Developmentally-Regulated Excision of the SPβ Prophage Reconstitutes a Gene Required for Spore Envelope Maturation in *Bacillus subtilis*

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Abstract

Temperate phages infect bacteria by injecting their DNA into bacterial cells, where it becomes incorporated into the host genome as a prophage. In the genome of *Bacillus subtilis* 168, an active prophage, SPβ, is inserted into a polysaccharide synthesis gene, *spsM*. Here, we show that a rearrangement occurs during sporulation to reconstitute a functional composite *spsM* gene by precise excision of SPβ from the chromosome. SPβ excision requires a putative site-specific recombinase, SprA, and an accessory protein, SprB. A minimized SPβ, where all the SPβ genes were deleted, except *sprA* and *sprB*, retained the SPβ excision activity during sporulation, demonstrating that *sprA* and *sprB* are necessary and sufficient for the excision. While expression of *sprA* was observed during vegetative growth, *sprB* was induced during sporulation and upon mitomycin C treatment, which triggers the phage lytic cycle. We also demonstrated that overexpression of *sprB* (but not of *sprA*) resulted in SPβ prophage excision without triggering the lytic cycle. These results suggest that *sprB* is the factor that controls the timing of phage excision. Furthermore, we provide evidence that *spsM* is essential for the addition of polysaccharides to the spore envelope. The presence of polysaccharides on the spore surface renders the spore hydrophilic in water. This property may be beneficial in allowing spores to disperse in natural environments via water flow. A similar rearrangement occurs in *Bacillus amyloliquefaciens* FZB42, where a SPβ-like element is excised during sporulation to reconstitute a polysaccharide synthesis gene, suggesting that this type of gene rearrangement is common in spore-forming bacteria because it can be spread by phage infection.

Introduction

Genetic information is normally preserved across generations in living organisms. However, genomic integrity is sometimes dramatically challenged by DNA rearrangement events, such as homologous recombination, viral genome integration, and transposon spreading. These DNA rearrangements contribute to genetic diversification in the evolutionary history of life on Earth. Importantly, some of these rearrangements are programmed to occur at specific sites and times during cellular differentiation and play crucial developmental roles in a variety of organisms. The best-known example is the rearrangement of immunoglobulin genes in the B lymphocytes of the vertebrate immune system. The assembly in different combinations of the variable (V), diversity (D), and joining (J) exons of the immunoglobulin gene generates antigen receptors with extremely diverse binding specificities [1].

DNA rearrangements also modulate gene expression in bacteria during cellular differentiation. For example, during differentiation to a heterocyst, which is a cell type that fixes atmospheric nitrogen, bacteria of the *Anabaena* genus have the ability to reconstitute the disrupted *nifD, fdsN*, and *hupL* genes that are normally inactive in photosynthetic cells [2–5]. In the sporulating Gram-positive bacterium *Bacillus subtilis*, the *sigK* gene, which encodes the sporulation sigma factor σ^K_, is interrupted by the phage-like element *skin*. During sporulation, *skin* is excised and a functional composite *sigK* gene is produced [6].

*B. subtilis* cells produce endospores in response to nutrient starvation. The *B. subtilis* spore envelope is characterized by a succession of concentric layers of chemically distinct composition: the cortex is a peptidoglycan layer assembled between the inner and outer spore membranes, while the coat is an external proteinaceous layer, which can be further subdivided into an inner coat layer and an outer coat layer [7,8]. An additional layer called the spore crust was recently discovered outside the outer coat [8–10]. Spore formation in *B. subtilis* has been studied extensively as a model system for cellular differentiation. The
process begins with an asymmetric division of the sporulating cell, thus producing two compartments of unequal size, each containing a copy of the genome. The two compartments will differentiate into specific cell types: the forespore and the larger mother cell. During sporulation, a cascade of sporulation sigma factors governs gene expression in a temporally controlled, cell-specific manner [11–13]. During the early stages of sporulation, gene expression is controlled by $\sigma^+$ in the forespore and $\sigma^-$ in the mother cell, whereas $\sigma^K$ (in the forespore) and $\sigma^K$ (in the mother cell) control the later stages of the developmental program. The $\sigma^K$-encoding gene, sigK, is disrupted by skin thereby splitting the gene into two protein coding sequences, spoIVCB (5’-end of sigK) and spoIICH (3’-end of sigK) [6]. A site-specific DNA recombinaise, SpoIVCA, promotes excision of skin from the chromosome and the joining in frame of spoIVCB and spoIICH to reconstitute a functional sigK gene [6,14–16]. The spoIVCA gene is located in the skin element and is expressed exclusively in the mother cell during sporulation under the control of $\sigma^-$. The rearranged mother cell chromosome is not transmitted to the progeny because the mother cell undergoes autolysis at the end of sporulation to release the mature spore (whose genome has not been rearranged) in the environment. A similar rearrangement of the sigK gene was observed in the pathogenic spore-forming bacterium Clostridium difficile [17].

This type of DNA rearrangement was thought to be a unique case because no examples other than sigK had been reported in spore-forming bacteria. However, we recently characterized two other cases of novel intervening sequence elements in mother cell-expressed sporulation genes, yodK in the spoVFB gene of Bacillus weihenstephanensis KBAB4 and yin in the spoYR gene of Geobacillus thermoglucosidasius C56-YS93 [18]. These findings suggest that DNA rearrangements may be common in the mother cell genome of spore-forming species, prompting us to embark in a systematic analysis of intervening sequence elements in spore-forming bacteria.

B. subtilis 168 contains 10 prophage-like elements [19]. Of these 10 elements, only skin and SPβ are inserted into protein-coding regions. SPβ is integrated into yodM (spore polysaccharide synthesis protein M), thus producing two gene fragments, yodU (5’-end of yodM) and ypqP (3’-end of yodM). The yodU and ypqP genes are expressed during sporulation under the control of $\sigma^K$ [20–22]. The most significant difference between skin and SPβ is that skin is a cryptic phage, whereas SPβ is an active prophage. SPβ usually stays in the dormant state (lysogenic cycle). However, when the SOS response is induced by DNA damage, specific genes in the SPβ genome are activated to generate virions that are released after lysis of the host cell (lytic cycle) [23]. A putative site-specific recombinase SpRA (SPβ site-specific recombination factor A; formerly yokA) encoded in the SPβ prophage region is a candidate to promote SPβ excision from the host genome [23]. Nevertheless, the requirement for SpRA in SPβ excision had not been investigated until now and the mechanism of excision is poorly understood. In the present study, we examined the fate of SPβ during sporulation. We showed that SPβ was excised from the mother cell genome, thus producing a composite yodM gene. We also investigated the biological function of SpsM and discovered significant changes in the surface properties of spores produced by mutant strains unable to reconstitute a functional yodM gene.

### Results

**yodU and ypqP Encode the N- and C-Terminal Portions of SpsM in B. subtilis 168**

In the genome of B. subtilis 168, the SPβ prophage is located between two open reading frames (ORFs), yodU (NCBI gene locus tag BSU19810) and ypqP (BSU21670). Amino acid (aa) sequence alignment and comparison to non-SPβ lysogenic B. subtilis strains, such as BEST195 (NCBI reference sequence no. NC_017196), showed that YodU (140 aa) and YpqP (207 aa) corresponded to the N- and the C-terminal portions of SpsM (Figure S1A). An overlapping 5-aa sequence “TDKAV” was observed at the C-terminus of YodU and at the N-terminus of YpqP. This sequence corresponds to the translation of the nucleotide sequence of the attachment site for SPβ. When the aa sequences of YodU and YpqP were joined at the overlapping sequence, the composite SpsM aa sequence was identical to that of strain BEST195, thereby indicating that B. subtilis 168 yodM does not contain any mutations (non-sense, missense, deletions or insertions). SpsM is a 341-aa protein, which contains a Polysacc._syt42_domain (Plam accession number, PF2719) in the 18–296-aa region. This domain was first observed in Staphylococcus aureus CapD [24], and is shared among bacterial polysaccharide biosynthesis proteins, such as Campylobacter jejuni WlaL (putative sugar epimerase/dehydrogenase) [25] and several sugar epimerases. SpsM shared 38% identity with a B. subtilis paralog, EpsC [26]. EpsC is an UDP-sugar epimerase encoded by the epsC locus and is essential for the production of extracellular polysaccharide (EPS) during biofilm formation [26]. B. subtilis SpsM has not been previously characterized, but the conserved domain and similarity to EpsC suggest that SpsM is a sugar epimerase likely to be involved in polysaccharide synthesis. However, a capsular polysaccharide has yet to be identified in vegetative cells of B. subtilis. Considering that yodU (the 5’-segment of yodM) and ypqP (the 3’-segment of yodM) were identified as sporulation genes in recent transcriptomic analyses of B. subtilis 168 [21,22], we postulated that yodM is involved in the synthesis of the spore polysaccharide. Similar transcriptional profiling results were obtained in the PY79 strain of B. subtilis, which is derived from 168, but cured of SPβ, where intact yodM was reported as a $\sigma^-K$-dependent gene [20]. As a whole, this information led us to hypothesize that in B. subtilis 168 the yodM rearrangement occurs during sporulation to allow production of spore polysaccharide.
Reconstitution of spsM upon MMC Treatment and during Sporulation

Figure 1A shows a diagram of the 134-kb long SPβ prophage from the B. subtilis 168 genome. sprA (formerly yokA; NCBI gene locus tag, BSU21660), which is located immediately upstream of ypqP, encodes a putative site-specific DNA recombinase, which shares 26% identity with SpoIYCA of the skin element. The attachment sites are indicated by triangles. When B. subtilis 168 vegetative cells are treated with mitomycin C (MMC), SPβ is excised (Figure 1B, left panel). Specifically, a wild-type culture was grown in Luria-Bertani (LB) medium and MMC (0.5 μg/ml) was added to the medium during the early exponential phase of growth (optical density at 600 nm [OD₆₀₀] = 0.25). DNA samples were extracted from the cells at different time points after MMC addition and digested with NdeI. From 0 to 120 min after MMC treatment, Southern blotting using the sprA-specific probe (sprA probe) detected a 9.9-kb band (corresponding to the DNA arrangement before SPβ excision). In addition to the 9.9-kb band, a second 5.6-kb band was detected at 60, 90, and 120 min after MMC treatment, which indicated SPβ excision and reconstitution of spsM. Subsequently, to examine spsM rearrangement during sporulation, we performed Southern blotting using DNA samples from sporulating B. subtilis 168 cells (Figure 1B, right panels). The wild-type cells were cultured at 37°C in liquid Difco sporulation medium (DSM) and harvested at successive time points one hour before, at the onset of stationary phase and every hour thereafter for 8 hours after the onset of stationary phase. Southern blotting using the sprA probe detected the 9.9-kb band from T₅₋₁ to T₃ (Figure 1B, right top panel). The 5.6-kb band was detected at T₃ and later, thereby indicating that SPβ was excised during sporulation without the need for MMC treatment (Figure 1B, right top panel). We also examined the spsM rearrangement using the ypqP-specific probe (ypqP probe) (Figure 1B, right bottom panel). In addition to the 9.9-kb band, a 6.1-kb band, which corresponded to the composite spsM, was detected at T₃ and later. To confirm spsM reconstitution, we determined the DNA sequences at the junction sites of the excised SPβ and composite spsM. The sequencing data showed that SPβ excision in the sporulating cells occurred at the same site as that in the MMC-treated vegetative cells (Figure S1B) [23]. The SPβ attachment sites contain 16-Bp core sequences (Figure S1B, nucleotides boxed in red) and 16-Bp inverted repeat sequences (Figure S1B, arrows). Next, we determined the compartment where the spsM rearrangement occurred, i.e., the mother cell or forespore. The mother cell DNA and forespore DNA were isolated from wild-type cells at T₆ and subjected to Southern blotting. The 5.6-kb and 6.1-kb bands were detected only in the mother-cell compartment, which indicated that SPβ excision during sporulation was a mother-cell-specific event and that the SPβ prophage DNA is maintained in the spore genome (Figure 1C). To evaluate the ability of the excised SPβ to form phage particles during sporulation, the supernatant of the DSM culture was filtered and spotted onto a lawn produced by a SPβ-sensitive strain CU1050 [27]. Plaques were not formed (Figure 1D), suggesting that SPβ excised during sporulation is not a phage particle. Nevertheless, we confirmed that the spsM rearrangement can occur during sporulation in a new SPβ lysogen, CU1050 (SPβ), which was obtained by infecting CU1050 cells with the SPβ phage lysate (Figure 1E). This result indicates that the spsM rearrangement system can be transferred to a new host via SPβ phage infection.

DNA Rearrangement of Bacillus amyloliquefaciens spsM

In addition to B. subtilis 168, several B. amyloliquefaciens strains carry a prophage sequence similar to SPβ at the spsM locus (Figure 2A and Table S1). The numbers of SPβ-related genes varied considerably among all of these strains. These SPβ-like elements are likely to be remnants of the SPβ prophage and have probably lost their ability to form infectious phage particles, because large parts of the SPβ-related genes were missing. Since the gene encoding the putative site-specific recombinase, sprA, was conserved in all of these elements, we examined whether the SPβ-like element was excised from the chromosome in B. amyloliquefaciens strain FZB42 (BGSC catalogue number, 10A6). Figure 2B shows a diagram of the SPβ-like element in B. amyloliquefaciens FZB42. First, we tested whether the element responded to MMC by analyzing a DNA sample prepared from MMC-treated vegetative cells of strain FZB42 and subjected to Southern blotting. The B. amyloliquefaciens sprA-specific probe (sprABam probe) detected a single 5.9-kb band from 0 to 120 min after MMC addition, indicating that excision of the element did not occur in the MMC-treated vegetative cells (Figure 2C, upper panel). Subsequently, Southern blotting was performed using a DNA sample obtained from sporulating cells of strain FZB42 (Figure 2C, lower panels). Bands indicating excision of the element (13 kb, left panel) and the generation of the composite spsM (3.6 kb, right panel) were detected using the sprABam and ypqPProbe probes, respectively. These data indicate that the SPβ-like element of B. amyloliquefaciens FZB42 exhibits a behavior distinct from the B. subtilis SPβ, but similar to skin, because it did not respond to MMC treatment and was excised only during sporulation.

sprA and spsM Expression and Regulation of SPβ Excision

Considering that in B. subtilis SPβ excision occurs both during sporulation and in response to DNA damage, whereas in B. amyloliquefaciens excision of the SPβ-like element only occurs during sporulation, it is likely that different mechanisms control prophage excision during sporulation and upon MMC treatment. To analyze how SPβ controlled its excision and to determine whether spsM expression always followed prophage excision, we constructed transcriptional lacZ fusions to yodU (5′-spsM) [YODUΔ; yodU::pMutinT3, PᵧodU−lacZ] and sprA (SPRAΔ; sprA::pMutinT3, PₛprsM−lacZ) using the pMutinT3 insertion plasmid (Figure S2A). Insertion of the pMutinT3 vector into a genome locus causes inactivation of the corresponding gene and allows analysis of its expression profile by measuring β-galactosidase activity, because the gene of interest is now transcriptionally fused to lacZ [28]. In the YODUΔ strain, PᵧodU−lacZ was expressed during the late stages of sporulation, consistent with the previously reported σA-dependency for yodU expression (Figure 3A, left panel, hour 8 and later). The timing of expression of yodU was delayed by 2 hours when compared to that of cotG, another σA-dependent gene [29]. This delay is likely due to the fact that yodU expression also requires the transcription factor GerE, which regulates gene expression in the mother cell during the ultimate stage of sporulation, as previously shown [20]. By contrast, PₛprsM−lacZ was not expressed in MMC-treated vegetative cells (Figure 3B, left panel), indicating that prophage excision does not systematically trigger spsM expression.

Analysis of the SPRAd mutant strain by Southern blotting did not reveal any difference in the band patterns of vegetative and sporulating cells (Figures 4A, middle panels and S2A), showing that sprA was necessary for spsM reconstitution. Nicolas et al. predicted a putative binding site for the housekeeping σ factor σb at positions −85 to −57 (TTGGTTT for the −33 box and TAAAAT for the −10 box) relative to the sprA start codon [22]. Consistent with a σb-dependent pattern of expression, but at odds with a specific role for SprA during the late stages of sporulation, the PₛprsM−lacZ activity kept increasing during vegetative growth.
peaked during the early stages of sporulation and gradually decreased as sporulation proceeded (Figure 3A, middle panel). The sprA expression level in vegetative cells was not increased by MMC addition (Figure 3B, middle panel). These unexpected results suggest that an additional factor(s) regulates the timing of prophage excision during sporulation and following DNA damage.

We observed that sprB (formerly yotN; NCBI gene locus tag, BSU19820), a SPβ gene located downstream of yodU, was conserved in all of the SPβ-like elements (Figure 2A). It encodes a 58-aa protein with no significant similarity to characterized proteins. To test whether sprB was required for excision, we constructed a sprB deletion mutant strain (SPRBd). Southern blotting revealed that SPRBd was defective in SPβ excision (Figures 4A, right panels and S2B), indicating that sprB was necessary for excision. As expected, P_sprB–lacZ was expressed during the middle and late stages of sporulation (Figure 3A, right panel) and was also induced by MMC addition to vegetative cells (Figure 3B, right panel, 60 min and later).

To examine the correlation between SPβ excision and sprA and sprB expression, we constructed the sprA-inducible strain (BsINDA) and the sprB-inducible strain (BsINDB), where sprA or sprB expression can be induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) addition. SPβ was excised when sprB expression was induced in BsINDB, but not when sprA was overexpressed in BsINDA (Figure S3). Combined with the results from Figure 3, showing that P_sprA–lacZ is expressed at significant levels during vegetative growth and the early stages of sporulation, we conclude that expression of sprA alone is not sufficient to excise SPβ from the chromosome (Figure S3A). By contrast, when sprB is induced, either in the presence of MMC, during sporulation, or artificially by IPTG addition, excision of SPβ will ensue (Figure S3B), provided that SprA is also present. In summary, both sprA

Figure 1. DNA rearrangement at the spsM locus. (A) Diagram showing SPβ excision in Bacillus subtilis 168. The thick lines indicate the location of the digoxigenin (DIG)-labeled probes used for Southern blotting. Nde indicates NdeI sites. Triangles point to the attachment sites for SPβ. (B) SPβ excision upon mitomycin C (MMC) treatment and during sporulation. Left panel shows induction of SPβ excision by MMC treatment. B. subtilis 168 cells were grown in LB medium. Vegetative cells in the early log phase (OD600 = 0.25) were treated with 0.5 μg/ml MMC. Time 0 indicates the time point immediately after MMC addition. Right panels show SPβ excision (top panel) and spsM reconstitution (bottom panel) during sporulation. B. subtilis 168 cells were grown in DSM, and samples were taken at the indicated times (h) after the onset of sporulation (T0). The DNA samples were digested with NdeI and subjected to Southern blotting. (C) Mother cell-specific SPβ excision. Chromosomal DNA from the vegetative cells (V) at T2, whole sporangia (W) at T8, and the forespores (FS) at T8 were isolated, digested with NdeI, and subjected to Southern blotting. (D) Lytic activity of SPβ phages. SPβ phage lysate, which was prepared by treating the Bacillus subtilis 168 vegetative cells with MMC, was spotted on the plate (MMC). The DSM culture of B. subtilis 168 at T6, T12, T24, and T48 was centrifuged and the supernatant was filtered with 0.44 μm Millex filter (Millipore). The filtrate was spotted on the lawn of a SPβ-sensitive strain CU1050 (DSM T24 and DSM T48). (E) Horizontal transfer of spsM rearrangement system. A new SPβ-lysogen, CU1050 (SPβ) was obtained by infecting CU1050 cells with the SPβ phage lysate. The CU1050 and CU1050 (SPβ) cells were induced to sporulate on DSM-agar plates at 37°C for 3 hours (V, vegetative cells) and 12 hours (S, sporulating cells). Chromosomal DNA of the CU1050 and CU1050 (SPβ) cells was subjected to Southern blotting.

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and sprB are necessary for excision, but the temporal control of excision is dependent on sprB. To determine whether SPβ genes other than sprA and sprB were also required for excision, we constructed a SPβ mutant strain (SPmini), where all the SPβ genes were deleted, except sprA and sprB. The SPmini strain retained the capacity for spsM rearrangement during sporulation (Figure 4C, left panels), indicating that sprA and sprB are necessary and sufficient for SPβ excision during sporulation. By contrast, SPmini did not undergo excision upon MMC treatment (Figure 4C, right panels), suggesting that an additional gene(s) or regulatory sequence present in SPβ but absent in SPmini may be required to promote excision and/or trigger sprB expression following DNA damage.

Regulation of sprB Expression

Since sprB is a key factor in the control of SPβ excision, we analyzed its transcriptional regulation (Figure 5). We performed Northern blotting using a sprB-specific probe (Figure 5A, thick black line). A major band of 5.0 kb and minor bands of 1.2 and 2.0 kb were detected in MMC-treated vegetative cells but not in untreated cells (Figure 5C, columns 1 and 2). By contrast, a single 0.2-kb band was detected during sporulation (Figure 5C, column 3). This result suggested that sprB was transcribed from distinct promoters upon MMC treatment and during sporulation. Lazarevic et al. reported that the yosX gene, which is located 5 kb upstream of sprB, possesses a σA-dependent promoter [23], but no other σA-dependent promoter was predicted between yosX and sprB (Figure 5A). Thus, it is likely that the major 5.0 kb band detected upon MMC treatment corresponds to a transcript originating from the yosX promoter, while the minor bands could correspond to truncated transcripts. Next, we performed RT-PCR using a sprB-specific reverse transcription primer (Figure 5A, RT primer, red arrow) followed by PCR amplification of the sprB cDNA using yosX, yotBCD, or sprB specific primers (Figure 5A, black arrows). When the sprB cDNA was obtained from the MMC-treated cells (Figure 5D, column 2) the yosX, yotBCD, and sprB regions were successfully amplified, whereas when the sprB cDNA was obtained from sporulating cells, only the sprB region...
could be amplified (Figure 5D, column 3). This result indicates that sprB is indeed co-transcribed with the upstream genes upon MMC treatment while it appears to be monocistronically transcribed during sporulation.

To determine the 5’ end of the sprB transcript during sporulation, we carried out 5’ RACE PCR with total RNA extracted at T₄. The sprB transcriptional start site (TSS) was found to be located 20 nt upstream of the start codon (Figures 5B and S4). Using DBTBS Search Tools (http://dbtbs.hgc.jp/) [30], a putative σ₅₄- or σ₇₀-binding site was found directly upstream of the TSS of sprB (Figure 5B). However, while the −10 element was a perfect match to the σ₅₄- or σ₇₀-consensus sequence, the putative −35 element of the sprB promoter was an imperfect match (Figure 5B). It is therefore possible that an additional mother cell transcription factor, such as SpoIIID, GerR or GerE, is required along with σ₅₄ or σ₇₀ for optimal expression of sprB. To test whether sprB expression is restricted to the mother cell, as would be expected if it is controlled by a σ₅₄ or σ₇₀, we constructed strain BsSPRBG, which harbors a plasmid carrying the translational fusion sprB–gfp without the upstream phage genes. As expected, GFP fluorescence in BsSPRBG was detected only in the mother cell (Figure 5E). Importantly, this observation is also consistent with the data presented above (Figure 3A), where P_sprB–lacZ activity was detected during the middle to the late stages of the sporulation, when σ₅₄ and σ₇₀ are most active.

Negative Staining of Spores Is Dependent on sprB

To investigate the functional role of sprB in sporulation, we used the YODUd (yodU) and SPRAd (sprA) strains. Since SPRBd exhibited the same phenotype as SPRAd, only the SPRAd strain will be considered further. We analyzed the morphologies of wild-type, YODUd, and SPRAd spores using phase-contrast microscopy and a negative staining procedure. When the spores were negatively-stained with Indian ink [31], which is a stain commonly used to reveal polysaccharide capsules, a clear halo was visible around the wild-type spores, but not around the YODUd and SPRAd spores (Figure 6A, top panels). The appearance of a halo is consistent with the presence of polysaccharides around the wild-type spore. Introduction of the composite sprB gene at the amyE locus of the mutant strains complemented the sprA and yodU mutations (SPRAc and YODUc) in the sense that the halo was restored (Figure 6A, top panels, sprA sprB+ and yodU sprB+). In addition, we observed that this putative polysaccharide layer of the wild-type spore was loose, because it can easily be removed from the spores by boiling in a buffer containing SDS. After this treatment, the wild-type and sprB− lacZ

Figure 3. Expression of sprM, sprA, and sprB in response to mitomycin C treatment and during sporulation. (A) β-galactosidase activity of B. subtilis strains carrying lacZ reporter constructs during sporulation. The B. subtilis strains, YODUd (yodU–lacZ), SPRAd (sprA–lacZ), and BsINDB (sprB–lacZ), were sporulated at 37°C in liquid DSM. Aliquots were collected at various time points during sporulation, and the β-galactosidase activity (Miller units, MU) was determined using o-nitrophenyl-β-D-galactoside (ONPG) as a substrate. To compare the expression pattern of yodU (S₄–spsM) to that of cotG, a previously-known σ₅₄-dependent sporulation gene [29], the β-galactosidase activity of cotG–lacZ (COTGd) is shown on the left panel (gray line, right axis) along yodU–lacZ (purple line, left axis). SPJ excision and sprM rearrangement occurred at T₃ and later time points (blue-shaded areas). The background activity was subtracted from the values. Error bars indicate ± standard deviations based on three independent experiments. (B) β-galactosidase activity of B. subtilis vegetative cells carrying the lacZ reporter construct fused transcriptionally to the promoters of sprA and sprB in response to MMC treatment. The B. subtilis strains, YODUd (yodU–lacZ), SPRAd (sprA–lacZ), and BsINDB (sprB–lacZ), were cultured in liquid LB medium. MMC was added to a final concentration of 0.5 µg/ml when the cells reached an OD₆₀₀ of 0.5. The culture was sampled at 0, 20, 40, 60, 80, 100, and 120 min after the addition of MMC. SPJ excision occurred at 60 min and later (blue-shaded areas). Error bars indicate ± standard deviations based on three independent experiments.

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SPRAd and YODUd spores, as none of the spores exhibited a halo (Figure 6A, bottom panels). These results suggest that the composite \( \text{spsM} \) is necessary for the production of an external spore structure most likely composed of polysaccharides.

Analysis of the Chemical Composition of the Spore Surface

Surface extracts from wild-type spores were loaded on a 5% polyacrylamide gel, separated by electrophoresis and stained with stains-All, a cationic carbocyanine dye that stains polysaccharides, nucleic acids, and acidic proteins. The spore surface component was detected as a bright blue band (Figure 6B), which indicated the presence of a high molecular weight substance. The blue band was not detected in extracts from SPRAd and YODUd \((\text{sprA} \text{ and } \text{yodU})\), whereas it was detected in extracts from SPRAc \((\text{sprA spsM}+)\) and YODUc \((\text{yodU spsM}+)\). These results imply that the formation of the spore surface component is dependent on the function of the composite \( \text{spsM} \). SpsM is a paralog of a polysaccharide synthesis protein, EpsC [26]. Thus, the high molecular weight substance from the spore surface is likely to be a polysaccharide, whose synthesis and/or attachment to the spore is dependent on SpsM. In addition, this high molecular weight substance was inferred to be produced in the mother cell because the composite SpsM protein fused to GFP was observed to reside in the mother cell during sporulation (Figure S5), consistent with its regulation by \( \sigma^E \) [20]. We quantified the amount of spore surface component using the method described by Hammerschmidt et al. [32]. The levels of high molecular weight substance in the SPRAd and YODUd spore surface extracts decreased to 12.5% and 5.0% of the amount isolated from wild-type spores (Figure 6C).

Next, we analyzed the monosaccharide composition of the wild-type spore surface extract. The extract was hydrolyzed and fluorescently labeled with 4-amino-benzoic acid ethyl ester (4-ABEE). HPLC analysis detected three major peaks. By comparison to fluorescently labeled monosaccharide standards, we infer that the two peaks detected in the extracts at retention times of 10.9 and 30.8 min corresponded to galactose and rhamnose.
respectively (Figure S6, peaks 3 and 12). A peak at 6.4 min, which did not correspond to any monosaccharide standard, was considered to be an unknown monosaccharide(s) or could result from an incomplete hydrolysis of oligosaccharides. The galactose and rhamnose peaks accounted for 21.1% and 68.1% of total monosaccharides detected by HPLC, respectively. The presence of rhamnose at the spore surface has been previously reported and was shown to be dependent on the enzymes SpsI, SpsJ, SpsK and SpsL, whose synthesis is dependent on σK during sporulation [20,33,34]. In conclusion, our experiments indicate that polysaccharides are present in spore surface extracts and that the spore polysaccharide are beneficial for the dispersal of the spores were left on the plates. Therefore, our results suggest that the spore polysaccharides are beneficial for the dispersal of B. subtilis spores through water and help prevent adherence to certain types of surfaces.

Properties of the spsM Mutant Spores

Subsequently, we investigated the functional roles of the spore polysaccharides. YODUd and SPRAd retained the ability to produce phase-bright and wet-heat resistant spores although spore titers in DSM cultures were slightly smaller than that of the wild type (Table S2). In addition, a SPβ-cured strain, SPβless, produced normal wet-heat resistant spores with a sporulation efficiency that was comparable to that of the wild type (Table S2). However, we noticed that the mutant spores exhibited significant differences in their properties. The purified mutant spores formed aggregates and displayed enhanced adhesion to solid surfaces, such as borosilicate glass and polypropylene. Figure 7A reports that mutant spores adhere to Pyrex tubes (13 x 100 mm, Corning), whereas wild-type and spsM+ spores do not. Figure 7B shows the result of an adhesion test using polypropylene tubes (see Materials and Methods). While 80%-90% of YODUd and SPRAd spores adhered to the tubes after five transfers, wild-type and spsM+ spores barely adhered to the tube even after ten transfers. Finally, we investigated the adhesive properties of the mutant spores on DSM-agar plates (Figure 7C). B. subtilis cells were cultured at 37°C on DSM plates for a week to allow sporulation. After this period, >95% cells on the plates became mature spores (Figure 7C, upper panels). After the plate was rinsed with water, the wild-type spores dispersed in water and disappeared from the plate (Figure 7C, lower panels). However, the SPRAd and YODUd spores were barely resuspended in water, and most of the spores were left on the plates. Therefore, our results suggest that the spore polysaccharides are beneficial for the dispersal of B. subtilis spores through water and help prevent adherence to certain types of surfaces.
Discussion

We demonstrated that both *B. subtilis* and *B. amyloliquefaciens* reconstitute a functional *spsM* gene during sporulation through developmentally-controlled excision of the SPβ prophage (Figures 1 and 2); however, while SPβ is an active prophage in *B. subtilis*, it has become a cryptic prophage in strains of *B. amyloliquefaciens* (Figure 2 and Table S1). The observation that the *spsM* rearrangement system can be transferred to a non-lysogenic strain via SPβ infection (Figure 1E) suggests that the element was originally acquired by the current lysogenic strains following an infection with an ancestral phage identical or very closely related to SPβ. We speculate that the strains of *B. amyloliquefaciens* have been infected with SPβ earlier than *B. subtilis* and have since lost most of the original phage genes, probably because they did not confer significant advantages or may even be harmful to the host (Figure 8A).

Prophage-mediated reconstitution of mother cell-specific sporulation genes is a common event since other intervening elements (e.g., *skin*, *vfbin*, and *vrin*) that carry phage-related genes have been previously observed in several spore-forming bacteria [18,35]. Similar to *B. amyloliquefaciens* SPβ, these elements are the descendants of ancestral prophages and have now become defective for producing phage particles, but are still being excised under specific conditions (Figure 8A). Importantly, the excision of these elements from the host genome is developmentally regulated and confined to a terminally differentiated cell type, the mother cell (Figure 1C) [6,16,18]. Limiting the DNA rearrangement to the mother cell genome ensures that the phage DNA is maintained in the spore genome (Figure 8B). Thus, after spore germination,

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**Figure 6. Analysis of *B. subtilis* spore surface components.** (A) Negative staining with Indian ink of the *B. subtilis* wild-type and mutants. The purified spores from strain 168 (WT), SPRAd (sprA), YODUd (yodU), SPRAc (sprA spsM*), and YODUc (yodU spsM*) were negatively stained with Indian ink and observed using phase-contrast microscopy. Untreated, native spores; boiled, heat-treated spores at 98 °C 10 min in SDS buffer. Scale bars, 4 μm. (B) Electrophoresis of *B. subtilis* spore surface extracts. Spore surface extracts from strain 168 (WT), SPRAd (sprA), YODUd (yodU), SPRAc (sprA spsM*), and YODUc (yodU spsM*) were loaded onto a 5% native polyacrylamide gel. The gel was stained with Stains-All after electrophoresis. (C) Quantification of the polysaccharides in spore surface extracts. The spore surface polysaccharides from *B. subtilis* spores were ethanol-precipitated. The precipitants were dissolved in water and reacted with Stains-All. The amounts of polysaccharides were determined by measuring the OD_{640} according to the method described by Hammerschmidt et al. [32]. Error bars indicate ± standard deviations based on three independent experiments.

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Figure 7. Spore properties. (A) Adhesion of the mutant and wild-type spores to glass tubes. The spores purified from strain 168 (WT), SPRAd (sprA), YODUd (yodU), SPRAc (sprA spsM*), and YODUc (yodU spsM*) were resuspended in water and the final OD600 was adjusted to 15. Each 30 μl of spore resuspension was added to a Pyrex tube (13 x 100 mm, Corning) and vortexed gently for 30 s. After removing the spore resuspensions, the glass tubes were briefly dried and images were acquired. (B) Adhesion of the mutant and wild-type spores to polypropylene tubes. Adhesion (%) was determined by 10 successive binding reactions of the spores to the tubes. Error bars indicate ± standard deviations based on three independent experiments. (C) The polysaccharide layer facilitates spore dispersal through water flow. Overnight cultures of B. subtilis cells grown in LB medium were spotted onto DSM-agar plates. The plates were incubated at 37°C for 1 week. Each colony was confirmed as containing >95% free spores using phase-contrast microscopy. The images show the spore colonies on the DSM plates before (upper panels) and after rinsing with 1 ml of DDW (lower panels). The wild-type spores on the plates were dispersed by water, whereas the mutant spores stuck to the plates.

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Figure 8. Model of the phage-mediated DNA rearrangement. (A) A model of the sporulation-specific phage-mediated gene rearrangement, based on the cases of SP1 in B. subtilis and B. amyloliquefaciens. (B) Maintenance of the intervening element in the host genome. Sporulation gene (spo gene), black box; attachment sites, triangle; intervening element, red line; sprA and sprB, red arrow; phage-related genes, red box; host genes, open box.

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SPβ is vertically transferred to the progeny upon cell division as a permanent element in the host genome sequence.

Insertion of prophages in sporulation genes is advantageous to the host for at least two reasons: (1) to add one level of control to the progression of sporulation; and (2) to acquire immunity against other phages. As an example of the first type of benefit, the presence of *skin* in the host genome was shown to be required for efficient sporulation in *Clostridium difficile* [17], even though it is dispensable in *B. subtilis* [36]. During sporulation in *B. subtilis*, the temporal control of σ^B^ activity is achieved by triggering the proteolytic removal of an inhibitory pro-sequence at its N-terminus [37]. Since σ^B^ does not possess the pro-sequence in *C. difficile*, another regulatory mechanism is required to control the timing of σ^B^ activation [17]. Regarding the second type of benefit, phages constitute an ideal vehicle for the host to acquire genes that provide selective advantages, especially as protection against other phage infections. In addition, sporulation genes are suitable locations for bacterial attachment sites because they are not essential for vegetative cell growth and viability. In general, lysogenic bacteria become immune to further infections by acquiring the ability to synthesize repressor proteins for closely related phages. Furthermore, in the case of SPβ, the prophage carries both *sunA*, which encodes sublancin, an antimicrobial that inhibits cell growth of non-SPβ lysogens [38], and *nonA*, which confers resistance to infections by the virulent phage SP10 [39–41]. Since the SPβ-cured strain, SPβless, produces normal spores (Table S2), the presence of SPβ in the *B. subtilis* genome is more likely to be beneficial to the host by providing immunity against other phages rather than adding a layer of control to sporulation progression. Recently, Rabinovich et al. have reported a similar prophage excision event in *Listeria monocytogenes* [42]. In this case, a functional *comK* gene is reconstituted to favor escape from phagocytosis. This observation suggests that prophage-mediated gene reconstitution is common among bacteria and is not limited to spore-formers.

Of the SPβ genes, we found that only *sprA* and *sprB* were required for excision (Figure 4). We have shown that *sprB* was expressed in response to MMC treatment (DNA damage) and is developmentally regulated during sporulation, whereas *sprA* was expressed irrespective of the host cell status (Figure 3). Moreover, overexpression of *sprB*, but not of *sprA*, successfully promoted *sprM* reconstitution in vegetative cells, even without MMC induction (Figure S3). Our results suggest that *sprB* is the factor that controls the timing of SPβ excision. *SprA* belongs to a family of large serine recombinases, which rely on recombination directionality factors (RDFs) to promote excision [43]. RDFs are small DNA-binding proteins that initiate the assembly of the recombinase–DNA complexes. *SprB* may serve as a RDF for the *SprA* recombinase during SPβ excision. Lazarevic et al. found putative SPβ repressor-binding sites called SPBRE in the promoter regions of *yorE*, *yorM*, *yorZ*, and *yosX* [23]. Repression is expected to be relieved upon MMC treatment. Thus, activation of *sprB* expression in response to DNA damage seems to result from derepression of *yosX* and its downstream genes, which include *sprB*. Importantly, *Spmini* is not subjected to excision upon MMC treatment (Figure 4C), since it is lacking the SOS-inducible phage genes upstream of *sprB*. In addition, we identified a mother cell-specific promoter immediately upstream of the *sprB* gene (Figures 5A-B). We propose that the reason why phage particles are not produced after SPβ excision during sporulation is because many SPβ genes lack sporulation-specific promoters, resulting in insufficient production of phage structural components. After SPβ excision, transcription of *sprM* is controlled by σ^B^ [20]. The σ^B^-encoding gene, *sigK*, is itself generated by excision of *skin* [6]. Therefore, expression of the composite *spsM* requires two DNA rearrangement events mediated by the phage elements SPβ and *skin*.

Our study also revealed an important connection between *spsM* function and *B. subtilis* sporule surface properties. In *Bacillus anthracis* and *Bacillus cereus* strains, spores are surrounded by an exosporium, which is a loose-fitting and balloon-like structure, containing glycoproteins and polysaccharides [44,45]. The exosporium is not observed in *B. subtilis* spores, but the crust can be considered to be an exosporium-like structure, even though it does not display the balloon-like structure of a typical exosporium. The protein composition of the crust has been characterized to some extent, in the sense that the coat proteins CgeA, Cog, and CogXYZ were identified as crust components [9,10]; however, beyond the fact that rhamnose, whose synthesis is dependent on *sprIJKL* [34], is a component of the spore surface [33], the spore polysaccharide composition in *B. subtilis* remains poorly characterized. Our analyses indicate that the *B. subtilis* spore polysaccharide also comprises galactose in addition to rhamnose and possibly another monosaccharide of unknown identity. In addition, we have shown that the production and/or attachment of the polysaccharide to the spore surface were *spsM*-dependent (Figure 6). Our results also indicated that although *spsM* mutant spores were as heat-resistant as wild type spores (Table S2), they were considerably more sticky and aggregated in water (Figure 7). The slight reduction of the spore numbers in the *spsM* mutant strains (Table S2) may be due to their increased adhesive properties. The hydrophobic phenotype of the *spsM* mutant spores may be attributable to the hydrophobic nature of the crust proteins CotXYZ and CgeA proteins [46]. In the absence of polysaccharide addition, these proteins become directly exposed at the spore surface, and the consequence may be a decrease in the solubility of spores in water. In natural environments, water flow, such as rainfall, rivers, and sea currents, is likely to play a role in spore dispersal. For an immobile spore, the ability to be transported to a different niche, where it can germinate and resume growth, constitutes a major advantage.

In conclusion, *B. subtilis* SPβ prophage has two pathways to excision. In response to host DNA damage, the SPβ prophage is excised from the host genome to form phage particles. By contrast, during sporulation, SPβ excision occurs in the mother cell to reconstitute a sporulation gene, *sprM*, a necessary event for spore polysaccharide synthesis. Although phage particle formation does not occur during sporulation, the SPβ prophage is propagated vertically to the progeny because phage excision is limited to the mother cell genome.

### Materials and Methods

#### Bacterial Strains and Genetic Manipulations

The primers used in this study are shown in Table S3. The bacterial strains and plasmids used in this study are listed in Table S4. Standard genetic manipulations of *B. subtilis* were performed as previously described [47].

#### Strain Construction

Internal segments of *yodU* (+28 to +244 relative to the first nucleotide of the start codon), *sprA* (+29 to +990), and *cotG* (+21 to +217) were amplified from the chromosome of *B. subtilis* 168 using primer pairs P01/P02, P03/P04, and P05/P06, respectively. PCR products were digested with *Hin*dIII and *Bam*HI, and inserted into the *Hin*dIII-*Bam*HI site of pMutiN3. The resulting pMUT-yodU, pMUT-sprA, and pMUT-cotG, plasmids were introduced into *B. subtilis* 168-competent cells to disrupt *yodU,*
sprA, and cotG, respectively. The resulting YODUd, SPRAd, and COTGd strains were selected on Luria-Bertani (LB) agar plates containing 0.5 μg/ml erythromycin.

To construct BslNDa and BslNDB, the 5‘ portions containing the SD sequence of sprA (−27 to +990) and of sprB (−20 to +499) were amplified using primer pairs P07/P04 and P08/P09, respectively. PCR products were digested with HindIII and BamHI and inserted into the HindIII-BamHI site of pMutinT3, which allows generation of a fusion transcript with a gene encoding β-galactosidase and placing genes downstream of an IPTG-inducible promoter P(βgal). The resulting plasmids, pMUT-sprAind and pMUT-sprBind, were introduced into B. subtilis 168-competent cells. The transformants were selected on LB-agar plates containing 0.3 μg/ml erythromycin.

To obtain the SPβ-cured strain (SPless), we cultivated BslNDB at 37°C in LB liquid medium in the presence of 0.5 mM IPTG overnight. The culture was spread on a LB-agar plate after dilution with fresh LB medium. The plate was incubated at 37°C overnight. The next day, SPβ-cured colonies were selected by colony PCR using primer pair P10/P11 and by erythromycin sensitivity.

A sprB-deletion mutant (SPBrd) and a strain harboring the minimized SPβ (SPmini) were constructed by double-crossover over recombination using the ermC gene cassettes. To construct SPBrd, DNA fragments corresponding to the upstream (−1126 to −1) and the downstream (+169 to +2246) flanking regions of sprB were amplified from the B. subtilis 168 genome using primer pairs P12/P13 and P14/P15. A DNA fragment containing the ermC gene was amplified from a pUCE191 plasmid vector using primer pair P16/P17. The DNA fragments were combined by over-extension PCR (OE-PCR) using the primer set P12/P15. The resulting PCR product was introduced into B. subtilis-168 competent cells and by double cross-over replacement of the sprB locus by the ermC cassette. The transformants were selected on the LB-agar plates containing 0.3 μg/ml erythromycin.

For construction of the SPmini strain, the primer sets P18/P19 and P20/P24 were used for amplification of the DNA fragments containing the sprB gene (−331 to +1301 relative to the first nucleotide of the sprB start codon) and the sprA gene (−84 to +990 relative to the first nucleotide of the sprA start codon) with their promoter regions and the attachment sites. The DNA fragments were combined with the ermC cassette by OE-PCR with the primer set P19/P24 and used for transformation of B. subtilis 168. Transformants were selected on erythromycin-containing LB plates.

Gene Complementation

DNA fragments containing a composite sfpM with its promoter region (−374 to +1080, relative to the first nucleotide of the start codon of the composite sfpM) were amplified from chromosomal DNA of B. subtilis 168 sporulating cells using primer pairs P21/P22. The PCR product was digested with EcoRI and BglII, and inserted into the EcoRI-BglII site of the integration vector pMF20 [48]. The resulting plasmid pMFspsM was linearized by BglII-digestion and subsequently integrated into amyE locus of YODUd and SPRAd by double crossover recombination. The resulting YODUAc and SPRAd strains were selected on LB agar plates containing 0.5 μg/ml erythromycin and 5 μg/ml chloramphenicol.

Sporulation of B. subtilis

Overnight cultures of B. subtilis strains grown at 37°C in liquid LB medium were diluted 1:100 with fresh liquid Difco sporulation medium (DSM) and incubated at 37°C with shaking. The CU1050 derivatives did not sporulate well in liquid DSM. Therefore, these strains were induced to sporulate on DSM-agar plates.

Isolation of Genomic DNA from B. subtilis and B. amyloliquefaciens

B. subtilis and B. amyloliquefaciens strains were cultured at 37°C in liquid DSM. We harvested 4 ml of the culture by centrifugation at various time points during sporulation. For induction of sporulation of the CU1050 derivatives, cells were spread on DSM-agar plates and incubated at 37°C for either 3 (vegetative phase) or 12 hrs (sporulation phase). Cell morphology was monitored by phase-contrast microscopy. After addition of 20 ml of deionized distilled water (DDW) to the plates, the cells were gently scraped from the plates and harvested by centrifugation. Genomic DNA was extracted as follows: cell pellets were suspended in 500 μl of TEN buffer [10 mM Tris-CH3 (pH 7.5), 10 mM EDTA, and 0.1 M NaCl] containing 250 μg/ml lysozyme and 10 μg/ml RNase A. The suspension was incubated at 37°C for 20 min, supplemented with 0.1% of sodium dodecyl sulfate (SDS), and incubation was continued for 5 min. Genomic DNA was isolated by phenol extraction and precipitated by ethanol. The DNA pellet was resuspended in TE buffer [10 mM Tris-CH3 (pH 8.0) and 1 mM EDTA].

To isolate B. subtilis forespore DNA, 50 ml of the DSM culture at T4 was harvested by centrifugation. The cell pellets were resuspended in TEN buffer containing 250 μg/ml lysozyme and 100 μg/ml DNase I and incubated at 37°C for 20 min to lyse the mother cells and non-sporulating cells. The suspension was centrifuged and the pellet washed five times by resuspension and re-centrifugation in 2 ml of TEN buffer. The forespore pellet was resuspended in SUTD buffer [1% (w/v) SDS, 8 M Urea, 50 mM Tris-CH3 (pH 8.0), and 50 mM dithiothreitol] [47,49] and incubated at 37°C for 90 min. The suspension was washed five times in 2 ml of TEN buffer. The forespore pellet was lysed with 250 μg/ml lysozyme, followed by phenol extraction and ethanol precipitation. The spore DNA pellet was resuspended in TE buffer.

Preparation of Digoxigenin (DIG)-Labeled Probes

To prepare the DIG-labeled probes, DNA fragments corresponding to the 358-bp sfpP probe, the 982-bp sprA probe, and the 535-bp sprB probe were amplified from the chromosomal DNA of B. subtilis using the primer pairs P23/P24, P03/P04, and P18/P25, respectively. DNA fragments corresponding to the 168-bp sprAerm and the 500-bp sfpPerm were amplified from the chromosomal DNA of B. amyloliquefaciens FZB42 using the primer pairs P26/P27 and P28/P29, respectively. The resulting PCR products were gel-purified and labeled using DIG-High Prime (Roche) according to the supplier’s instructions.

Southern Blotting

Chromosomal DNA (2.5 μg) was digested with 20 U of restriction enzymes at 37°C for 16 hours, separated by 0.8% agarose gel electrophoresis and blotted onto a Hybond-N+ membrane (GE Healthcare) using Alkaline solution (10× SSC and 0.2 N NaOH). Hybridization and detection were performed according to the DIG Application Manual (Roche). Signals were detected by a nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) reaction using the DIG Nucleic Acid Detection Kit (Roche).
β-galactosidase Assay

YODUd (yodU::pMutinT3, P_{yodU}-lacZ), SPRAd (sprA::pMutinT3, P_{sprA}-lacZ), and BdINDB (sprB::pMutinT3, P_{sprB}-lacZ, P_{pac}-sprB) were used to monitor the yodU, sprA, and sprB promoter activities, respectively. The B. subtilis strains were sporulated at 37°C in liquid DSM. The samples were collected at various time points after the end of the exponential phase of growth. β-galactosidase activity was determined using the method described by Miller [50].

Northern blotting

B. subtilis 168 cells were grown at 37°C in 50 ml LB medium up to the early log phase (OD_{600} = 0.25). The culture was further incubated at 37°C for 60 min in the presence or absence of MMC (0.5 μg/ml), and harvested by centrifugation. For preparation of the sporulating cells, the B. subtilis 168 cells were cultured at 37°C in 50 ml of liquid DSM and harvested at T_4 by centrifugation. Total RNA was isolated as described previously [51]. Five micrograms of total RNA were mixed with two volumes of denaturing buffer [50% formamide, 6% formaldehyde, 20 mM morpholinopropanesulfonic acid (MOPS) (pH 7.0), 5 mM sodium acetate, 1 mM EDTA, 0.05% bromophenol blue, and 10% glycerol] and incubated at 55°C for 10 min. The denatured RNA sample was loaded to a 2% denaturing agarose gel containing 2% formaldehyde, separated by electrophoresis in 1× MOPS buffer [20 mM MOPS (pH 7.0) and 5 mM sodium acetate], and capillary-transferred to Hybond N+ membrane (GE Healthcare) overnight in 10× SSC buffer [1.5 M NaCl and 150 mM sodium citrate (pH 7.0)]. The resulting membrane was baked at 80°C for 2 hrs and stained with a methylene blue solution [0.03% methylene blue and 0.5 M sodium acetate (pH 5.6)]. Hybridization and detection were performed according to the DIG Application Manual (Roche). Signals were detected using CDP-Star (Roche).

RT-PCR

Total RNA from B. subtilis 168 vegetative cells with or without MMC treatment and sporulation cells at T_4 were prepared as described above. The sprB cDNA was synthesized from 5 μg of the total RNA by an AMV reverse transcriptase XL (Takara) using the sprB-specific primer P90, according to the manufacturer’s instructions. Internal segments of the sprB, yosX, and yotBCD coding regions were amplified from the cDNA by 25-cycled PCR reactions using ExTaq (Takara) and the primer sets P08/P09, P31/P32, and P33/P34, respectively. PCR products were analyzed by 2% agarose gel electrophoresis.

Construction of a B. subtilis Strain Expressing SprB-GFP

To construct the pUBsprBgp plasmid carrying the sprB gene translationally fused to gfp, a DNA fragment containing sprB ORF and its sporulation-specific promoter was amplified from the B. subtilis 168 chromosomal DNA using primers P18/P25. An 858-bp DNA fragment of gfp was amplified from the PM20 vector [48] using primer pair P35/P36. The PUB110 plasmid vector [52] was linearized by PCR using the primer set P37/P38. The sprB DNA fragment, the gfp DNA fragment and the linearized pUB plasmid were combined by OE-PCR and amplified using the primer set P18/P37. The PCR product was self-ligated with T4 DNA ligase (Takara) in the presence of polynucleotide kinase (Takara) and introduced into B. subtilis 168-competent cells as described previously [18]. The transformants were selected by addition of 10 μg/ml kanamycin on LB-agar plates.

Fluorescence Microscopy

B. subtilis strains, BspRPBG and BspRPSM, were cultured at 37°C in liquid DSM containing FM4-64 (0.25 μg/ml). For the cultivation of the BspRPBG strain carrying the pUBsprBgp plasmid, kanamycin was added to the medium at a final concentration of 10 μg/ml. Sporulating cells were observed using fluorescence microscopy as previously described [53].

Preparation of B. subtilis Spores

Overnight cultures of B. subtilis strains in LB medium were spread on 90-mm DSM-agar plates. The DSM plates were incubated at 37°C for 6 days and kept at 4°C for a day. After the addition of 20 ml of DDW to the plates, the spores were gently scraped from the plates. The spores were centrifuged at 8,000 × g for 30 min. The spore pellets were resuspended in 20 ml of DDW and kept overnight at room temperature. The spores were further purified as described by Carrera et al. [54].

Indian Ink Staining

B. subtilis spores were negatively stained with Indian ink, as previously described [31] with a slight modification. The purified spores were resuspended in DDW, and 2 μl of the suspension was mixed with an equivalent volume of Indian ink (Daiso Sogyo, Japan) on a slide glass. A cover glass was placed on the slide glass and any excess fluid was pushed out using thumb pressure. The negatively stained outermost layer of the spore was observed using phase-contrast microscopy.

Preparation and Detection of Spore Surface Extracts

The purified spores were resuspended in DDW and the final OD_{600} was adjusted to 50. Next, 100 μl of the spore suspension were added to 100 μl of the SDS buffer [125 mM Tris–HCl (pH 6.8), 2% SDS, and 4% β-mercaptoethanol] and incubated at 98°C for 10 min. The supernatant was collected after centrifugation at 20,400 × g for 5 min and 40 μl of the supernatant was loaded onto a 5% native polyacrylamide gel, which was separated by electrophoresis at 100 V for 30 min in 1× TBE buffer [44.5 mM Tris, 44.5 mM borate, and 1 mM EDTA (pH 8.0)]. The resulting gel was stained overnight at room temperature using dye solution [0.025% (w/v) Stains-All (Sigma-Aldrich), 7.5% formamide, 3% acetic acid, and 25% 2-propanol]. The polysaccharide components in the spore surface extract were quantified as described by Hammerschmidt et al. [32]. The monosaccharide composition of the spore polysaccharide was determined as described in Supporting text S1.

Adhesion Assay

The purified B. subtilis spores were resuspended in DDW, and the final OD_{600} was adjusted to 0.5. Each 1 ml of the spore resuspensions was added to a polypropylene tube (3.8×40 mm; Safe-Lock tube 2.0 ml; Eppendorf). The spore resuspension was vortexed gently for 10 s and transferred to a fresh polypropylene tube. This operation was repeated 10 times. Total amount of the spores bound to the tubes [Adhesion (‰)] was calculated from the percentage decrease in OD_{600} of the spore resuspension as follows: 100 × (OD_{i} – OD_{f}) / OD_{i}, where OD_{i} and OD_{f} are the initial OD_{600} (= 0.5) and OD_{600} of each binding reaction, respectively.

Supporting Information

Figure S1 Amino-acid sequence alignment of SpS proteins. (A) Multiple alignments of SpS proteins. Amino-acids sequences of YodU, YpqP, and SpM proteins are shown. The over-lapped amino-acids sequences between YodU and YpqP of B. subtilis 168
are boxed in red. Bs168, B. subtilis 168 (YodU, NCBI locus tagBSU19810; YpqP, BSU21670); BsBEST195, B. subtilis BEST195 (SpSM, BSN03232; BaFZB42, B. amyloliquefaciens FZB42 (YodU, RBAM019560; YpqP, RBAM019840); BaY2, B. amyloliquefaciens Y2 (SpSM, MUS2345). (B) Nucleotides sequences of the attachment sites of SPβ and the joint site of spsM gene. Nucleotides sequences of the attachment sites of SPβ and the joint site of spsM gene. The 16-bp inverted repeat sequences of the attachment sites of SPβ and the joint site of spsM gene were indicated by arrows. The sequences boxed in red are core sequences.

Figure S2 Strain constructs. Schematic drawing showing the yodU, sprA, and cotG gene disruptions by pMutinT3 [upper line: yodU (YODU); middle line: sprA (SPRA); bottom line: cotG (COTGd)] (A), and the sprB gene deletion by the ermC cassette (SPRBd) (B). Thick lines indicate the sprA and ypqP probes for Southern blotting. NdeI denotes restriction sites.

Figure S3 Effects of overexpression of sprA and sprB on the SPβ excision. A schematic above shows the construct of BdINDA (A) and BsINDB (B). The B. subtilis strains carrying the constructs of Pspac–sprA (BsINDA) and Pspac–sprB (BsINDB) were cultured at 37°C in LB medium. IPTG (0.2 mM) was added to the medium when the cells reached mid-log phase of cell growth (OD600 = 0.5). DNA was extracted from the cells at various time points after addition of IPTG and digested with NdeI and subjected to Southern blot using the ypqP probe.

Figure S4 Determination of the transcriptional start site of sprB. 5' RACE was performed using total RNA from B. subtilis 168 sporulating cells (T3) and the sprB-specific primers. The sequence of the 5' end of the sprB cDNA is shown. Nucleotides boxed in blue indicate the protein-coding region. The transcriptional start site (TSS) is shown as the nucleotide boxed in red. The black arrow denotes the primer sequence used for the reverse transcription reaction. The predicted sprB promoter region is shown in Figure 5B.

Figure S5 Compartmentalization of SpsM–GFP expression. The B. subtilis strain carrying ypqP–gfp (BsPSMG) was induced to sporulate at 37°C in liquid DSM containing FM4-64 (0.25 μg/ml). The sporulating cells at T8 were harvested and observed by phase-contrast microscopy, PC, phase-contrast; Membrane, cell membranes stained with FM4-64; GFP, SpsM–GFP; Merge, merged image of Membrane and GFP. Scale bar, 2 μm.

Table S1 Genes in B. amyloliquefaciens SPβ-like elements.

Table S2 Sporulation frequencies of B. subtilis strains.

Table S3 Primers used in this study.

Table S4 Strains and plasmids used in this study.

Text S1 Supporting materials and methods and references.

Author Contributions

Conceived and designed the experiments: KAb TS. Performed the experiments: KAb YK KL. Analyzed the data: KAb PE TS. Contributed reagents/materials/analysis tools: KAb YK Ak YMY TS. Wrote the paper: KAb PE TS.

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Prophage-Mediated Gene Reconstitution

PROPHAGE-REGULATED GENES AND THEIR ROLE IN SPORULATION

Gene expression during sporulation is tightly controlled, and various regulatory mechanisms are involved. In Bacillus subtilis, the sporulation process involves the coordinated expression of genes that control the development of the spore and the mother cell. This process is highly regulated to ensure the formation of a viable spore that can withstand environmental stresses and germinate under appropriate conditions.

One of the key regulatory factors during sporulation is the σF factor. The σF factor is transcribed from the σF promoter, which is located on the prophage SP8 genome. The σF protein is synthesized in the mother cell in the early stages of sporulation and is essential for the transcription of sporulation-specific genes.

The σF factor regulates the expression of various genes involved in sporulation, including those encoding the main sporulation sigma factors (σE, σK), as well as genes encoding proteins involved in the formation and maturation of the spore coat. The σF promoter is activated by the σF factor, and its expression is controlled by the σF-RsbU complex, which is involved in the regulation of the RsbRS two-component system.

The σF factor plays a crucial role in the transition from vegetative growth to sporulation, as it is required for the activation of the σE factor, which in turn controls the expression of the σK factor. The σK factor is a master regulator of sporulation and is involved in the transcription of genes encoding spore-specific proteins.

In addition to the σF factor, other regulatory mechanisms, such as the RsbRS two-component system and the TCS (two-component system), are involved in the regulation of sporulation. The RsbRS system senses changes in the cell's nutrient status and regulates the expression of genes involved in nutrient acquisition and metabolism, which are essential for sporulation.

The TCS system, on the other hand, is involved in the regulation of genes encoding proteins involved in the formation of the spore coat. The TCS system senses changes in the cell's osmotic stress and regulates the expression of genes involved in the synthesis and secretion of the spore coat proteins.

In conclusion, the σF factor, along with other regulatory mechanisms, plays a crucial role in the regulation of gene expression during sporulation in Bacillus subtilis. Understanding the molecular mechanisms involved in the regulation of sporulation will help in the development of new strategies for the control of bacterial sporulation, which is important for both environmental and industrial applications.