamA) were only 63% similar (comparison percentage generated using tBLASTx), whereas those of SPB (spsM) and φ3T (kamA) were 69% similar. Thus, the degree of genomic similarity between prophages containing heterogeneous SSR units (φ3T versus φ3T) is higher than that between homogeneous SSR units (φ3T versus φ12-5) (Figure 4C). The presence of heterogeneous SSR units in similar prophages (ϕβ and φ3T) suggests that the correspondence between prophages and their cognate SSR units is not absolute.

The kamA Gene Is Reconstituted during Sporulation by Excision of the SPβ-like Phage φ3T

Previous studies have shown that phage φ3T is integrated between positions 2,106,060 and 2,106,064 in the B. subtilis BEST7003 genome (Goldfarb et al., 2015). This site (i.e., the putative DNA breakpoint for integration of φ3T) corresponds to a CCTAC sequence in the kamA gene. The N- and C-terminal encoding portions of kamA were named S’-kamA and kamA-3’, respectively (Figure 4A). Imperfect inverted repeat sequences (23 24 bp long) were found adjacent to the CCTAC site and may provide binding sites for a site-specific recombinase (Figure S1D). As mentioned above, kamA is a member of the σE regulon (Feucht et al., 2003; Eichenberger et al., 2003, 2004), suggesting that φ3T is excised during sporulation to reconstitute the composite kamA gene in the mother cell genome. We confirmed that φ3T was excised from attLφ3T

| Phages | Phage Titer (pfu) | Integration Frequency | Integrated at attB Sites (%) |
|--------|------------------|----------------------|-----------------------------|
| SPβ   | 1.3 (±0.8) × 10^6 | ~                    | ~                           |
| SPβkam | 8.4 (±2.8) × 10^7 | 4.2 (±3.2) × 10^-4  | 100                         |
| SPβspsM | 1.2 (±0.5) × 10^6 | 8.4 (±2.3) × 10^-4  | 100                         |
| SPβspsMa | 4.1 (±0.9) × 10^7 | 1.4 (±1.0) × 10^-5  | 100                         |

Table 1. Phage Titration and Lysogenic Frequency

*The data shown are the average of three independent experiments ±SD.
Infected by MOI = 0.1
Twenty lysogens were investigated.
Figure 3. Excision of SPbLys Using Site-Specific Recombination (SSR) Units from a Defective Prophage

(A) Excision mechanisms for SPb, skin, and SPbLys (from left to right). SPb and SPbLys excisions were induced either after MMC treatment or during sporulation, whereas skin excision was observed only during sporulation, as previously reported (Kimura et al., 2010). Pν, σ^A-dependent promoter; PSt, stress-inducible promoter; PE/K, mother cell-specific σE/K-dependent sporulation promoter; PE/SpoIIID, mother cell-specific σE and SpoIIID-dependent sporulation promoter. Horizontal black arrowheads indicate the positions of primers for PCR amplification.

(B) Excision in the presence of MMC. SPb and SPbLys genomes were excised from host genomes after MMC treatment, whereas the defective prophage skin (no stress-inducible promoter) was not. B. subtilis 168 cells containing SPb, skin, or SPbLys lysogens were grown in LB medium. Vegetative cells in the early log phase (OD600 ~ 0.2) were treated with 0.5 μg/mL MMC and were harvested at indicated times and analyzed by PCR amplification (top panel) and qPCR (bottom panel). N.D., not detected.

(C) Excision during sporulation. Schematics of SPb and skin excision during sporulation are shown above figure. B. subtilis 168 sporulating cells divided asymmetrically to produce mother cells and forespores at 1–2 h after the initiation of sporulation (T1–T2). Subsequently, skin and SPb excision was specifically induced in mother cells at approximately 3 h after the initiation of sporulation (T3) (Abe et al., 2014; Sato et al., 1990). B. subtilis 168 cells and SPbLys lysogens were grown in DSM, and samples of vegetative cells were collected (OD600 ~ 0.2; T/C0 = 1) at indicated times at 1-h intervals (T1–T5). Middle and bottom panels represent PCR amplification and qPCR analysis, respectively. In (B) and (C), Amplification of attBSPb (PCR and qPCR, 305 bp) and attBskin (PCR and qPCR, 221 bp) by PCR and qPCR analysis. Data are mean ± SD; n = 3 independent experiments. See also Figure S3 and Table S4.
Figure 4. Gene Organization of SPβ-Related Phages Inserted into the kamA Gene

(A) Synteny of SPβ-related phages residing in kamA; the top diagram shows the positions of the integrase (int) and putative recombination directionality factor (rdf) genes. Host strains and phage names are indicated on the left of the diagram and lengths on the right. Integrase amino acid sequence homologies (%) to Int$_{412-5}$ or SprA (Int$_{SPβ}$) are shown on the right column.

(B) Comparisons between the SPβ and φ3T phage genomes. The enlarged views on the left-hand side show the genes flanking the rdf. Vertical black arrowheads indicate the position of the integration site.

(C) Synteny of the SPβ, φ3T, and φ12-5 genomes. Genome data were extracted from the genome database at the national center for biotechnology information (NCBI). Sequence comparisons were performed using tBLASTx; red-blue lines indicate regions with 60%–100% identity. Genome alignment figures were created using Easyfig (Easyfig 2.2.2 for generating tBLASTx alignment files and visualization). Genetic homology (tBLASTx) percentages are included on the right of the diagram.

See also Figures S4 and S5 and Table S2.
and attR3T sites upon MMC treatment and also during sporulation (Figures 5A and 5B). When the φ3T lysogen was treated with MMC, φ3T was excised from the kamA gene after 30 min (Figure 5B). We confirmed φ3T excision during sporulation by analyzing DNA samples from sporulating φ3T lysogens. In these experiments, φ3T was excised at hour 3 of sporulation in the absence of MMC (Figures 5B and S6A). Next, we sequenced and identified the flanking sequences at the junctions (attLφ3T and attRφ3T). Because the attRφ3T sequence is conserved between attLφ3T and attRφ3T, its presence was determined by comparison of DNA sequences before (attLφ3T and attRφ3T) and after (attBφ3T) excision of φ3T. These analyses showed that φ3T excision combines the S’-kamA and kamA-3’ frame during sporulation (Figure 5C). We then identified an int gene and its cognate rdf gene as components of the SSR unit of φ3T (attLφ3T-rdfφ3T-intφ3T-attRφ3T) by replacing the native promoter of each gene with the IPTG-inducible promoter Pspac. In both intφ3T- (ESI-φ3T) and rdfφ3T- (ESR-φ3T) inducible strains, no excision was detected in the absence of IPTG, neither following MMC treatment nor during sporulation (Figures S6B and S6C). But in the presence of IPTG, the excision pattern of ESI-φ3T strains was similar to that of the wild-type φ3T lysogen, whereas excision in ESR-φ3T strains was detected regardless of induction of sporulation or SOS response (via addition of MMC). In agreement, growth inhibition was observed only in ESR-φ3T (Figure S6D). These data suggest that the erm gene is lost upon addition of IPTG, because the rdfφ3T gene was induced, thus producing the RDF that regulates φ3T prophage excision. Next, we examined the expression of intφ3T, rdfφ3T, and kamA from lacZ fusion constructs. Strains harboring intφ3T-lacZ (in ESI-φ3T), rdfφ3T-lacZ (in ESR-φ3T), and kamA-lacZ (in IND(kamA) were constructed and analyzed. During vegetative growth and sporulation, intφ3T-lacZ was constitutively expressed. Yet, rdfφ3T-lacZ and kamA-lacZ were expressed concomitantly 2 h after the initiation of sporulation, indicating that rdfφ3T controls the timing of φ3T excision (Figures 5D and S7). Collectively, these data demonstrate that the φ3T prophage, which is highly similar to the SPB prophage except for its SSR units, regulates kamA expression by excision of the φ3T prophage during sporulation. Even though the SPB and φ3T prophages were highly similar, they integrated into specific attB sites. Importantly, the specificity of integration depended solely on the nature of the corresponding SSR unit. These findings are consistent with the hypothesis that SSR units are adaptable between MGEs.

DISCUSSION

In bacteria, mobilization of lysogenic phages and ICEs consists of a series of successive steps, starting with excision, followed by intercellular transfer and finally integration of the genetic material into new host cells. Excision and integration are mediated by SSR units (attL-int-rdf-attR). Therefore, acquisition of individual SSR units is a key factor driving MGE evolution. Many different types of SSR units have been found in bacterial genomes. Each SSR unit carries a gene encoding an individual member of the integrase family (either a Tyr- or a Ser-type enzyme) that recognizes cognate attP and attB sites with high selectivity. Some site-specific recombination systems, including P1 (Cre), Bxb1, TP901-1, R4, and φC31 integrases, have been widely used as tools to introduce foreign genes, carried, for instance, on site-specific integration plasmids, in a range of organisms, including other microbes, plants, and mammalian cells (Hirano et al., 2011; Fogg et al., 2014; Meinke et al., 2016). In this study, we demonstrate that, after artificial exchange of SSR units between a lysogenic phage and an ICE, these units remain functional and specific to the attB site recognized by their respective integrase. Specifically, SPB and ICEBsI are two MGEs in B. subtilis that recognize different attB sites based on the SSR unit they carry. Yet, these SSR units are not restricted to their MGE; on the contrary, they are interchangeable and remain fully functional when inserted into other lysogenic phages and ICEs (Figure 6A). Only slight reductions in phage titers and integration frequencies were observed with non-native SSR units. This in contrast with other phage elements, like tails or capsids. In a recent study, it was demonstrated that the exchange of phage tails altered the host ranges (Ando et al., 2015). Although capsids or tails can be exchanged from phage to phage, adaptation to unrelated virion proteins is an issue. Since the SSR unit is not a structural part of the virion, distinct SSR units that vary in their recognition sites (attB) can be viewed as highly adaptable phage components. By extension, adaptability of SSR units may constitute an important factor modulating plasticity among MGEs in their interaction with host genomes.

To allow adaptation of SSR units to a new MGE, the corresponding int and rdf genes must be expressed at appropriate times in the host life cycle (Ghosh et al., 2006; Fogg et al., 2014; Merrick et al., 2018). Considering that int is required for both integration and excision, int genes are often constitutively expressed. In contrast, regulation of RDF production is more elaborate, presumably because the role of RDF is limited to excision. Furthermore, because excision often occurs early in the lytic cycle, rdf is often among the first
Figure 5. DNA Rearrangement of kamA in the φ3T Lysogen

(A) Diagram of φ3T prophage excision and kamA rearrangement. During the lytic cycle, excised φ3 transfer DNA is packaged into phage capsids to produce virions and promote host cell lysis. During sporulation, prophage excision generates a functional kamA gene. Horizontal arrowheads indicate positions and directions of putative promoters, and red arrowheads represent the active promoter of rdf43T; V, vegetative promoter; Pspo, sporulation-specific promoter; Pst, stress-inducible promoter.

(B) Excision of φ3T, bar graphs show qPCR analyses of attBφ3T (229-bp) generated by φ3T excision upon MMC treatment (left) and during sporulation (right).

(C) Nucleotide sequences of φ3T attachment sites before and after genome excision; the 5-bp long overlapping nucleotide sequence is indicated with red letters. Translated amino acid sequences are shown above or below nucleotide sequences.

(D) β-Galactosidase activity of intφ3T, rdfφ3T, and kamA–lacZ reporter constructs during sporulation; intφ3T, rdfφ3T, and kamA genes were transcriptionally fused to the lacZ reporter gene in ESI-φ3T, ESR-φ3T, and INDkamA, respectively. φ3T excision occurred at 3 h after the initiation of sporulation (T3; blue-shaded areas).

In (B) and (D), data are mean ± SD; n = 3 independent experiments. See also Figures S1, S6, and S7 and Tables S2 and S5.
transcription units induced by SOS responses (Khalee et al., 2011; Jain and Hatfull, 2000; Ghosh et al., 2006). In addition to regulating prophage excision, these responses also condition ICE transfer. In *B. subtilis*, the *immR-immA* operon is the regulatory module integrating SOS response and cell density signals to promote the mobilization of ICEBs1. Although *immR-immA* is constitutively transcribed (along with the downstream gene *intICEBs1*), expression of the RDF (xisICEBs1) is repressed by ImmR, whose degradation by ImmA is dependent on SOS and cell density signals (Auchtung et al., 2005, 2007; Lee et al., 2007; Bose et al., 2008). In the present study, we had to place sprB (the gene encoding the RDF of SPβ) immediately downstream of P\_xis to ensure control by the *immR-immA* system. As a result, excision of the chimeric SPβ\_ICEBs1 prophage in the lysogen was detected following addition of MMC and induction of the SOS response, thus showing that regulation by the *immR-immA* module can be co-opted for prophage excision. This result agrees with a previous report that the *B. subtilis* lysogenic phage 4\_105 relied on a similar *immR-immA* system (Bose et al., 2008). Thus, at least some induction systems regulated by RDFs are common among MGEs, regardless of prophage or ICE origin.

Other lysogenic phages containing SSR units similar to those of SPβ and φ3T were found to be present in multiple organisms, especially variations on the basic SSR unit: attL, int, rdf, and attR. As shown in Figure 4B, in the SPβ and φ3T prophages, int and rdf are located at both ends of the element (close to attL or attR). After excision, however, int and rdf are only separated by the attP locus. In this circular genome state,
RecA-mediated homologous recombination could promote exchange of int-attP-rdf cassettes between MGEs, especially between circular phage genomes. The presence of several lysogenic phages (SPB and φ3T) in a single strain further supports our hypothesis that SSR units are transferable between MGEs (Figure 6B). One advantage of having lysogenic phages with different SSR units may be that prophages could gain the ability to integrate into other attB sites in the host genome.

When certain genes, like those encoding restriction enzymes, methyltransferases, toxin-antitoxin modules, or drug resistance enzymes, are carried by an MGE, they may influence the stability of that element. A key factor favoring maintenance of SSR units in intervening elements interrupting sporulation genes is that gene reconstitution is necessary for survival through sporulation. This might constitute an even larger evolutionary advantage than prophage ability to excise in response to the SOS system. This could explain why most of the small intervening elements in sporulation genes are no longer functional prophages but are maintained in the host genome because of the role they play in gene reconstitution (Figure S5). SSR units that split sporulation genes act to rejoin interrupted genes specifically during sporulation, and the genes are usually dispensable during growth. However, mutations that prevent reconstitution of the interrupted gene into a functional gene during sporulation would likely be eliminated by natural selection, because of the survival advantage provided by the ability to sporulate. Furthermore, it should be noted that almost all intervening elements in sporulation genes were integrated into mother cell-specific genes (Abe et al., 2013, 2014, 2017a). Mother cells are killed by lysis at the end of sporulation, whereas the intact spore genome is protected in a highly resistant dormant cell, thus the intervening element is maintained in its genome. For all these reasons, SSR units represent an advantageous platform for functional lysogeny and are especially favored in spore-forming bacteria. In this report, we also showed that the SSR unit of the defective prophage skin becomes active after introduction into the SPB genome (SPB\textsubscript{skm}). Thus, although the degradation of a prophage causes defectiveness, defective prophages can turn back into lysogenic phages by addition of SSR units (and possibly adjacent genes) under natural conditions (Figure 6A). Through this mechanism, phages can reacquire lysogen function by coordinating certain SSR units, implying that SSR units from lysogenic phages and ICEs share a common foundation.

We also identified an RDF gene called skr in the skin element and found that its 3' half overlapped with the S' end of spoIVCA encoding the N-terminal region of Int\textsubscript{skin}. We have not yet determined how the +1 shift in reading frame regulated expression of skr-spoIVCA during sporulation and the consequences on Skr activity. A similar gene encoding an RDF from an intervening sequence interrupting sigK has been identified in Clostridioides difficile (Serrano et al., 2016). However, the SSR units of the two skin elements differ between B. subtilis and C. difficile, and although both recognize attB sites, the corresponding sequences are located in different regions of the sigK gene.

Finally, we characterized the SSR unit of φ3T that recognized an attB site in kamA, a composite gene that is also reconstituted during sporulation. The kamA gene encodes an L-lysine 2,3-aminomutase (Chen et al., 2000), an enzyme that converts L-lysine to L-β-lysine, which is the first metabolite of the lysine degradation pathway in Bacillus sp (Zhang et al., 2014). In Escherichia coli, post-translational modification by β-lysylation is required for the activity of elongation factor P (EF-P) (Park et al., 2012). We have not yet determined the precise role of β-lysylation in B. subtilis, but considering that kamA expression is controlled by the mother cell-specific sigma factor σ\textsubscript{I}, it may play a role in sporulation, even though deletion of kamA does not appear to significantly impair sporulation (Eichenberger et al., 2003).

**Limitations of the Study**

Although we identified closely related prophages with distinct SSR unit, such ICEs were not found in genomic databases. These data were results based on the basic local alignment search tool (BLAST) analysis utilizing the database from the national center for biotechnology information (NCBI). To clarify the detailed SSR unit distribution, more advanced bioinformatics approaches would be required.

**METHODS**

All methods can be found in the accompanying Transparent Methods supplemental file.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.100805.
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AUTHOR CONTRIBUTIONS
S.S., D.I., K.A., and T.S. designed the experiments. S.S. and M.Y. conducted the experiments. S.S. analyzed the data. S.S., D.I., P.E., and T.S. wrote the paper. T.S. supervised the project.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

Compatibility of Site-Specific Recombination Units between Mobile Genetic Elements

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Figure S1. Nucleotide sequences of attachment sites, related to Figures 1A and 5C. Attachment site sequences of SPβ, (A); ICE Bs1, (B); skin, (C); and φ3T, (D). Core sequences that are sites of strand exchanges are indicated by bold characters. Horizontal arrowheads show inverted repeat sequences. Uppercase and lowercase letters indicate host sequences and prophage/ICE sequences, respectively.
Figure S2. Integration junction sequences of SPβICEB1, ICEBs1SPβ, and SPβskin, related to Figure 1. 
attL and attR sequences of chimeric SPβICEB1, (A); ICEBs1SPβ, (B); and SPβskin, (C). Above diagrams show the integrated phage or ICE genome into the target genes. Flanking region sequences of each DNA breaking point are shown by the figure below. Core sequences were represented by red character.
Figure S3. Identification of rdfskin (skr) of the skin element, related to Figure 3. (A), Genomic structure and flanking sequences of skr, rdfskin (skr) and intskin (spolVCA) genes are arranged in tandem on the chromosome. Vertical arrowheads indicate the insertion positions of the Ps pac promoter. The 3' terminal sequence of skr overlapped with 101 bp of the spolVCA gene. Skr and SpolVCA coding regions are highlighted red and yellow, respectively. (B), Schematic of DNA deletion assays for identification of the skr gene. An inducible Ps pac promoter was inserted into the positions +47, +18, and −17 from the first nucleotide of the putative skr gene and the resulting strains were designated IVCAΔ1, IVCAΔ2, and IVCAΔ3, respectively. (C), Excision of skin following induction of skr. Schematics of skin element excision by IPTG are represented in the left panel. The erythromycin resistance gene was excised from the host genome with skin after induction of the intact skr gene by IPTG treatment. Subsequently, the host strain became sensitive to erythromycin. Growths of IVCAΔ1, IVCAΔ2, and IVCAΔ3 strains on LB agar plates containing erythromycin (Em) with or without IPTG are shown in the right panel. (D), Detection of reconstructed attBskin. IVCAΔ1, IVCAΔ2, and IVCAΔ3 were grown in LB medium with or without 1 mM IPTG for 16 h. Excision of the skin element was confirmed using PCR with the primers P85/P86 and extracted genome templates.
Figure S4. Synteny plots of SPβ related phages using Easyfig tBLASTx, related to Figure 4. Genome comparison of sixteen SPβ related phage genomes possessing an SSR unit homologous to that of SPβ (B. subtilis 168) and residing in the spsM gene. Host names and accession numbers are indicated on the left column. Blue-Red lines indicate region with 60-100 % identity.
Figure S5. Synteny plots of degenerate SPβ phages from Easyfig tBLASTx, related to Figure 4. Genome comparisons of fifteen degenerate SPβ phage genomes carrying SSR units homologous to that of SPβ (*B. subtilis* 168) and residing in the *spsM* gene; host names are indicated on the left. Blue–red lines indicate regions with 60%–100% identity.
**Figure S6.** \( \Phi3T \) excision upon induction of \( \text {int}_{\Phi3T} \) or \( \text {rdf}_{\Phi3T} \), related to Figure 5. (A) Detection of \( \Phi3T \) excision. \( \text {attB}_{\Phi3T} \) (229-bp) was amplified using PCR with the primers P79/P80. Detection of ESI-\( \Phi3T \) excision, (B) and ESR-\( \Phi3T \) excision, (C), upon MMC treatment and during sporulation with or without IPTG. \( \text {attB}_{\Phi3T} \) (1447-bp) was PCR amplified using the primers P65/P81 for B and C. Positions and directions of native and \( P_{spac} \) promoters are represented by black and blue arrowheads, respectively. (D), Detection of \( \Phi3T \) genome excision by antibiotic selection on plates. Ten-fold serial dilutions of cultures were spotted onto LB agar plates containing erythromycin with or without IPTG. Horizontal arrowheads represent positions and directions of transcriptional promoters; \( P_v \), vegetative promoter; \( P_{spac} \), sporulation-specific promoter; \( P_{so} \), stress inducible promoter; \( P_{spac} \), IPTG-inducible promoter.
Figure S7. Construction of ESI-ϕ3T, ESR-ϕ3T, and INDkamA, related to Figure 5. Schematics of construction of ESI-ϕ3T, (A); ESR-ϕ3T, (B); and INDkamA, (C), strains. Gene names are indicated. The horizontal gray arrowheads and horizontal black arrowheads indicate the position of Psac promoter and of primers used for construction of pMutin-int_{ϕ3T}, pMutin-rdf_{ϕ3T}, and pMutin-kamA, respectively. The horizontal gray arrowheads indicate putative position and direction of native transcriptional promoters. P_{V}, σ^{V}-dependent promoter; P_{Spo}, sporulation specific promoter; P_{St}, stress inducible promoter. Transcriptional terminators are presented by open circles. Single and double asterisks indicate disrupted genes and putative promoters, respectively. Open boxes are represented ϕ3T phage genes excluding int_{ϕ3T} and rdf_{ϕ3T}. 

ESI-ϕ3T genome

\[ \text{pMutin-int}_{\phi 3T} \]

ESR-ϕ3T genome

\[ \text{pMutin-rdf}_{\phi 3T} \]

INDkamA genome

\[ \text{pMutin-kamA} \]
### Supplemental Tables

#### Table S1. Relevant characteristics of chimeric phages and ICEs, related to Figure 1.

| Mobile element | Genome size kb | Marker | **attL** | **rdf** | **int** | **attR** |
|----------------|----------------|--------|----------|---------|---------|---------|
| SPβ            | 134            |        | **attL_{SPβ}** | **sprB** | **sprA** | **attR_{SPβ}** |
| ICEBsl         | 20             |        | **attL_{ICEBsl}** | **xis_{ICEBsl}** | **int_{ICEBsl}** | **attR_{ICEBsl}** |
| skin           | 48             |        | **attL_{skin}** | **skr** | **spoIVCA** | **attR_{skin}** |
| SPβ_{kan}      | 136            | **kan** | **attL_{SPβ}** | **sprB** | **sprA** | **attR_{SPβ}** |
| SPβ_{ICEBsl}   | 137            |        | **erm** | **attL_{ICEBsl}** | **xis_{ICEBsl}** | **int_{ICEBsl}** | **attR_{ICEBsl}** |
| ICEBsl_{cat}   | 22             | **cat** | **attL_{ICEBsl}** | **xis_{ICEBsl}** | **int_{ICEBsl}** | **attR_{ICEBsl}** |
| ICEBsl_{SPβ}   | 22             | **cat** | **attL_{SPβ}** | **sprB** | **sprA** | **attR_{SPβ}** |
| SPβ_{skin}     | 136            | **spc** | **attL_{skin}** | **skr** | **spoIVCA** | **attR_{skin}** |
**Table S2. Information of integrase and rdf protein sequences, related to Figures 1, 4, and 5.**

| Phage/ICE | Protein  | Accession ID               |
|-----------|----------|----------------------------|
| SPβ       | SprA     | CAB14084.1                  |
| SPβ       | SprB     | CAB13873.1                  |
| ϕ3T       | Int₃₃T   | APD21144.1                  |
| ϕ3T       | RDF₃₃T   | APD21343.1                  |
| ϕ12-5     | Intₙ₌₁ₕ | AMR46776.1                  |
| ϕ12-5     | RDFₙ₁₂₅* | CP014858                    |
|           |          | From 2027172 to 2027318     |
| ICEBs₁    | Int      | CAB12287.1                  |
| ICEBs₁    | Xis      | CAB12290.1                  |
| skin      | SpoIVCA  | CAB14518.2                  |
| skin      | Skr      | AL009126                    |
|           |          | From 2654774 to 2654968     |

* Predicted
Table S3. Mating frequency of ICEBs1cat and chimeric ICEBs1sep, related to Figure 1.

| ICEs      | Conjugation freq.\(^a\) | Integrated at attB sites (\(\%\))\(^b\) |
|-----------|--------------------------|------------------------------------------|
| ICEBs1cat | 3.3 (± 0.7) × 10\(^{-2}\) | 100                                      |
| ICEBs1sep | 3.3 (± 1.7) × 10\(^{-2}\) | 100                                      |

\(^a\) The data shown are the average of three independent experiments ± SD.

\(^b\) 10 transconjugants were investigated.
| Strains     | Genotype and/or Characteristics | Source or Reference |
|------------|--------------------------------|-------------------|
| HSS001     | trpC2 SPβ ICEBs1 ΔsprB::erm    | This study        |
| HSS002     | trpC2 SPβ cured strain, yddM::cat | This study        |
| HSS003     | trpC2 SPβ cured strain, ICEBs1 yddM::cat | This study        |
| HSS004     | trpC2 SPβΔsin yokB::spc        | This study        |
| HSS005     | trpC2 SPβΔkan yokB::kan        | This study        |
| ESI-φ3T    | trpC2 φ3T Δint::pMutinT3-intφ3T erm | This study        |
| ESR-φ3T    | trpC2 φ3T Δrdf::pMutinT3-rdfφ3T erm | This study        |
| INDkamA    | trpC2 ΔkamA::pMutinT3-kamA erm | This study        |
| Δ2         | trpC2 SPβ and ICEBs1 cured strain | This study        |
| Δ3         | trpC2 SPβ, ICEBs1, and skin element cured strain | This study        |
| Δ2CK       | trpC2 ICEBs1 and SPβ cured strain, ΔcomK::kan | This study        |
| IVCAΔ1     | trpC2 skr::pIVCAΔ1 erm         | This study        |
| IVCAΔ2     | trpC2 skr::pIVCAΔ2 erm         | This study        |
| IVCAΔ3     | trpC2 skr::pIVCAΔ3 erm         | This study        |
Transparent methods

Growth media

Standard genetic manipulations of *B. subtilis* were performed as described previously (Harwood and Cutting, 1990). Cells were grown at 37°C with shaking in Luria-Bertani medium (LB) (Sambrook and Russell, 2001), Difco sporulation medium (DSM) (Harwood and Cutting, 1990), and defined minimal medium (Auchtung et al., 2005) supplemented with 50 μg/ml tryptophan. When required, antibiotics were added at the following concentrations: chloramphenicol, 5 μg/ml; erythromycin, 0.5 μg/ml; kanamycin, 5 μg/ml; spectinomycin, 100 μg/ml; ampicillin, 100 μg/ml.

Strain construction

All strains were derived from *B. subtilis* 168. The primers and the bacterial strains used in this study are listed in Tables S4 and S5, respectively. Q5 High-Fidelity DNA polymerase (NEB, U.S.A) was used to construct donor DNA fragments and plasmids.

To construct chimeric SPβICEBs1 phages, primer pairs P1/P2, P3/P4, and P5/P6 were used to amplify regions from *cgeB* to 5'-*spsM* genes, from *attLICEBs1* to *ydcO* genes, and from *attLSPβ* to *yotJ* genes in the SPRBd (Abe et al., 2014) genome, respectively. The *B. subtilis* 168 genome was used as a template for P1/P2 and P3/P4 amplifications. The obtained DNA fragments were simultaneously used as templates for PCRs with the primers P1/P6. The four primer pairs P7/P8, P9/P10, P11/P12, and P13/P14 were used to amplify the region from *yokC* to *yokB*, *attRICEBs1*, a spectinomycin resistant gene of pUCS191 (Hosoya et al., 2002), and a region from *spsM*-3’ to *msrA*, respectively. The *B. subtilis* 168 genome was used as a template for PCR amplification with primers P7/P8, P9/P10, and P13/P14. The obtained DNA fragments were simultaneously used as templates for PCR with the primer pair P7/P14. The resulting products from P1/P6 and P7/P14 primer pairs were used to transform *B. subtilis* 168 and erythromycin- and spectinomycin resistant transformants were selected, resulting in strain HSS001.

To select conjugated ICEBs1, a chloramphenicol resistance gene was introduced within the *yddM* gene and the *attRICEBs1* region in ICEBs1. The primer pairs P15/P16, P17/P18, and P19/P20 were used to amplify *rapI* to *yddM*, a chloramphenicol resistant gene (*cat*) from pMF20 (Murakami et al., 2002), and *attRICEBs1* to *lrpA*, respectively. The *B. subtilis* 168 genome was used as template for PCR with primer pairs P15/P16 and P19/P20. PCR products were then simultaneously used for amplification with the primers P15/P20. The
resulting products were used to transform SPβless. Chloramphenicol resistant cells were selected and the resulting strain was designated HSS002.

To construct chimeric ICEBs1SPβ, the primer pairs P21/22, P23/P24, P25/P26, P27/P28, P29/P30, P31/P32, and P33/P34 were used to amplify the region from ydcI to trnS-leu1, the erythromycin resistant gene of pUCE191 (Abe et al., 2014), spacer sequences between trnS-leu1 and attL-ICEBs1, from sprA to attRspβ, from immA to immR, the sprB gene, and from ydcL to ydcQ, respectively. The B. subtilis 168 genome was used as template for P21/P22, P25/P26, P27/P28, P29/P30, P31/P32, and P33/P34 amplifications. The resulting DNA fragments were simultaneously used in PCR amplifications with primers P21/P34. Primer pairs P35/P36, P37/P38, and P39/P40 were used to amplify the region from rapI to the cat gene in the HSS002 genome, attLSPβ, and yddN and IrpA genes, respectively. The B. subtilis 168 genome was used as template for PCR with P37/P38 and P39/P40 primers. The resulting DNA fragments were simultaneously used in PCR with the primer pair P35/P40. The resulting DNA fragments were used to transform SPβless cells. The erythromycin and chloramphenicol resistant strain was selected and designated HSS003.

To eliminate the possibility of ICEBs1 integration in B. subtilis cells, the comK gene was disrupted. To this end, we used the genome of the 8G32 (ΔcomK::kan) (Ogura and Tanaka, 1997) strain to transform the ICEBs1-less SPβless strain (Δ2), which was designated Δ2CK.

To construct chimeric SPβskin, the primer pairs P41/P42, P43/P44, P45/P49, and P50/P6 were used to amplify the region from phy to 5'-spsM, the kanamycin resistant gene (kan) of pJM114 (Perego, 1993), the skr gene of the skin element, and the region from yotM to yotJ, respectively. The B. subtilis 168 genome was used as template for PCR with P41/P42, P45/P49, and P50/P6 primer pairs. The fragment from PCR with the P45/P49 primer pair was used as a template for step-by-step PCR using primer pairs P46/P49, P47/P49, and P48/P49 in order to attach an attLskin sequence to the 5' end of the fragment. These fragments were simultaneously used as template in PCR with the primers P41/P6. The primer pairs P51/P52, P11/P12, P53/P54, P55/P56, and P57/P14 were used to amplify the region from yokC to yokB, the spectinomycin resistant gene of pUCS191, the promotor region of sprA, from spoIVCA to attRskin, and from spsM-3' to mrsA, respectively. The B. subtilis 168 genome was used as template for PCR with the primer pairs P51/P52, P53/P54, P55/P56, and P57/P14. These fragments were simultaneously amplified using primer pair P51/P14. The fragments from P41/P6 and P51/P14 primer pairs were used to transform the B. subtilis 168 strain, resulting in the strain designated HSS004.

SPβskin was constructed as follows: primer pairs P7/P58, P43/P44, and P59/P60 were used to amplify the region from yokC to yokB, the kan gene of pJM114, and the region from sprA to mrsA, respectively. The B.
*subtilis* 168 genome was used as template for PCR with the primer pairs P7/P58 and P59/P60. The resulting fragments were simultaneously used as templates in PCR with the primer pair P7/P60. The resulting DNA fragment was used to transform the *B. subtilis* 168 strain to produce the strain HSS005.

The ϕ3T lysogen was constructed by infecting SPβless cells with ϕ3T phages. Integration of ϕ3T was confirmed by PCR amplification using the primers P65/P82 for *attL*ϕ3T and P81/P87 for *attR*ϕ3T, followed by DNA sequence analyses. To construct *int*ϕ3T or *rdf*ϕ3T-inducible strains, *int*ϕ3T (−31 to +958 relative to the first nucleotide of the start codon) and *rdf*ϕ3T (−24 to +91) were amplified from the chromosome of ϕ3T lysogens using the primers P61/P62 and P63/P64, respectively. The obtained DNA fragments were digested using BamHI and HindIII and were inserted into the BamHI-HindIII site of pMutinT3 (Vagner et al., 1998). The resulting pMutinT3-*int*ϕ3T and pMutinT3-*rdf*ϕ3T constructs were used to transform the ϕ3T lysogen and the corresponding *int*ϕ3T or *rdf*ϕ3T-inducible strains were designed ESI-ϕ3T and ESR-ϕ3T, respectively (Figures S7A and B). To construct a kamA-inducible strain, the kamA gene was PCR amplified using the primer pair P65/P66 and the amplicon was digested by HindIII and BamHI and were ligated into the HindIII/BamHI sites of linearized pMutinT3 plasmid. The resulting plasmid construct was used to transform SPβless. Transformants were selected according to erythromycin resistance on LB agar plates containing 1-mM IPTG for 16 h. Erythromycin resistance was confirmed by curing ICEBs1 by PCR amplification using the primer pair P75/P76. The resultant strain was designated Δ2 (trpC2 SPβless ICEBs1less).

SPβ and ICEBs1 cured strains (Δ2) were constructed by amplifying *xis*ICEBs1 to *ydiL* genes using the primers P67/P68. Amplicons were then digested using HindIII and BamHI and were ligated into the HindIII/BamHI sites of linearized pMutinT3 plasmid. The resulting plasmid construct was used to transform SPβless. Transformants were selected according to erythromycin resistance on LB agar plates containing 1-mM IPTG for 16 h. Erythromycin resistance was confirmed by curing ICEBs1 by PCR amplification using the primer pair P75/P76. The resultant strain was designated Δ2 (trpC2 SPβless ICEBs1less).

Strains for identification of *rdf*skin were constructed as follows: The primer pairs P69/P70, P69/ P71, and P69/P72 were used to amplify upstream of *spoIVCA* from −47 to +282, −76 to +282, and −111 to +282 relative to the first nucleotide of *spoIVCA*, respectively. The *B. subtilis* 168 genome was used as a template. Amplified DNA fragments were digested with HindIII and BamHI and were ligated into the HindIII/BamHI sites of linearized pMutinT3 plasmid. The resulting plasmids, pIVCAΔ1, pIVCAΔ2, and pIVCAΔ3 plasmids were used to transform *B. subtilis* 168. Transformants were selected according to erythromycin resistance and were designated IVCAΔ1, IVCAΔ2, and IVCAΔ3 respectively.

SPβ-less ICEBs1-less and skin-less (Δ3) strains were constructed as follows: initially the Δ2 strain was transformed with the plasmid pIVCAΔ3 and transformants were then grown on LB agar plates containing 1-
mM IPTG for 16 h. The resulting erythromycin resistant strain was confirmed as SPβless, ICEBs1-less, and skin-less strain using PCR and was designated Δ3 (trpC2 SPβless ICEBs1-less skin-less).

These constructed strains were confirmed by PCR and DNA sequence analysis.

Phage preparation

SPβ, SPβkan, SPβICEBs1, SPβskin, and φ3T phage lysates were prepared from 168, HSS005, HSS001, HSS004, and BGSC 1L1 (CU1065 φ3T) strains, respectively. Phage lysogens were precultured overnight in LB medium at 30°C with shaking. Overnight cultures were then diluted 100-fold in LB medium and were grown to the early log phase (OD600 ~ 0.2). Cultures were then incubated at 37°C in the presence of MMC (0.5 μg/ml) and when the optical density was decreased to around OD600 ~ 0.1, cells were removed by centrifugation at 7,000 × g at 4°C and lysates were filtered through 0.45-μm membrane filters. Phage lysates were then stored at 4°C and were spotted onto lawns of Δ3 strains to evaluate their abilities to form phage plaques.

Measurements of integration frequencies

The Δ3 strain was grown to early log phase (OD600 ~ 0.2) in LB medium and was infected with the obtained phages at a multiplicity of infection (MOI) of 0.1. Cells were then incubated for 1 h at room temperature without shaking, and were then plated onto LB plates containing antibiotics. Integration frequencies were calculated as described by Tal et al. (2014). Insertion of the phages into attB sites was verified using colony PCR. In these analyses, colonies were picked using sterilized toothpicks and were transferred to PCR tubes. PCR tubes were then irradiated in a microwave oven for 1.5 min and PCR reaction mixture was added to the PCR tubes.

Mating experiments

Mating experiments were performed using previously published methods (Auchtung et al., 2005) with some modifications. Briefly, donor cells were grown in defined minimal medium and treated with MMC. Transconjugants were selected according to the presence of kanamycin and chloramphenicol resistance genes in ICEBs1cat and ICEBs1sp. Mating frequencies were calculated by dividing numbers of transconjugants by numbers of recipient cells. Transfer frequencies are reported as means ± standard errors of the mean from at least three independent biological replicates. Insertion of phages into attB sites was verified using colony PCR.
**β-galactosidase assays**

Insertion of pMutinT3 plasmid into target genes inactivates the gene and allows analysis of its transcriptional profile by measuring β-galactosidase activity. To measure transcriptional activity of int₆₃T, rdf₆₃T, and kamA, the strains ESI-φ₃T, ESR-φ₃T, and INDkamA strains, respectively, were precultured in LB medium at 30°C for 16 h. Cells were then inoculated into Difco sporulation medium (DSM) at OD₆₀₀ ~ 0.04 and were incubated at 37°C for indicated times. β-galactosidase activity was determined using the method described by Miller (Miller, 1972).

**Excision assay**

To evaluate phage and ICE excision from host genomes, attB, but not attP, was used as a target DNA region for PCR amplification, because under these conditions, phage and ICE genomes were spontaneously excised at low frequencies and were amplified into multiple copies of DNA. PCR amplification was performed using Prime taq (GenetBio, Korea) with 100-ng aliquots of extracted genomic DNA. Primer sequences are listed in Table S4. PCR cycle numbers were adjusted to avoid reaching plateaus as follows: P73/P74 for attB₃₉₃, SPβkan and SPβ, 25 cycles; ICEBs₁₆₃, 27 cycles; P75/P76 for attBICEBI, SPβICEBI, 27 cycles; ICEBs₁cat, 25 cycles; P77/P78 for attBskin, skin and SPβskin, 25 cycles.

**Quantitative PCR assays**

Quantitative PCR assays were performed using previously published methods (Abe et al., 2017) with some modifications. Briefly, the qPCR reactions were performed using the KOD SYBR qPCR Mix (TOYOBO, Japan) with 50-ng aliquots of genomic DNA. To prepare a DNA standard for absolute quantification of attB, the attB₃₉₃, attBICEBI, attBskin, and attB₃₉₃T were amplified by PCR from the chromosomal DNA of Δ₃ strain, using P73/P74, P75/P76, P77/P78, and P79/P80, respectively. The quantitative PCR assay was conducted at 98°C for 2 min and then 40 cycles of 98°C for 10 sec, 60°C for 10 sec, and 68°C for 35 sec. The reaction specificity was verified using a melt curve analysis. As an internal control for the quantification, the copy number of yodT, which is a single-copy gene in the B. subtilis genome with no involvement in the phage excision, was quantified by the same method as described above, using the P83/P84 primers. The phage and ICE excision frequency were calculated as a ratio of the copy number of the attB site to that of yodT.
Syntenic plots of SMGC–Easyfig

Syntenic plots were generated using Easyfig version 2.2.2 (Sullivan et al., 2011). All GenBank files describing the clusters to be compared were obtained from the nucleotide database of the National Center for Biotechnology Information (NCBI). The tBLASTx option was used with an e− ≤ 0.001 and an alignment identity of ≥ 60.
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