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NOTES

An α/β-Type, Small, Acid-Soluble Spore Protein Which Has Very High Affinity for DNA Prevents Outgrowth of *Bacillus subtilis* Spores

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A derivative of SspC, a minor α/β-type, small, acid-soluble spore protein (SASP) from *Bacillus subtilis*, was generated that has a very high affinity for DNA. This protein ($SspC^{\Delta 11\text{-}D13K}$) was able to confer UV resistance on spores lacking α/β-type SASP, and spores with $SspC^{\Delta 11\text{-}D13K}$ triggered germination normally. However, $SspC^{\Delta 11\text{-}D13K}$ blocked outgrowth of ≥90% of germinated spores, and $SspC^{\Delta 11\text{-}D13K}$ persisted in these germinated spores, whereas wild-type SspC was almost completely degraded. The outgrowth phenotype of spores with $SspC^{\Delta 11\text{-}D13K}$ is proposed to be due to the high stability of the $SspC^{\Delta 11\text{-}D13K}$-DNA complex, which prevents rapid degradation of this α/β-type SASP early in germination. The persistence of this protein on spore DNA then interferes with transcription during spore outgrowth.

Spores of *Bacillus* and *Clostridium* species contain a number of small, acid-soluble spore proteins (SASP) which comprise 7 to 20% of total spore protein (25). One subset of these proteins, the α/β-type SASP, are encoded by multiple genes and comprise a large protein family whose amino acid sequences are very highly conserved within and between species (25). The α/β-type SASP are nonspecific DNA binding proteins which saturate the spore chromosome and protect spore DNA from damage caused by UV radiation, heat, and peroxides (2, 10, 19, 20; reviewed in references 23 and 24). In addition to the α/β-type SASP, another type of SASP, termed SASP-γ, is also found at very high levels within dormant spores. In contrast to the α/β-type SASP, the γ-type SASP are encoded by a single gene, do not bind to DNA, and also do not share extensive sequence homology with the α/β-type SASP (25). However, both types of SASP are cleaved during spore germination by the same sequence-specific endoproteinase, termed the germination protease, or GPR. This cleavage initiates the degradation of the α/β- and γ-type SASP to amino acids which support protein synthesis during this period of development (3, 16). The rapid degradation of α/β-type SASP during spore germination is essential to allow for DNA transcription and eventually DNA replication during spore outgrowth, the period between spore germination and the resumption of vegetative growth (16).

The α/β-type SASP are essentially unstructured in the absence of DNA and consequently are very sensitive to proteolysis (6, 21). However, α/β-type SASP are much more resistant to protease cleavage when bound to DNA and may be completely resistant to GPR cleavage while bound to DNA (21). Indeed, the current model of α/β-type SASP degradation during spore germination (16) suggests that the rapid rehydration and volume expansion of the spore core early in germination result in the partial dissociation of α/β-type SASP from the chromosome. The dissociated α/β-type SASP are then cleaved by GPR, and this cleavage depletes the pool of free α/β-type SASP and leads to further dissociation and further cleavage (16). One corollary of this model is that α/β-SASP should not bind to spore DNA too tightly, or their degradation during germination could be impaired and thus spore outgrowth inhibited.

While studying N-terminal deletion mutant forms of SspC, a minor α/β-type SASP from *Bacillus subtilis*, we generated a protein ($SspC^{\Delta 11}$) which lacks amino acid residues Gln2 through Asn12 (Fig. 1) (4). Spores of *B. subtilis* that express $SspC^{\Delta 11}$ as their major α/β-type SASP are more sensitive to UV radiation and heat than spores expressing wild-type SspC (4). $SspC^{\Delta 11}$ also binds to pUC19 plasmid DNA with 30-fold-lower affinity and to poly(dA-dT) · poly(dA-dT) with >50-fold-lower affinity than does wild-type SspC (4). We sought to increase the affinity of $SspC^{\Delta 11}$ for DNA by changing Asp13 to a lysine residue, thus generating $SspC^{\Delta 11\text{-}D13K}$ (Fig. 1). This additional change was chosen because α/β-type SASP with positively charged amino acid residues near the N terminus tend to bind to DNA with higher affinity (4). This effect is presumably due to previously identified protein-protein interactions that occur between adjacent DNA-bound α/β-type SASP, in which the positively charged N terminus of one α/β-type SASP interacts with the negatively charged GPR cleavage sequence of an adjacent DNA-bound α/β-type SASP (Fig. 1) (8). Based on the information noted above, we reasoned that $SspC^{\Delta 11}$ may bind to DNA with low affinity due to unfavorable protein-protein interactions arising from electrostatic repul-

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SspC - AQQSRSRSNNNNNDLLIPQAASAIEQMKLEIASEFGVQLGAETTSRANGSVGGEITKRLVRLAQQNMGGQFH

Δ11 -              ADLLIPQAASAIEQMKLEIASEFGVQLGAETTSRANGSVGGEITKRLVRLAQQNMGGQFH

Δ11-D13K -         AKLLIPQAASAIEQMKLEIASEFGVQLGAETTSRANGSVGGEITKRLVRLAQQNMGGQFH
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FIG. 1. Amino acid sequence alignment of wild-type and mutant SspC proteins. The amino acid sequences for wild-type SspC, SspC$^{\Delta 11}$, and SspC$^{\Delta 11\text{-}D13K}$ are given in one-letter code (1, 25). Asterisks above amino acid residues indicate residues which are conserved in all α/β-type SASP identified from *Bacillus, Sporosarcina*, and *Thermoactinomyces* species (1, 25). The downward-pointing arrow indicates the peptide bond which is cleaved by GPR.

sion between Asp13 near the N terminus and the two glutamate residues in the GPR cleavage region (Fig. 1). The electrostatic repulsion model is also based upon data which indicate that SspC$^{\Delta 14}$ (which lacks residues Gln2 through Leu16) binds to DNA with higher affinity than SspC$^{\Delta 11}$ (4); note that SspC$^{\Delta 14}$ has only uncharged residues in the N-terminal region (Fig. 1). Equilibrium binding studies indicated that as predicted, SspC$^{\Delta 11\text{-}D13K}$ has >350-fold-higher affinity for pUC19 than does SspC$^{\Delta 11}$, and surprisingly, SspC$^{\Delta 11\text{-}D13K}$ was also found to bind to pUC19 with 12-fold-higher affinity than wild-type SspC does (4). In addition, a complex of SspC$^{\Delta 11\text{-}D13K}$ and poly(dG) · poly(dC) is remarkably more stable to thermal denaturation than an SspC-poly(dG) · poly(dC) complex (4), and the SspC$^{\Delta 11\text{-}D13K}$-poly(dG) · poly(dC) complex only dissociates at temperatures slightly below the melting temperature of poly(dG) · poly(dC) (4).

Spores which contain SspC$^{\Delta 11}$ as their major α/β-type SASP are not as resistant to UV or heat as spores containing wild-type SspC, presumably at least in part because of the weaker binding of the variant protein to DNA compared to that of wild-type SspC (4). Although SspC$^{\Delta 11\text{-}D13K}$ binds to DNA with higher affinity than wild-type SspC in vitro, we wanted to determine if this protein is a functional α/β-type SASP in vivo. Therefore, we examined whether the D13K change increased the ability of SspC$^{\Delta 11}$ to confer UV resistance to spores. SspC, SspC$^{\Delta 11}$, and SspC$^{\Delta 11\text{-}D13K}$ were overexpressed to similar high levels in spores lacking the two major α/β-type SASP (termed $\alpha^-\beta^-$ spores) of *B. subtilis* (SspC, SspC$^{\Delta 11}$, and SspC$^{\Delta 11\text{-}D13K}$ spores, respectively) as described previously (4), and the spores were purified as described previously (13). As predicted, $\alpha^-\beta^-$SspC$^{\Delta 11\text{-}D13K}$ spores were more resistant to UV radiation than were $\alpha^-\beta^-$SspC$^{\Delta 11}$ spores and were almost as resistant as $\alpha^-\beta^-$SspC spores (Fig. 2), indicating that the additional sequence change in SspC$^{\Delta 11\text{-}D13K}$ complements the slightly UV-sensitive phenotype of $\alpha^-\beta^-$SspC$^{\Delta 11}$ spores. However, we noted that $\alpha^-\beta^-$SspC$^{\Delta 11\text{-}D13K}$ spore preparations consistently gave only 5 to 10% of the CFU per unit of optical density at 600 nm (OD_{600}) on Luria-Bertani (LB) plates that $\alpha^-\beta^-$, $\alpha^-\beta^-$SspC, or $\alpha^-\beta^-$SspC$^{\Delta 11}$ spores gave. Resporulation of an $\alpha^-\beta^-$SspC$^{\Delta 11\text{-}D13K}$ colony which had arisen from a spore and purification and analysis of the spores showed again that the colony-forming ability of these spores was 10- to 20-fold lower than expected. Expression of SspC$^{\Delta 11\text{-}D13K}$ in wild-type spores also conferred the same low-viability phenotype (data not shown).

One explanation for the apparent low viability of $\alpha^-\beta^-$SspC$^{\Delta 11\text{-}D13K}$ spores is that germination itself is defective in these spores. To test this explanation, $\alpha^-\beta^-$ spores expressing SspC, SspC$^{\Delta 11}$, or SspC$^{\Delta 11\text{-}D13K}$ were examined for their ability to germinate after heat shock (70°C, 30 min) under a variety of conditions, including 20 mM Tris-HCl (pH 8.0)–100 mM KCl–8 mM L-alanine and rich medium (LB or 2× yeast-tryptone [YT]) (7) supplemented with 8 mM L-alanine. In general, $\alpha^-\beta^-$SspC$^{\Delta 11}$ spores germinated with greater efficiency (80 to 90%) than $\alpha^-\beta^-$SspC or $\alpha^-\beta^-$SspC$^{\Delta 11\text{-}D13K}$ spores (50 to 60%) as determined by the percentage of phase-dark spores present after 60 min of germination as observed by phase-contrast microscopy (data not shown). The germination efficiencies observed by microscopy were in good agreement with the percentages of total spore dipicolinic acid (DPA) released by each strain upon germination; $\alpha^-\beta^-$SspC$^{\Delta 11}$ spores released almost all their DPA, whereas $\alpha^-\beta^-$SspC and $\alpha^-\beta^-$SspC$^{\Delta 11\text{-}D13K}$ spores released only ~50% of their total DPA after 1 h in 10 mM Tris-HCl (pH 8.0)–100 mM KCl–8

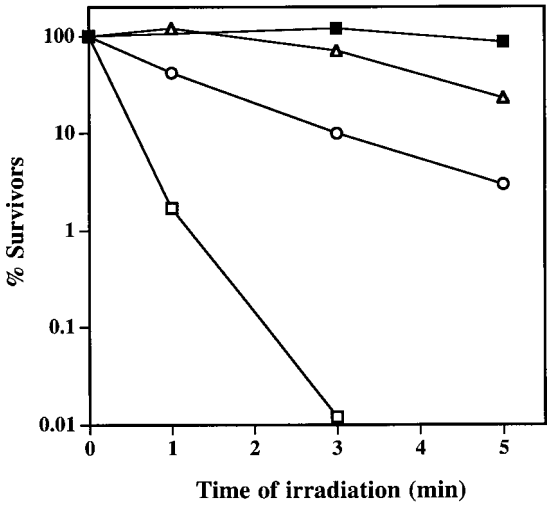

FIG. 2. Resistance of $\alpha^-\beta^-$ spores overexpressing SspC, SspC$^{\Delta 11}$, and SspC$^{\Delta 11\text{-}D13K}$ to UV radiation. Spores of various strains were purified, and their resistance to UV radiation at 254 nm was determined as described earlier (14) under conditions in which the spores were exposed to 45 J of UV radiation per m^2 at 254 nm for 1 min. This experiment was performed twice with independent spore preparations. The relative resistances of the strains were the same in both experiments, with average D90 values (time to kill 90% of the initial spore population) of 30 s for $\alpha^-\beta^-$ spores, 3 min for SspC$^{\Delta 11}$ spores, 8 min for SspC$^{\Delta 11\text{-}D13K}$ spores, and >20 min for SspC spores. Symbols: ■, $\alpha^-\beta^-$SspC; △, $\alpha^-\beta^-$SspC$^{\Delta 11\text{-}D13K}$; ○, $\alpha^-\beta^-$SspC$^{\Delta 11}$; and □, $\alpha^-\beta^-$pUB110.

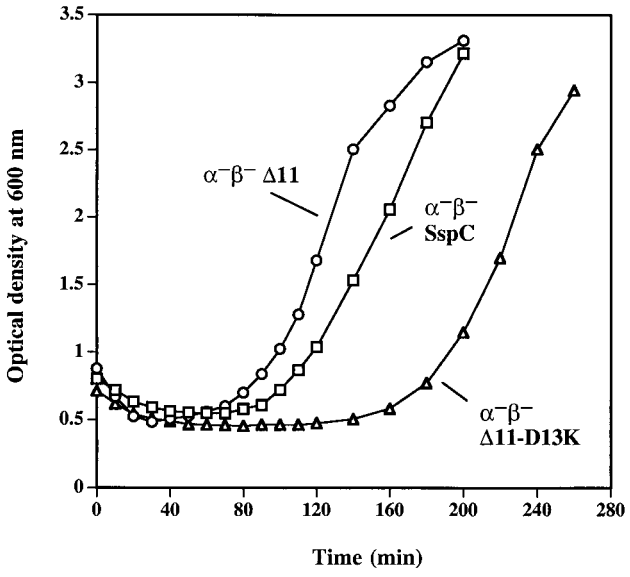

FIG. 3. Germination and outgrowth of $\alpha^-\beta^-$ spores overexpressing SspC, $SspC^{\Delta 11}$, and $SspC^{\Delta 11\text{-}D13K}$. Spores were heat shocked for 30 min at 70°C followed by resuspension at an OD_{600} of 0.75 to 0.9 in 2× YT medium supplemented with 8 mM L-alanine and cultured at 37°C with shaking. This experiment was performed twice with independent spore preparations, with similar results. Symbols: ○, $\alpha^-\beta^-SspC^{\Delta 11}$; □, $\alpha^-\beta^-SspC$; and △, $\alpha^-\beta^-SspC^{\Delta 11\text{-}D13K}$.

mM L-alanine at 37°C. The germination kinetics of $\alpha^-\beta^-SspC$ and $\alpha^-\beta^-SspC^{\Delta 11\text{-}D13K}$ spores were also identical as measured by the initial decrease in OD_{600} after dilution into germination medium (Fig. 3 and data not shown). These data indicate that $\alpha^-\beta^-SspC^{\Delta 11}$ spores germinate more efficiently in liquid media than $\alpha^-\beta^-SspC$ and $\alpha^-\beta^-SspC^{\Delta 11\text{-}D13K}$ spores for reasons not known; more importantly, these data also indicate that $\alpha^-\beta^-SspC$ and $\alpha^-\beta^-SspC^{\Delta 11\text{-}D13K}$ spores germinate equally well, and thus that the low viability of $\alpha^-\beta^-SspC^{\Delta 11\text{-}D13K}$ spores is not due to a germination defect. The germinated $\alpha^-\beta^-SspC$ and $\alpha^-\beta^-SspC^{\Delta 11\text{-}D13K}$ spores also swell significantly as observed in a phase-contrast microscope, but the great majority of the germinated $\alpha^-\beta^-SspC^{\Delta 11\text{-}D13K}$ spores remain round and never divide (data not shown). Consequently, the resumption of the vegetative growth of $\alpha^-\beta^-SspC^{\Delta 11\text{-}D13K}$ spores at 37°C in 2× YT medium supplemented with 8 mM L-alanine is delayed by about 80 min compared to that of $\alpha^-\beta^-SspC$ spores (Fig. 3). This apparent difference in the times for the resumption of vegetative growth between $\alpha^-\beta^-SspC$ and $\alpha^-\beta^-SspC^{\Delta 11\text{-}D13K}$ spores is ~150 min when germination and outgrowth at 37°C are carried out in a slightly poorer medium, LB medium with 8 mM L-alanine (data not shown). The significant delay seen in the resumption of growth of $\alpha^-\beta^-SspC^{\Delta 11\text{-}D13K}$ spores (Fig. 3 and data not shown) is consistent with the low viability of these spores; because only 5 to 10% of germinated $\alpha^-\beta^-SspC^{\Delta 11\text{-}D13K}$ spores are able to successfully reinitiate vegetative growth, the time at which the OD_{600} of cultures of these spores can be seen to increase will be delayed relative to that for $\alpha^-\beta^-SspC$ spores, 10- to 20-fold more of which are viable.

Because the $SspC^{\Delta 11\text{-}D13K}$ protein has a higher affinity for DNA than does wild-type SspC in vitro, a second possible explanation for the apparent low viability of $SspC^{\Delta 11\text{-}D13K}$ spores is that $SspC^{\Delta 11\text{-}D13K}$ is not efficiently degraded by GPR during spore germination, and thus, the germinated spore dies because it cannot reinitiate vegetative growth appropriately. Indeed, it is known that the overexpression of α/β-type SASP in *Escherichia coli* results in cell death most likely due to the interruption of transcription and DNA metabolism (18), and possibly the same phenomenon occurs in germinating $SspC^{\Delta 11\text{-}D13K}$ spores. Previous work has shown that DNA-bound α/β-type SASP are very resistant to digestion by GPR compared to free α/β-type SASP (21). To determine whether the increased affinity of $SspC^{\Delta 11\text{-}D13K}$ for DNA results in reduced degradation by GPR in vitro compared to that of wild-type SspC, purified protein-DNA complexes were made of each α/β-type SASP and supercoiled pUC19 plasmid DNA followed by the addition of partially purified recombinant *Bacillus megaterium* GPR (9). Under these conditions (10 mM Tris-HCl [pH 7.4]–150 mM NaCl–2 mM $CaCl_2$ at 37°C), approximately 90% of wild-type SspC is digested in 30 min, while less than 75% of $SspC^{\Delta 11\text{-}D13K}$ is cleaved in the same time (Fig. 4A). That the difference in GPR cleavage of the two proteins is due to differences in their DNA binding affinity was shown by both the similar rates of cleavage and the complete cleavage (within 1 min) of both SspC and $SspC^{\Delta 11\text{-}D13K}$ by GPR in the absence of added plasmid DNA (data not shown).

The data on the thermal stability and in vitro GPR digestion of α/β-type SASP-DNA complexes indicate that $SspC^{\Delta 11\text{-}D13K}$ has a significantly higher affinity for DNA than do $SspC^{\Delta 11}$ and wild-type SspC, and that this tight binding to DNA can result in less than complete cleavage of $SspC^{\Delta 11\text{-}D13K}$ by GPR. If DNA-bound $SspC^{\Delta 11\text{-}D13K}$ is indeed preventing spore outgrowth in $\alpha^-\beta^-SspC^{\Delta 11\text{-}D13K}$ spores, then significant amounts of uncleaved $SspC^{\Delta 11\text{-}D13K}$ protein should be present in these germinated spores. To test this prediction, purified $\alpha^-\beta^-$, $\alpha^-\beta^-SspC$, and $\alpha^-\beta^-SspC^{\Delta 11\text{-}D13K}$ spores were germinated at 37°C in LB medium supplemented with 8 mM L-alanine for 100 min; this time was chosen to give maximum spore germination without significant spore outgrowth. Because $\alpha^-\beta^-$ spores overexpressing wild-type SspC and $SspC^{\Delta 11\text{-}D13K}$ germinate less efficiently than $\alpha^-\beta^-$ spores, germinated spores were purified from ungerminated spores by centrifugation through a solution of 50% metrizoic acid as described earlier (13), and total SASP were extracted from disrupted dormant spores and purified germinated spores as described previously (5). Proteins from equivalent amounts of dormant spores and germinated spores (based on the OD_{600} and taking into account the 50% decrease in spore OD_{600} upon germination) were resolved by polyacrylamide gel electrophoresis at low pH (Fig. 4B). Dormant spores of all three strains had roughly the same levels of SASP-γ, and 100 min after initiating spore germination virtually all the SASP-γ had been degraded in all three strains (Fig. 4B). The $\alpha^-\beta^-SspC$ and $\alpha^-\beta^-SspC^{\Delta 11\text{-}D13K}$ dormant spores also contained the same level of these α/β-type SASP, and the great majority of wild-type SspC (and $SspC^{\Delta 11}$; data not shown) was degraded during germination (Fig. 4B, lanes 2, 3, and 5). In contrast, substantial amounts of $SspC^{\Delta 11\text{-}D13K}$ (~30 to 40%) remained long after germination had initiated (Fig. 4B, lane 6). Presumably the $SspC^{\Delta 11\text{-}D13K}$ remaining in germinated spores is bound to DNA and therefore is resistant to GPR as well as degradation by other pro-

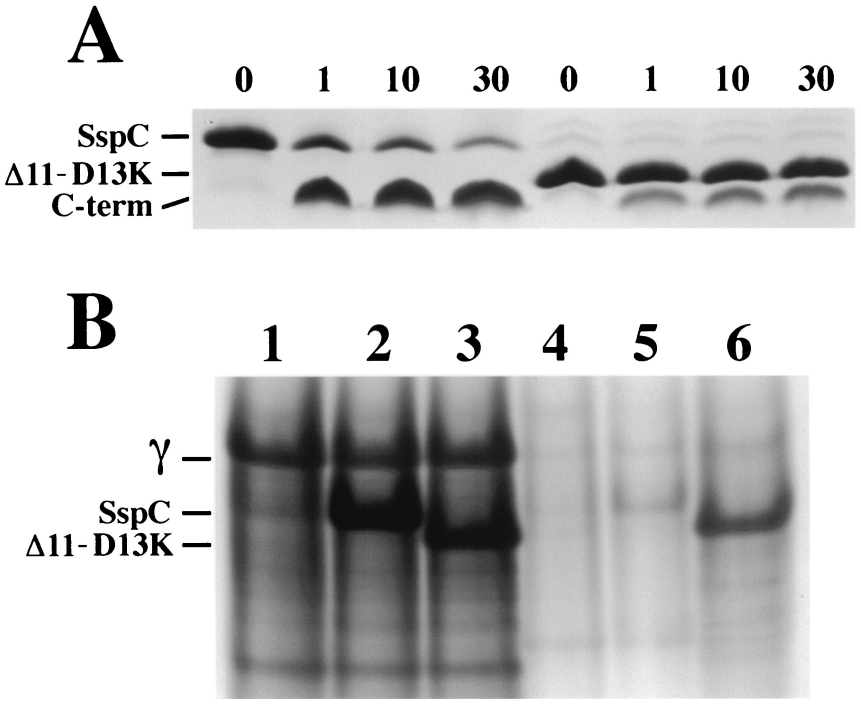

FIG. 4. Degradation of SspC and SspC$^{\Delta 11\text{-}D13K}$ in vitro and in vivo. (A) SspC and SspC$^{\Delta 11\text{-}D13K}$ (50 μM) were equilibrated with supercoiled pUC19 plasmid DNA (0.13 mg/ml) in 10 mM Tris-HCl (pH 7.4)–150 mM NaCl–2 mM $CaCl_2$ at 37°C for 90 min prior to addition of partially purified *B. megaterium* GPR to 20 μg/ml. Samples were removed before (0 min) and after addition of GPR (1, 10, and 30 min) for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on Tris-tricine gels (17). The positions of full-length proteins and the C-terminal GPR cleavage peptide (C-term) are indicated on the left of the figure, and the minutes of incubation with GPR are given above the lanes. (B) Total SASP were acid extracted from dry, ruptured dormant spores (lanes 1 through 3) and purified germinated spores (germinated for 100 min as described in the text) (lanes 4 through 6), and samples from equivalent amounts of spores and germinated spores were run on polyacrylamide gel electrophoresis at low pH (15). The samples in each lane are as follows: lane 1, $\alpha^-\beta^-$ dormant spores; lane 2, $\alpha^-\beta^-$SspC dormant spores; lane 3, $\alpha^-\beta^-$SspC$^{\Delta 11\text{-}D13K}$ dormant spores; lane 4, $\alpha^-\beta^-$ germinated spores; lane 5, $\alpha^-\beta^-$SspC germinated spores; and lane 6, $\alpha^-\beta^-$SspC$^{\Delta 11\text{-}D13K}$ germinated spores. The positions of SASP-γ (γ) SspC, and SspC$^{\Delta 11\text{-}D13K}$ are indicated on the left.

teases (see below). Purified germinated spores were also plated on LB agar with kanamycin to determine plating efficiency, and these spores again gave only ~8% of the colonies per unit of OD_{600} of germinated spores as compared to $\alpha^-\beta^-$ or $\alpha^-\beta^-$SspC germinated spores (data not shown).

The continued presence of significant levels of SspC$^{\Delta 11\text{-}D13K}$ in germinated spores expressing this protein and this protein's high affinity for DNA in vitro strongly suggests that outgrowth of these spores is inhibited due to the persistence of this protein on germinated spore DNA, although some of these germinated spores (5 to 10%) are able to degrade SspC$^{\Delta 11\text{-}D13K}$ sufficiently to allow vegetative growth to resume. However, successful degradation of SspC$^{\Delta 11\text{-}D13K}$ and resumption of vegetative growth appears to be a stochastic event, because resporulated clones of SspC$^{\Delta 11\text{-}D13K}$ spore survivors still show the low-spore-viability phenotype.

In contrast to the decrease in spore viability due to undegraded SspC$^{\Delta 11\text{-}D13K}$, slowing degradation of wild-type α/β-type SASP during spore germination by inactivation of GPR causes no noticeable decrease in spore viability (16). While the reason(s) for this difference between the effects of undegraded SspC$^{\Delta 11\text{-}D13K}$ and wild-type α/β-type SASP on spore viability is not clear, there are at least two possible explanations, which are not mutually exclusive. First, wild-type α/β-type SASP bind relatively weakly to DNA; in fact, the major α/β-type SASP of *B. subtilis* (α and β) have a much lower affinity than does SspC for DNA (11, 22). Consequently, even though 50% of the genome remains complexed with α/β-type SASP early in germination of *gpr* spores (16), the proteins are likely rapidly dissociating and reassociating with the DNA such that essentially all regions of the genome are available for transcription at least some of the time. In contrast, the much tighter binding of SspC$^{\Delta 11\text{-}D13K}$ may keep some regions of the germinated spore genome constantly covered with protein, thus precluding their transcription and resulting in spore death. This may especially be the case if some regions of the genome must be transcribed at an appropriate time relative to other regions to ensure an orderly progression through spore outgrowth. A second possible explanation is based on the fact that while degradation of wild-type α/β-type SASP is slowed during germination of *gpr* spores, this degradation still takes place, presumably due to other nonspecific proteases (16); indeed, degradation of SASP-α and -β is largely complete after 90 min of germination of *gpr* spores in a rich medium (16). In contrast, SspC$^{\Delta 11\text{-}D13K}$ persists in wild-type spores germinated for >100 min (Fig. 4B, lane 6). Since the viability of $\alpha^-\beta^-$SspC$^{\Delta 11\text{-}D13K}$ spores is not further reduced by introduction of a *gpr* mutation (data not shown), presumably SspC$^{\Delta 11\text{-}D13K}$ bound to DNA is also resistant to other nonspecific proteases that can degrade α/β-type SASP. Consequently, SspC$^{\Delta 11\text{-}D13K}$ persists much

longer in germinated spores than do wild-type α/β-type SASP in germinated *gpr* spores, and thus SspC$^{\Delta 11\text{-}D13K}$ causes significant spore death.

A major conclusion drawn from the data in this communication is that to be effective, α/β-type SASP must bind to DNA tightly enough to effect the global change in chromosome DNA conformation required for spore resistance to UV radiation and other damaging treatments (10–12) but not so tightly that dissociation of α/β-type SASP from all parts of the spore chromosome does not occur efficiently and rapidly during spore germination. Interestingly, many of the minor α/β-type SASP (such as SspC) studied to date have higher affinity for DNA than do the major α/β-type SASP which comprise ~75 to 85% of the total α/β-type SASP pool (6, 22). It may be that the low-affinity major α/β-type SASP are sufficient to bind to most of the chromosome in spores, but that certain regions of the chromosome may require higher-affinity minor α/β-type SASP for efficient binding. This might explain why spores contain several different α/β-type SASP. In this manner, the spore chromosome could be saturated with these proteins without the need for an excessive amount of high-affinity α/β-type SASP, as the latter proteins could persist after initiation of spore germination and significantly retard or even prevent spore outgrowth.

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