JOURNAL OF BACTERIOLOGY, Dec. 2000, p. 6906–6912 Vol. 182, No. 24
0021-9193/00/$04.00+0

Effects of Major Spore-Specific DNA Binding Proteins on *Bacillus subtilis* Sporulation and Spore Properties

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Sporulation of a *Bacillus subtilis* strain (termed α^- β^-) lacking the majority of the α/β-type small, acid-soluble spore proteins (SASP) that are synthesized in the developing forespore and saturate spore DNA exhibited a number of differences from that of the wild-type strain, including delayed forespore accumulation of dipicolinic acid, overexpression of forespore-specific genes, and delayed expression of at least one mother cell-specific gene turned on late in sporulation, although genes turned on earlier in the mother cell were expressed normally in α^- β^- strains. The sporulation defects in α^- β^- strains were corrected by synthesis of chromosome-saturating levels of either of two wild-type, α/β-type SASP but not by a mutant SASP that binds DNA poorly. Spores from α^- β^- strains also exhibited less glutaraldehyde resistance and slower outgrowth than did wild-type spores, but at least some of these defects in α^- β^- spores were abolished by the synthesis of normal levels of α/β-type SASP. These results indicate that α/β-type SASP may well have global effects on gene expression during sporulation and spore outgrowth.

A key event in the process of sporulation in the bacterium *Bacillus subtilis* is an unequal cell division that splits the sporulating cell into the larger mother cell and the smaller forespore, which is destined to become the spore. Following this sporulation septation, the two compartments exhibit very different patterns of transcription, determined by the activation and synthesis of different sigma (σ) factors for RNA polymerase in the mother cell and forespore (4, 27). While both compartments of the sporulating cell contain identical genomes, the structure of their nucleoids is quite different (14). Throughout the later stages of sporulation, the mother cell nucleoid retains the diffuse lobular structure of the vegetative cell nucleoid, while the forespore nucleoid is initially rather condensed and then assumes a ringlike structure (14, 19). The conversion of the forespore nucleoid to a ringlike structure is due to the synthesis of a group of forespore-specific DNA binding proteins termed α/β-type small, acid-soluble spore proteins (SASP), which saturate the forespore chromosome (14, 21, 22, 23). These proteins also saturate the dormant spore chromosome. The chromosome retains its ringlike shape in the first minutes of spore germination but reverts to a slightly condensed spherical form after the α/β-type SASP are degraded early in spore germination (15, 21, 23). As spore outgrowth proceeds, the nucleoid eventually returns to the diffuse lobular shape of the vegetative cell nucleoid.

Previous work has shown that mutants lacking SASP-α and -β (termed α^- β^- strains), which make up the majority of the spore's α/β-type SASP, do not have significant levels of ringlike nucleoids in either forespores or germinated spores (14, 15). Despite this drastic difference in forespore and spore nucleoid structure in wild-type and α^- β^- strains, α^- β^- strains do sporulate and α^- β^- spores go through outgrowth. However, there are several observations suggesting that there might be some differences in sporulation and spore properties between wild-type and α^- β^- strains. First, recent work has found slight differences in wild-type and α^- β^- spore resistance to several chemicals (iodine and glutaraldehyde) which do not kill spores by DNA damage and probably kill spores at least in part by inactivating some protein present in the spore's outer layers involved in spore germination (28). The α/β-type SASP protect spore DNA against damage (21, 23), but it is not clear why they should play any direct role in protecting proteins in the spore's outer layers. Second, the outgrowth of α^- β^- spores is significantly slower than that of wild-type spores, even in media likely containing far more amino acids than are provided by α/β-type SASP degradation (9). Given these observations, as well as the likelihood that nucleoid structure will globally affect transcription in a cell, we have investigated in detail the role of α/β-type SASP in the processes of sporulation and spore outgrowth and have found that the presence of wild-type levels of α/β-type SASP is necessary for both normal sporulation and spore properties.

MATERIALS AND METHODS

Bacterial strains and spore preparation and outgrowth. The *B. subtilis* strains used in this work are listed in Table 1; all are derivatives of strain PS832, which is derived from strain 168. Strains were constructed by transformation of appropriate strains with chromosomal DNA from strains carrying *lacZ* fusions or with plasmid DNA or by infection with a lysogenic SPβ phage as described (2, 31).

Sporulation was initiated at 37°C without antibiotics by using either the resuspension method (26) or nutrient exhaustion in 2× SG medium (12). Samples (1 ml) were harvested by centrifugation and stored frozen prior to analyses, and spores were harvested, cleaned, and stored as described (12). All spores used for analysis of spore outgrowth or glutaraldehyde resistance were free (>97%) of growing or sporulating cells or germinated spores. Spore outgrowth was preceded by a heat shock (30 min, 70°C) of spores in water. After cooling on ice, spores were germinated at an optical density at 600 nm (OD_{600}) of ~0.4 and 37°C in 2× YT medium (in grams per liter: yeast extract, 10; tryptone, 16; NaCl, 5) containing 4 mM L-alanine to stimulate initiation of spore germination.

Analytical procedures. Samples were extracted and analyzed for dipicolinic acid (DPA) and DNA as described (16, 18). Aliquots of sporulating cells were permeabilized with lysozyme and assayed for β-galactosidase with orthonitrophenyl-β-D-galactoside as the substrate, as described (12). In some experiments, the coats of dormant spores were first removed with urea and sodium dodecyl sulfate to inactivate external enzymes and allow spore lysozyme disruption. Those spores were assayed for β-galactosidase and glucose dehydrogenase as described (12). β-Galactosidase-specific activities are expressed in Miller units unless otherwise noted (10).

Cultures sporulating in resuspension medium as described above were fixed and treated. The DNA was stained with 4′,6′-diamidino-2-phenylindole (DAPI), and nucleoids were examined with a fluorescence microscope as described (14). Spores germinated for 2 to 5 min as described above were stained with DAPI,

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TABLE 1. *B. subtilis* strains, plasmids, and SPβ phage used

Strain, plasmid, or phage	Genotype and phenotype[a]	Source and/or reference
B. subtilis strains		
AD52	*cotD*::*cotD-lacZ* Cm^r	Adam Driks (31)
AD56	*cotC*::*cotC-lacZ* Cm^r	Adam Driks (31)
PNZ-7	SPβ::*sspF-lacZ* Cm^r	Scott Panzer (13, 25)
780[b]	*trpC2 spoVFA*::*spoVFA-lacZ* MLS^r	Jeff Errington (3)
PS346	*sspA*::*sspA-lacZ* Cm^r	8
PS356	Δ*sspA* Δ*sspB* α^- β^-	9
PS361	Δ*sspA* Δ*sspB sspA*::*sspA-lacZ* α^- β^- Cm^r	8
PS499	Δ*sspE* γ^-	5
PS533	[pUB110] Km^r	29
PS549	[pUB-A] Km^r	pUB-A→PS832
PS578	Δ*sspA* Δ*sspB* [pUB110] α^- β^- Km^r	29
PS579	Δ*sspA* Δ*sspB* [pUB-A] α^- β^- Km^r	pUB-A→PS356
PS832	Wild-type derivative of strain 168	Laboratory stock
PS848	SPβ::*sspF-lacZ* MLS^r	PNZ-7→PS832
PS850	SPβ::*sspF-lacZ* Δ*sspA* Δ*sspB* α^- β^- MLS^r	PNZ-7→PS356
PS1450	Δ*sspA* Δ*sspB* [$pSspC^{wt}$] α^- β^- Km^r	29
PS1485	[$pSspC^{wt}$] Km^r	29
PS1612	SPβ::*sspF-lacZ* [$pSspC^{wt}$] Km^r MLS^r	$pSspC^{wt}$→PS848
PS1613	SPβ::*sspF-lacZ* [$pSspC^{ala}$] Km^r MLS^r	$pSspC^{ala}$→PS848
PS1614	SPβ::*sspF-lacZ* Δ*sspA* Δ*sspB* [$pSspC^{wt}$] α^- β^- Km^r MLS^r	$pSspC^{wt}$→PS850
PS1615	SPβ::*sspF-lacZ* Δ*sspA* Δ*sspB* [$pSspC^{ala}$] α^- β^- Km^r MLS^r	$pSspC^{ala}$→PS850
PS3196	SPβ::*spoIVCB-lacZ* Cm^r	SPβ::*spoIVCB-lacZ*→PS832
PS3197	SPβ::*spoIVCB-lacZ* Δ*sspA* Δ*sspB* Cm^r	SPβ::*spoIVCB-lacZ*→PS356
PS3215	*sspA*::*sspA-lacZ* [pUB-A] Cm^r Km^r	pUB-A→PS346
PS3216	Δ*sspA* Δ*sspB sspA*::*sspA-lacZ* [pUB-A] α^- β^- Cm^r Km^r	pUB-A→PS361
PS3226	*cotD*::*cotD-lacZ* [pUB110] Cm^r Km^r	AD52→PS533
PS3227	*cotC*::*cotC-lacZ* [pUB110] Cm^r Km^r	AD56→PS533
PS3228	*cotD*::*cotD-lacZ* Δ*sspA* Δ*sspB* [pUB110] α^- β^- Cm^r Km^r	AD52→PS578
PS3229	*cotC*::*cotC-lacZ* Δ*sspA* Δ*sspB* [pUB110] α^- β^- Cm^r Km^r	AD56→PS578
PS3231	*cotC*::*cotC-lacZ* [pUB-A] Cm^r Km^r	AD56→PS549
PS3233	*cotC*::*cotC-lacZ* Δ*sspA* Δ*sspB* [pUB-A] α^- β^- Cm^r Km^r	AD56→PS579
PS3236	SPβ::*spoIVCB-lacZ* [pUB-A] Cm^r Km^r	pUB-A→PS3196
PS3237	SPβ::*spoIVCB-lacZ* Δ*sspA* Δ*sspB* [pUB-A] Cm^r Km^r	pUB-A→PS3197
PS3276	*cotC*::*cotC-lacZ* Cm^r	AD56→PS832
PS3277	*cotC*::*cotC-lacZ* Δ*sspE* γ^- Cm^r	AD56→PS499
PS3355	*spoVFA*::*spoVFA-lacZ* [pUB110] Km^r MLS^r	780→PS533
PS3356	*spoVFA*::*spoVFA-lacZ* Δ*sspA* Δ*sspB* [pUB110] Km^r MLS^r	780→PS578
Plasmids		
$pSspC^{wt}$	pUB110 with gene for $SspC^{wt}$ under *sspB* promoter control Km^r	29
$pSspC^{ala}$	pUB110 with gene for $SspC^{ala}$ under *sspB* promoter control Km^r	29
pUB-A	pUB110 with gene for SASP-α under control of its own promoter Km^r	8
Phage		
SPβ::*spoIVCB-lacZ*	SPβ carrying *spoIVCB-lacZ* Cm^r	Irina Bagyan (7)

[a] MLS, erythromycin plus lincomycin.
[b] Note that the *spoVFA* gene is also called *dpaA* (3).

and nucleoids were examined as described (15). Cleaned spores were analyzed for resistance to 0.9% glutaraldehyde at room temperature as described (28); for individual strains, the variation in the slopes of the spore-killing curves varied by less than 10% in replicate experiments.

RESULTS

Effect of α/β-type SASP on DPA accumulation and forespore gene expression during sporulation. Although previous studies did not note any qualitative differences in the sporulation of wild-type and α^- β^- strains, there were several observations suggesting that there might be subtle differences in the sporulation of these two strains (8, 9, 28). However, in these latter studies sporulation was induced by nutrient exhaustion, which is harder to study on a quantitative basis, as it is difficult to be sure of the precise time for initiation of sporulation. Consequently, we turned to the resuspension method (26) to induce sporulation, as with this method the time for initiation of sporulation is precisely defined. Analysis of DPA accumulation during sporulation of a number of wild-type and α^- β^- strains showed that the wild-type strains accumulated DPA significantly earlier than did the α^- β^- strains (Fig. 1). However, the α^- β^- spores accumulated ~25% more DPA than did the wild-type spores when spore DPA levels were expressed relative to levels of spore DNA (Table 2). Spore DPA levels were expressed in this way since *B. subtilis* spores contain only a single genome (6). Introduction of plasmids expressing high levels of either SASP-α or $SspC^{wt}$, a normally minor wild-type, α/β-type SASP (29), reversed the effect of losing SASP-α and -β on DPA accumulation, while these plasmids had essentially no effect on DPA accumulation during sporulation of the wild-type strains (Fig. 1 and Table 2; also data not shown). Control experiments showed that expression of high

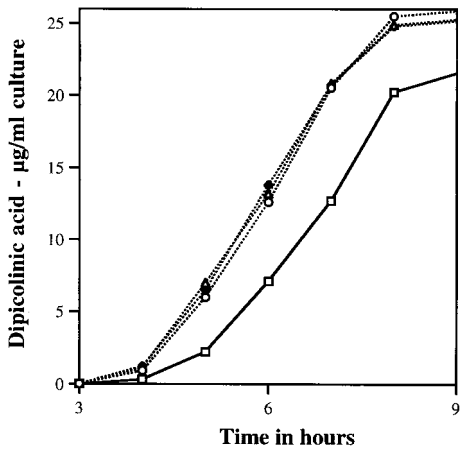

FIG. 1. DPA levels during sporulation of various strains. Strains were sporulated by the resuspension method, and samples were taken and analyzed for DPA. Zero time is the time of initiation of sporulation. Each curve is the average of DPA determinations from strains without a *lacZ* fusion or with one of three *lacZ* fusions (*sspA-lacZ*, *spoIVCB-lacZ*, or *cotC-lacZ*); within each group of four strains, the values for individual strains varied by <10% from the average value. Symbols: ○, wild-type strains PS346, PS533, PS3196, and PS3227; □, α^- β^- strains PS361, PS578, PS3197, and PS3229; △, wild-type strains with plasmid pUB-A (PS549, PS3215, PS3231, and PS3236); ●, α^- β^- strains with plasmid pUB-A (PS579, PS3216, PS3233, and PS3237). Average values for micrograms of DPA per milliliter of culture for the four groups of strains at 22 h were as follows: wild type, 30.3; α^- β^-, 38.8; wild type plus pUB-A, 29.8; α^- β^- plus pUB-A, 29.6.

levels of SASP-α from pUB-A or $SspC^{wt}$ from $pSspC^{wt}$ restored wild-type or near-wild-type levels of ringlike nucleoids to developing forespores (data not shown); these proteins also restore normal levels of ringlike chromosomes to α^- β^- spores (reference 15 and data not shown). In contrast to the delay in DPA accumulation during sporulation of α^- β^- strains, loss of the most abundant *B. subtilis* SASP, SASP-γ (21, 22), had no effect on DPA accumulation during sporulation (data not shown). While SASP-γ is synthesized at the same time as SASP-α and -β and is also degraded early in spore germination, SASP-γ is not bound to spore DNA (21, 22).

In contrast to the delay in DPA accumulation in the α^- β^- strains, there was no difference between wild-type and α^- β^- strains in the timing of the onset of β-galactosidase accumulation from an *sspA-lacZ* fusion (Fig. 2A). Since *sspA* encodes SASP-α, this is not particularly surprising. However, the α^- β^- strain carrying the *sspA-lacZ* fusion accumulated significantly higher levels of β-galactosidase during sporulation than did the wild-type strain, and this was reflected in higher levels of β-galactosidase in α^- β^- spores (Fig. 2A; Table 2). This difference was again abolished by synthesis of either SASP-α from plasmid pUB-A (Table 2; data not shown) or $SspC^{wt}$ from plasmid $pSspC^{wt}$ (data not shown). There was also a small increase in the specific activity of glucose dehydrogenase, the product of a gene expressed in parallel with *sspA* (8) in α^- β^- spores. This increase was also abolished by synthesis of high levels of SASP-α (Table 2). Another gene expressed in the forespore is the *sspF* gene (originally called 0.3 kb), but this gene is expressed ~1 h later than is *sspA* (13, 22). Analysis of the spore levels of β-galactosidase from an *sspF-lacZ* fusion showed that α^- β^- spores had ~3 times as much β-galactosidase as did wild-type spores (Table 3). The true amount of *sspF-lacZ*-driven β-galactosidase in α^- β^- spores relative to that in wild-type spores is probably even higher, since in this experiment β-galactosidase specific activity was calculated relative to glucose dehydrogenase activity and the amount of the latter enzyme is elevated in α^- β^- spores (Tables 2 and 3). Again, the difference in *sspF-lacZ*-driven β-galactosidase specific activity between wild-type and α^- β^- spores was abolished by synthesis of high levels of $SspC^{wt}$ but not by high levels of $SspC^{ala}$, a variant of $SspC^{wt}$ that binds DNA very poorly (29) (Table 3).

Effect of lack of α/β-type SASP synthesis on mother cell gene expression. Since there were effects on forespore gene expression in the absence of the majority of the forespore's α/β-type SASP, it seemed worthwhile to examine the effects of a lack of α/β-type SASP on mother cell gene expression, as there are several examples of regulatory cross talk whereby forespore-specific events modulate gene expression in the mother cell (4, 27). We therefore analyzed the expression of *lacZ* fusions to four mother cell-specific genes: *spoIVCB*, which encodes a part of the mother cell-specific σ factor for RNA polymerase (σ^K); *spoVFA*, which encodes one subunit of DPA synthetase; and *cotC* and *cotD*, which encode components of the proteinaceous coat layers that surround the mature spore (4, 22, 27, 31). The expression of the *spoIVCB*-, *spoVFA*-, and *cotD-lacZ* fusions was relatively similar in both wild-type and α^- β^- strains (Fig. 2B to D), even though DPA accumulation was 45 to 60 min slower in α^- β^- strains and ultimately ~25% higher in α^- β^- spores (Table 2 and data not shown). However, with the *lacZ* fusion expressed latest in sporulation, *cotC-lacZ*, there was a long delay in expression of this *lacZ* fusion in an α^- β^- strain, although this delay was abolished by synthesis of high levels of SASP-α from pUB-A (Fig. 2E). In contrast to the effect of losing SASP-α and -β on mother cell gene expression during sporulation, loss of SASP-γ had no noticeable

TABLE 2. Levels of DPA, glucose dehydrogenase, and β-galactosidase from an *sspA-lacZ* fusion in spores of various strains[a,b]

pUB-A presence	Level of spore DPA (μg/μg of DNA)[c]		Sp act of *sspA-lacZ* product[d]		Sp act of glucose dehydrogenase[d]	
	Wild-type strains	α^- β^- strains	Wild-type strains	α^- β^- strains	Wild-type strains	α^- β^- strains
Without	7.4 (7.1–8.2)	10.4 (9.7–12.2)	0.18 ± 0.02	0.27 ± 0.3	0.023 ± 0.002	0.030 ± 0.004
With	7.0 (6.0–7.8)	7.2 (5.6–8.4)	0.16 ± 0.02	0.15 ± 0.02	0.022 ± 0.002	0.024 ± 0.002

[a] DPA and DNA analyses were done on clean spores as described in Materials and Methods. Assays for β-galactosidase and glucose dehydrogenase were performed as described in Materials and Methods after removal of spore coats prior to lysozyme rupture of spores.

[b] Spores were prepared by the resuspension method.

[c] Values are the average (range) from duplicate assays with spores of wild-type strains without pUB-A (PS346, PS533, PS3196, and PS3227), α^- β^- strains without pUB-A (PS533, PS578, PS3197, and PS3229), wild-type strains with pUB-A (PS549, PS3215, PS3231, and PS3236), and α^- β^- strains with pUB-A (PS579, PS3216, PS3233, and PS3237).

[d] Values given are the ΔOD_{420} (*sspA-lacZ*) or ΔOD_{340} (glucose dehydrogenase) per minute per microgram of DNA in spores. The values were calculated based on the DPA content of the spores (without coats) used for preparation of enzyme extracts. These analyses were carried out in duplicate on spores from two different preparations of strains: PS346 (wild type), PS361 (α^- β^-), PS3215 (wild type with pUB-A), and PS3216 (α^- β^- with pUB-A).

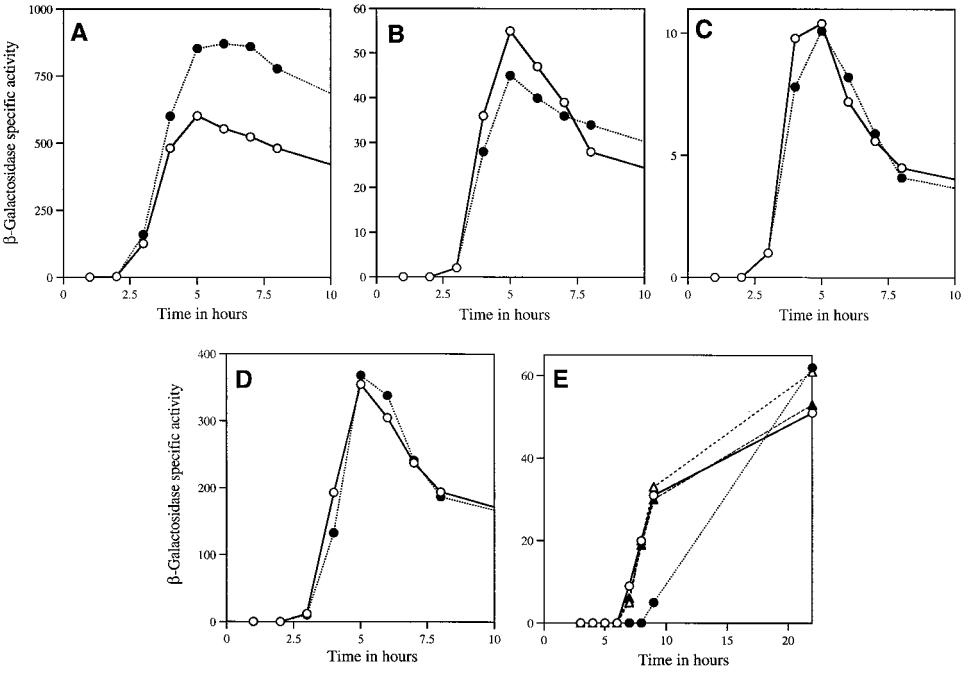

FIG. 2. Levels of β-galactosidase from various *lacZ* fusions in strains with or without α/β-type SASP. Strains were sporulated, and samples were taken and assayed for β-galactosidase. The *lacZ* fusions and strains used were *sspA-lacZ* (PS346 and PS361) (A), *spoIVCB-lacZ* (PS3196 and PS3197) (B), *spoVFA-lacZ* (PS3355 and PS3356) (C), *cotD-lacZ* (PS3226 and PS3228) (D), and *cotC-lacZ* (PS3227, PS3229, PS3231, and PS3233) (E). Symbols: ○, wild-type strains without pUB-A; ●, α^- β^- strains without pUB-A; △, wild-type strain with pUB-A; ▲, α^- β^- strain with pUB-A. β-Galactosidase activity is given in Miller units.

effect on *cotC-lacZ* expression during sporulation (data not shown).

Effects on spore properties of loss of α/β-type SASP. The finding of significant quantitative differences in the sporulation of strains with and without maximum levels of α/β-type SASP suggested that there might also be significant differences in the properties of wild-type and α^- β^- spores. Such differences have been well documented in a number of studies, but in most of these studies this is because α/β-type SASP protect spore DNA from various types of damage; α^- β^- spores are thus much more sensitive than wild-type spores to killing by DNA damage from agents such as UV radiation, heat, and some chemicals (23). However, a recent study found that among spores prepared by nutrient exhaustion, α^- β^- spores were more sensitive to both glutaraldehyde and the iodine-based disinfectant Betadine than were wild-type spores (28). This was surprising, as it was clear that (i) these agents did not kill spores by DNA damage and (ii) coats were extremely important in protecting spores from these agents (28). One obvious possibility is that the defect in the sporulation of α^- β^- strains results in slightly altered spore coats, which in turn slightly decrease resistance to chemical agents such as Betadine and glutaraldehyde. Using spores prepared by the resuspension method, we also found that the α^- β^- spores were significantly more sensitive to glutaraldehyde than were wild-type spores (Fig. 3). However, this decreased glutaraldehyde resistance of α^- β^- spores was abolished by synthesis of saturating levels of SASP-α from pUB-A, and both wild-type and α^- β^- spores with pUB-A had almost identical resistance to glutaraldehyde (Fig. 3).

TABLE 3. Comparison of specific activity of *sspF-lacZ* product from spores of different *B. subtilis* strains

Strain	Major SASP[a]	Sp act of *sspF-lacZ* product[b]
PS848	α and β	1.8 ± 0.3
PS850	None	5.6 ± 1.2
PS1612	α, β, and $SspC^{wt}$	1.7 ± 0.3
PS1613	$SspC^{wt}$	1.8 ± 0.3
PS1614	α, β, and $SspC^{ala}$	2.2 ± 0.4
PS1615	$SspC^{ala}$	6.8 ± 1.5

[a] The major SASP noted are only the α/β-type SASP.

[b] Aliquots of 36-h cultures in 2× SG medium were extracted with urea and sodium dodecyl sulfate to remove spore coats and inactivate enzymes not in spores. Spores were then disrupted with lysozyme, and extracts were assayed for β-galactosidase and glucose dehydrogenase. The *sspF-lacZ* product-specific activities are given as the ratio of the ΔOD_{420}/30 min/0.3 ml extract in the β-galactosidase assay to the ΔOD_{340}/2 min/0.1 ml extract in the glucose dehydrogenase assay and are averages of duplicate assays on spores from three separate experiments. Values for β-galactosidase in spores without a *lacZ* fusion have been subtracted from those for β-galactosidase-specific activity; in all cases this value was less than 10% of that in spores with an *sspF-lacZ* fusion.

Germination and outgrowth of spores with and without α/β-type SASP. Since spore properties are certainly affected by the presence or absence of α/β-type SASP during sporulation, it was possible that spore germination would also be affected by the presence or absence of α/β-type SASP. However, both previous work (9) and studies noted below found no difference in the initiation of germination of spores with or without α/β-type SASP. Despite this lack of effect on the initiation of spore germination, it seemed possible that the presence of α/β-type SASP might influence spore outgrowth significantly, since in the early minutes of spore germination and outgrowth the nucleoid has a ringlike shape which is only slowly transformed into a slightly more condensed form. In contrast, germinated α^- β^- spores contain only slightly condensed nucleoids (15). Since it seems likely that the drastic difference found in nucleoid morphology between germinated wild-type and α^- β^- spores would have some global effects on transcription, we

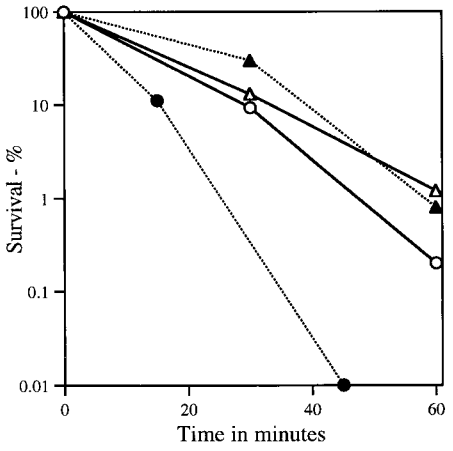

FIG. 3. Glutaraldehyde resistance of spores with and without α/β-type SASP. Spores were incubated in 0.9% glutaraldehyde at room temperature, and survival rates were measured. Symbols: ○, PS533 (wild type); ●, PS578 (α^- β^-); △, PS549 (wild type plus pUB-A); ▲, PS579 (α^- β^- plus pUB-A).

might then expect that there would also be some defect in the outgrowth of α^- β^- spores. This has been observed previously in not particularly rich media and was ascribed in large part to the lack of generation of free amino acids by degradation of α/β-type SASP in α^- β^- spores (9). However, upon spore germination and outgrowth in a rich medium (2× YT) with a much higher concentration of amino acids (~0.1 M) than is generated by α/β-type SASP degradation (~10 μM), there was still a significant delay in α^- β^- spore outgrowth compared to wild-type spore outgrowth (Fig. 4). Unfortunately, spores from strains carrying pUB-A initiated germination extremely asynchronously, so we could not assess the effect of synthesis of high levels of SASP-α on the outgrowth of wild-type and α^- β^- spores.

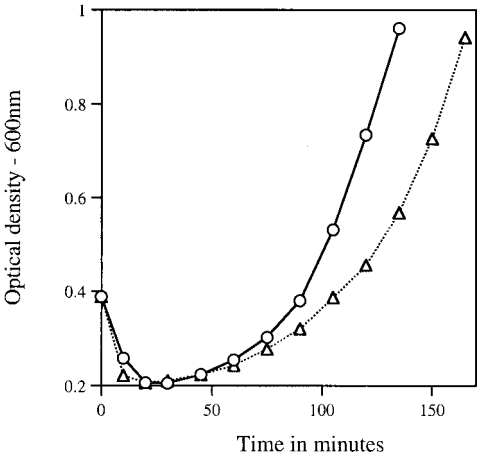

FIG. 4. Germination and outgrowth of spores with and without α/β-type SASP. Spores prepared in resuspension medium were heat shocked and then cooled, and spore germination and outgrowth were carried out as described in Materials and Methods. Symbols: ○, PS533 (wild type); △, PS578 (α^- β^-). Similar results were obtained with spores prepared in 2× SG medium and with other wild-type and α^- β^- pairs.

DISCUSSION

It is clear from the results in this study that the absence of the majority of α/β-type SASP has a significant effect on the sporulation of *B. subtilis*, including increased accumulation of several spore core-specific products (DPA and β-galactosidase from *sspA*- and *sspF-lacZ*) and delayed production of at least one mother cell-specific product (β-galactosidase from *cotC-lacZ*). These effects were largely reversed by synthesis of genome-saturating levels of either SASP-α or $SspC^{wt}$ but not by synthesis of $SspC^{ala}$, an SspC variant that binds DNA poorly and does not affect DNA properties significantly (29). While high levels of either SASP-α or $SspC^{wt}$ suppress the effects of loss of SASP-α and -β, α^- β^- spores containing high levels of SASP-α or $SspC^{wt}$ are likely not identical to wild-type spores, as SASP-β is absent from spores with high SASP-α levels and $SspC^{wt}$ has some differences in its interaction with DNA from that of SASP-α and -β (20, 28). Indeed, as noted above, the initiation of germination of spores (either wild type or α^- β^-) carrying pUB-A was much more asynchronous than that of spores of strains without this plasmid.

SASP-α and -β normally comprise ~5% of total protein in the dormant spore, and synthesis of these proteins utilizes a large amount of the forespore's translational and transcriptional capacity (20). Thus one explanation for the increased levels of other forespore-specific gene products in α^- β^- spores is the utilization of the forespore's transcriptional and translational capacity made available in the absence of genes encoding SASP-α and -β. The increased levels of glucose dehydrogenase and β-galactosidase from *sspA-lacZ* and *sspF-lacZ* in α^- β^- spores might simply then be a reflection of the increased forespore capacity to synthesize these proteins. While this reasoning may suffice to explain a small fraction of the increased accumulation of some proteins in α^- β^- spores, synthesis of $SspC^{ala}$, whose level in spores is identical to that of $SspC^{wt}$ (29), did not reverse the effects of loss of SASP-α and -β. Similarly, loss of SASP-γ, a major SASP that does not bind to DNA and whose level in wild-type spores is almost equal to that of SASP-α plus -β (21), had no significant effect on sporulation, particularly on DPA accumulation and *cotC-lacZ* expression. Thus, the increased transcriptional and translational capacity of α^- β^- forespores cannot explain all the increased protein accumulation in α^- β^- spores and certainly not the other effects of loss of SASP-α and -β on sporulation and spore properties. Interestingly, α^- β^- spores prepared by nutrient exhaustion in 2× SG medium did not contain significantly higher levels of DPA or β-galactosidase from *sspA-lacZ*. In this medium the pattern of *cotC-lacZ* expression was similar during the sporulation of wild-type and α^- β^- strains (although the level of *cotC-lacZ* expression was significantly lower than in resuspension medium, as seen previously) (8, 9, 31; data not shown). These data suggest that the magnitude of the effects of loss of SASP-α and -β depends on the sporulation medium. However, there are altered levels of β-galactosidase from *sspF-lacZ* in α^- β^- spores prepared in 2× SG medium, and these α^- β^- spores also have altered glutaraldehyde resistance and outgrowth (28). Thus, sporulation of α^- β^- strains by nutrient exhaustion also appears to be aberrant, although perhaps not as aberrant as in resuspension medium.

If, as discussed above, the absence of SASP-α and -β does not alter sporulation or spore properties simply because of an increase in available forespore protein synthetic capacity, how might the absence of α/β-type SASP alter sporulation and spore properties? The drastic change in forespore nucleoid morphology in α^- β^- spores (14, 15), as well as data indicating that α/β-type SASP can have striking inhibitory effects on tran-

scription (presumably by blocking access of RNA polymerase to the DNA template) (17, 20), suggests that α/β-type SASP may have global effects on forespore transcription. In this scenario, during the sporulation of a wild-type strain, synthesis of α/β-type SASP results in repression of further transcription as the genome becomes covered with these DNA binding proteins. However, in the absence of synthesis of the majority of these proteins, i.e., in an α^- β^- strain, much less of the genome becomes covered with α/β-type SASP (23) and thus transcription of at least some genes may increase and/or continue for slightly longer. The actual amount of the increase in expression of any individual gene in α^- β^- forespores would then depend on the relative affinities of RNA polymerase and SASP-α and -β for a particular gene or region of the chromosome. For example, transcription of the genes encoding glucose dehydrogenase and SASP-α might normally be repressed by α/β-type SASP only late in forespore development, while *sspF* expression, which takes place well after initiation of synthesis of SASP-α and -β (13), might be much more sensitive to repression by these DNA binding proteins. Thus, in an α^- β^- strain there would be a much larger increase in β-galactosidase expression from *sspF-lacZ* than the increase in glucose dehydrogenase or β-galactosidase expression from *sspA-lacZ*. One other fact that must be kept in mind in this type of analysis is that in addition to likely repression of transcription by α/β-type SASP, there is also the depletion of high-energy compounds, including ATP, in the developing forespore (24), which will also eventually shut down all transcription. However, this ATP depletion cannot take place until α/β-type SASP accumulation is complete. If the scenario given above is correct, then in an α^- β^- strain there will be significant changes in the relative amounts of expression of forespore-specific genes, with one example being *sspF*. There are also several forespore proteins involved in modulating forespore-specific gene expression (1, 30), and transcription of the genes encoding these regulatory proteins might also be affected by the absence of most α/β-type SASP, thus exacerbating even further the transcriptional anomalies in α^- β^- forespores.

As described above, it is relatively straightforward to understand disruption of forespore-specific events in α^- β^- strains. What about our observation that the lack of α/β-type SASP synthesis has very little effect on expression of the *spoIVCB*, *spoVFA*, and *cotD* genes? These genes are transcribed in the mother cell compartment under control of the RNA polymerase σ factors σ^E plus σ^K (*spoIVCB*) or σ^K alone (*spoVFA* and *cotD*) (4, 27). While forespore-specific transcription is needed for conversion of pro-σ^E and pro-σ^K to their active forms in the mother cell, the necessary forespore transcription takes place either prior to or in parallel with synthesis of α/β-type SASP under control of the forespore-specific σ factor σ^G (4, 27). Since the initiation of *sspA-lacZ* expression is essentially normal in α^- β^- strains, although the level of expression achieved is elevated, it is not surprising that *spoIVCB*, *spoVFA*, and *cotD* expression is relatively normal in α^- β^- strains. However, in contrast to *spoVFA* and *cotD*, which are expressed in parallel and require only σ^K for their expression, *cotC* is expressed significantly later, as its transcription requires not only σ^K but also the transcriptional activator GerE, whose coding gene is also expressed under σ^K control (4, 27). The striking delay in *cotC-lacZ* expression in an α^- β^- background and the suppression of this delay by forespore synthesis of high levels of SASP-α thus strongly suggest that there is an additional modulation of late mother cell gene expression by late events in the forespore, although the precise nature of this additional regulatory cross talk between the forespore and the mother cell compartments is presently unknown.

It is notable that expression of *spoVFA* appears normal in α^- β^- strains, as *spoVFA* encodes one subunit of DPA synthetase, with the other encoded by *spoVFB*, which is cotranscribed with *spoVFA* (3). This suggests that the level of DPA synthetase protein is likely to be similar during sporulation of both wild-type and α^- β^- strains, although DPA accumulation is clearly delayed during sporulation of α^- β^- strains. This further suggests that the activity of DPA synthetase may be subject to some type of feedback regulation ensuring that DPA synthesis in the mother cell is largely coupled to DPA uptake in the developing forespore, and thus it may be DPA uptake by the forespore that is delayed in α^- β^- strains. Unfortunately, at present neither the mechanism of nor the gene products involved in forespore DPA uptake are known.

With late mother cell gene expression being delayed in α^- β^- strains, as exemplified by our results with *cotC-lacZ* and with other mother cell-specific genes expressed late in sporulation likely encoding spore coat proteins, it would not be surprising if complete spore coat assembly was slightly delayed in α^- β^- strains, possibly resulting in slightly aberrant spore coats. The production of slightly aberrant spore coats in α^- β^- strains might also be promoted by slight alterations in the relative levels of many spore coat proteins, due to slight alterations in late mother cell gene expression. While α^- β^- spores are lysozyme resistant (8), indicating that there is no major defect in the coats of α^- β^- spores, the decreased glutaraldehyde and Betadine resistance of α^- β^- spores (28) is certainly consistent with there being slight defects in the outer layers of α^- β^- spores. In addition, a delay in spore coat assembly might allow DPA accumulation in the forespore to continue slightly longer in an α^- β^- strain and thus allow α^- β^- spores to accumulate more DPA than do wild-type spores. However, the precise defect in the outer layers of α^- β^- spores is not clear.

There is no difference in the vegetative growth rates of otherwise isogenic wild-type and α^- β^- strains (9), and α^- β^- spores have no defect in the initiation of spore germination, consistent with there being no major defect in the spore's outer layers. However, spore outgrowth is slowed in α^- β^- spores even when large amounts of free amino acids are present in the medium. One possible reason for this outgrowth defect is that α^- β^- spores have some slight imbalance in the levels of one or more proteins that are needed for outgrowth. A second possible reason may be that the absence of α/β-type SASP results in a nucleoid in the first minutes of spore germination that is significantly different from that in a wild-type spore (15). Indeed, significant levels of ring-shaped nucleoids persist in germinated wild-type spores for 20 to 30 min after the initiation of germination (15), during which time there is significant RNA and protein synthesis (17). If the presence of α/β-type SASP on the forespore nucleoid is expected to modify transcription at this time in development, it would also be expected to affect transcription of the germinating spore nucleoid. This altered transcription would then be expected to influence spore outgrowth, almost certainly in a negative way. Previous work has shown that α/β-type SASP have huge effects on spore properties, in particular by protecting spore DNA from damage. Given the dramatic effects on nucleoid properties of α/β-type SASP, it is not surprising that the absence of these proteins also has significant effects on gene expression.

ACKNOWLEDGMENTS

This work was supported by grants from the Army Research Office and the National Institutes of Health, GM19698.

REFERENCES

1. **Bagyan, I., J. Hobot, and S. Cutting.** 1996. A compartmentalized regulator of developmental gene expression in *Bacillus subtilis*. J. Bacteriol. **178:**4500–4507.
2. **Connors, M. J., J. M. Mason, and P. Setlow.** 1986. Cloning and nucleotide sequence of genes for three small, acid-soluble proteins of *Bacillus subtilis* spores. J. Bacteriol. **166:**417–425.
3. **Daniel, R. A., and J. Errington.** 1993. Cloning, DNA sequence, functional analysis and transcriptional regulation of the genes encoding dipicolinic acid synthetase required for sporulation in *Bacillus subtilis*. J. Mol. Biol. **232:**468–483.
4. **Errington, J.** 1993. *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. Microbiol. Rev. **57:**1–33.
5. **Hackett, R. H., and P. Setlow.** 1988. Properties of spores of *Bacillus subtilis* strains which lack the major small, acid-soluble protein. J. Bacteriol. **170:**1403–1404.
6. **Hauser, P. M., and D. Karamata.** 1992. A method for the determination of bacterial spore DNA content based on isotopic labeling, spore germination and diphenylamine assay: ploidy of spores of several *Bacillus* species. Biochimie **74:**723–733.
7. **Kunkel, B., K. Sandman, S. Panzer, P. Youngman, and R. Losick.** 1988. The promoter for a sporulation gene in the *spoIVC* locus of *Bacillus subtilis* and its use in studies of temporal and spatial control of gene expression. J. Bacteriol. **170:**3513–3522.
8. **Mason, J. M., R. H. Hackett, and P. Setlow.** 1988. Regulation of expression of genes coding for small, acid-soluble proteins of *Bacillus subtilis* spores: studies using *lacZ* gene fusions. J. Bacteriol. **170:**239–244.
9. **Mason, J. M., and P. Setlow.** 1986. Essential role for small, acid-soluble spore proteins in the resistance of *Bacillus subtilis* spores to ultraviolet light. J. Bacteriol. **167:**174–178.
10. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Nicholson, W. L., and P. Setlow.** 1990. Dramatic increase in negative superhelicity of plasmid DNA in the forespore compartment of sporulating cells of *Bacillus subtilis*. J. Bacteriol. **172:**7–14.
12. **Nicholson, W. L., and P. Setlow.** 1990. Sporulation, germination and outgrowth, p. 391–450. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biological methods for Bacillus. John Wiley & Sons, Chichester, United Kingdom.
13. **Panzer, S., R. Losick, D. Sun, and P. Setlow.** 1989. Evidence for an additional temporal class of gene expression in the forespore compartment of sporulating *Bacillus subtilis*. J. Bacteriol. **171:**561–564.
14. **Pogliano, K., E. Harry, and R. Losick.** 1995. Visualization of the subcellular location of sporulation proteins in *Bacillus subtilis* using immunofluorescence microscopy. Mol. Microbiol. **18:**459–470.
15. **Ragkousi, K., A. E. Cowan, M. A. Ross, and P. Setlow.** 2000. Analysis of nucleoid morphology during germination and outgrowth of spores of *Bacillus* species. J. Bacteriol. **182:**5556–5562.
16. **Rotman, Y., and M. L. Fields.** 1967. A modified reagent for dipicolinic acid analysis. Anal. Biochem. **22:**168.
17. **Sanchez-Salas, J.-L., M. L. Santiago-Lara, B. Setlow, M. D. Sussman, and P. Setlow.** 1992. Properties of *Bacillus megaterium* and *Bacillus subtilis* mutants which lack the protease that degrades small, acid-soluble proteins during spore germination. J. Bacteriol. **174:**807–814.
18. **Schneider, W. C.** 1957. Determination of nucleic acids in tissues by pentose analysis. Methods Enzymol. **3:**680–684.
19. **Setlow, B., N. Magill, P. Febbroriello, L. Nakhimovsky, D. E. Koppel, and P. Setlow.** 1991. Condensation of the forespore nucleoid early in sporulation of *Bacillus* species. J. Bacteriol. **173:**6270–6278.
20. **Setlow, B., D. Sun, and P. Setlow.** 1992. Studies on the interaction between DNA and α/β-type small, acid-soluble spore proteins: a new class of DNA binding protein. J. Bacteriol. **174:**2312–2322.
21. **Setlow, P.** 1988. Small, acid-soluble spore proteins of *Bacillus* species: structure, synthesis, genetics, function, and degradation. Annu. Rev. Microbiol. **42:**319–338.
22. **Setlow, P.** 1993. Spore structural proteins, p. 801–809. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D.C.
23. **Setlow, P.** 2000. Resistance of bacterial spores, p. 217–230. *In* G. Storz and R. Hengge-Aronis (ed.), Bacterial stress responses. American Society for Microbiology, Washington, D.C.
24. **Singh, R. P., B. Setlow, and P. Setlow.** 1977. Levels of small molecules and enzymes in the mother cell compartment of sporulating *Bacillus megaterium*. J. Bacteriol. **130:**1130–1138.
25. **Stephens, M. A., N. Lang, K. Sandman, and R. Losick.** 1984. A promoter whose utilization is temporally regulated during sporulation in *Bacillus subtilis*. J. Mol. Biol. **176:**333–348.
26. **Sterlini, J. M., and J. Mandelstam.** 1969. Commitment to sporulation in *Bacillus subtilis* and its relationship to development of actinomycin resistance. Biochem. J. **113:**29–37.
27. **Stragier, P., and R. Losick.** 1996. Molecular genetics of sporulation in *Bacillus subtilis*. Annu. Rev. Genet. **30:**297–341.
28. **Tennen, R., B. Setlow, K. L. Davis, C. A. Loshon, and P. Setlow.** 2000. Mechanisms of killing of spores of *Bacillus subtilis* by iodine, glutaraldehyde and nitrous acid. J. Appl. Microbiol. **89:**1–10.
29. **Tovar-Rojo, F., and P. Setlow.** 1991. Effects of mutant small, acid-soluble spore proteins from *Bacillus subtilis* on DNA in vivo and in vitro. J. Bacteriol. **173:**4827–4835.
30. **Wu, L. J., and J. Errington.** 2000. Identification and characterization of a new prespore-specific regulatory gene, *rsfA*, of *Bacillus subtilis*. J. Bacteriol. **182:**418–424.
31. **Zheng, L., and R. Losick.** 1990. Cascade regulation of spore coat gene expression in *Bacillus subtilis*. J. Mol. Biol. **212:**645–660.