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Different Small, Acid-Soluble Proteins of the α/β Type Have Interchangeable Roles in the Heat and UV Radiation Resistance of *Bacillus subtilis* Spores

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Spores of *Bacillus subtilis* strains which carry deletion mutations in one gene (*sspA*) or two genes (*sspA* and *sspB*) which code for major α/β-type small, acid-soluble spore proteins (SASP) are known to be much more sensitive to heat and UV radiation than wild-type spores. This heat- and UV-sensitive phenotype was cured completely or in part by introduction into these mutant strains of (i) one or more copies of the *sspA* or *sspB* genes themselves; (ii) multiple copies of the *B. subtilis sspD* gene, which codes for a minor α/β-type SASP; or (iii) multiple copies of the SASP-C gene, which codes for a major α/β-type SASP of *Bacillus megaterium*. These findings suggest that α/β-type SASP play interchangeable roles in the heat and UV radiation resistance of bacterial spores.

Spores of various *Bacillus* species contain a number of small, acid-soluble spore proteins (SASP), which are synthesized midway in sporulation and are rapidly degraded during spore germination (6, 14). In *B. subtilis* one group of these SASP has been termed the α/β type (3). The α/β-type SASP are coded for by at least seven genes, and the proteins coded for show greater than 70% amino acid sequence homology (3). Two of the α/β-type SASP genes (termed *sspA* and *sspB*) code for two of the three major SASP (termed α and β) found in *B. subtilis* spores; the other closely related genes, two of which (termed *sspC* and *sspD*) have been cloned, code for SASP found at much lower levels (3, 4). The third major SASP found in *B. subtilis* spores (termed γ) is coded for by the *sspE* gene and is quite different from the α/β-type SASP (8).

Work on this system over the past years has shown that a major function of these SASP is to be degraded during spore germination, generating amino acids for new protein synthesis during spore germination and outgrowth (14). However, more recent work has suggested that α/β-type SASP, in particular SASP-α, also play key roles in the dormant spore's resistance to heat and UV radiation (10, 13). Thus, spores of an *sspA* deletion strain are much more heat and UV sensitive than are wild-type spores, while spores of the *sspA sspB* double mutant are even more UV sensitive (10). However, the UV and heat resistance of spores of an *sspB* deletion strain is similar to that of wild-type spores (10). This difference in the phenotype of spores of the *sspA* and *sspB* deletion strains is surprising, since the products of these genes are almost identical proteins (3). However, the observed difference might have been due to an increased level of SASP-α in the *sspB* deletion strain compared with the level of SASP-β in the *sspA* deletion strain (10). To make a definitive assessment of the role of various α/β-type SASP in spore UV and heat resistance, we have introduced one or more copies of different wild-type SASP genes into various *B. subtilis* strains and determined the UV and heat resistance of the spores produced by the resulting strains.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The sources of the wild-type *B. subtilis* 168 and its derivatives, including the chloramphenicol-resistant and -sensitive *sspA* deletion strains (α^- strains) and the chloramphenicol-resistant *sspA* and *sspB* double deletion strain (α^- β^- strain), were described previously (10). The chloramphenicol resistance in the preceding strains is due to the integration of plasmid JH101 into the chromosome. A chloramphenicol-sensitive derivative of the α^- β^- strain which had lost the integrated plasmid pJH101 was also isolated as described previously (10).

SASP genes were cloned in either plasmid pHV33 (11) or pJH101 (5) by previously published procedures (2, 3, 9). The fragments cloned contained the complete SASP coding sequences, as well as 200 to 700 base pairs (bp) of upstream sequence (Table 1); where it has been measured, this upstream sequence encompasses the SASP gene promoters (3). Plasmid pJM1, containing the complete *B. megaterium* SASP-C gene as well as its promoter cloned in pHV33, was obtained as described previously (9). All plasmids were initially grown in *Escherichia coli* RR101, and plasmid DNA prepared as described by Birnboim and Doly (1) was used to transform various chloramphenicol-sensitive *B. subtilis* strains to chloramphenicol resistance (7) (3 μg/ml for pJH101 derivatives; 5 μg/ml for pHV33 derivatives). Strains carrying integrated pJH101 derivatives are designated by addition of the name of the integrating plasmid without the p; if the strain was resistant to 50 μg of chloramphenicol per ml, a subscript 50 is added to the name of the integrating plasmid.

Amplification of chromosomally integrated SASP genes and determination of their copy number. Strains in which chloramphenicol resistance was due to integration of pJH101 plus an associated SASP gene were streaked sequentially on L broth plates (4) containing increasing chloramphenicol concentrations (10, 20, 30, 40, and 50 μg/ml) until strains resistant to 50 μg/ml were obtained, in order to amplify the integrated pJH101 and the SASP gene it carried (16). The strategy for determination of the copy number of an integrated SASP gene associated with pJH101 was to isolate chromosomal DNA and digest it with a restriction enzyme which would give bands of similar yet separable size from

TABLE 1. Plasmids containing cloned SASP genes

Plasmid	Gene cloned	Fragment (kb)	Cloning site(s) in parental plasmid
pHVA[a] or pJA[b]	*sspA*	*Hin*cII-*Hae*III (0.69)[c]	*Eco*RV
pHVB[a] or pJB[b]	*sspB*	*Pvu*II-*Pst*I (0.9)[c]	*Eco*RV-*Pst*I
pHVD[a] or pJD[b]	*sspD*	*Pst*I (1.2)[c]	*Pst*I
pJM1[a]	SASP-C	*Hae*III (2.1)	*Eco*RI-*Pst*I[d]

[a] Parental plasmid was pHV33.
[b] Parental plasmid was pJH101.
[c] See reference 3.
[d] See reference 9.

the initial chromosomal SASP gene and the integrated SASP gene (12). Equal amounts of the digested DNA from different strains (as determined by chemical assay of DNA [12]) were run through agarose gel electrophoresis, and the DNA was transferred to nitrocellulose (15). The resulting blots were hybridized under restrictive conditions (3) to an SASP gene probe which was contained completely within both the initial and integrated SASP gene-containing band. The blots were then washed and autoradiographed, and hybridizing bands were cut out and counted in a scintillation counter. The enzymes used to digest the chromosomal DNA of various SASP gene integrates and the sizes of the hybridizing fragments were: *sspA*—*Bam*HI plus *Eco*RI, giving 1.2- and 0.66-kilobase (kb) fragments from the initial and integrated *sspA* gene, respectively; *sspB*—*Bam*HI plus *Eco*RI, giving 0.8- and 0.65-kb fragments from the initial and integrated *sspB* gene, respectively; and *sspD*—*Eco*RI, giving 1.2- and 1.1-kb fragments from the initial and integrated *sspD* gene, respectively (3). The probes used for hybridizations were: *sspA*—a 0.48-kb *Hin*cII-*Eco*RI fragment including the amino-terminal region of the coding sequence; *sspB*—a 0.45-kb *Pvu*II-*Eco*RI fragment including the amino-terminal region of the coding sequence; and *sspD*—a 0.42-kb *Pst*I-*Eco*RI fragment including the amino-terminal region of the coding sequence (3).

Other methods. *B. subtilis* strains were sporulated in 2× SG medium at 37°C as described previously (7). The medium for growth of strains containing pHV33 or unamplified integrated pJH101 contained 5 or 3 μg of chloramphenicol per ml, respectively. Strains with amplified pJH101 integrates were grown in 50 μg of chloramphenicol per ml. Spores were cleaned as described previously (10), and all spores used for measurement of heat or UV resistance were >98% free of sporulating cells or germinated spores.

Spore heat and UV resistance was measured as previously described at a temperature of 90°C and a UV flux of 1,000 to 1,200 ergs/mm^2 (10). Strains carrying pHV33 were plated on L broth plates containing chloramphenicol (5 μg/ml). Strains carrying integrated pJH101 were plated on L broth plates with and without chloramphenicol with identical results.

Sporulating cells for analysis of SASP were harvested, washed, lyophilized, disrupted, and extracted as described previously (7). The extracted SASP were dialyzed, lyophilized, redissolved, and separated by acrylamide gel electrophoresis at low pH, and the proteins were transferred to nitrocellulose (7). After the paper was baked for 1 h at 80°C, α- and β-type SASP were detected with anti-SASP-α/β immunoglobulin G (IgG) as described previously (7). Quantitation of the SASP on these immunoblots was done by reflectance densitometry as described previously (7). For quantitation of relative SASP levels in spores of various strains, at least two different amounts of spore extract were run on individual immunoblots, along with extract from equivalent amounts of wild-type spores. Enough extract was run so that the relationship between the densitometry signal and the amount of antigen run on the gel was approximately linear. In addition, the anti-SASP-α/β IgG we used had essentially identical efficiency in detecting SASP-α and SASP-β, since the relative amounts of these proteins determined in wild-type spores by immunoblot analysis were identical to those determined by direct analysis of the stained gels (data not shown).

RESULTS

Previous work has shown that the heat and UV resistance of spores of α^- and β^- strains is very different (10), suggesting a different function for the products of the *sspA* and *sspB* genes. This seemed surprising in view of the similarity between SASP-α and -β. To determine whether there was a true difference in the function of these proteins or the observed phenotypic difference was due only to a difference in the levels of SASP-α and -β, we reintroduced genes coding for α/β-type SASP into various *B. subtilis* strains. Initial experiments utilized the replicative plasmid pHV33 as a vector to carry different SASP genes. Strikingly, either the *sspA* or *sspB* gene carried on pHV33 could cure the heat- and UV-sensitive phenotypes of spores of the α^- and $\alpha^-\beta^-$ strains (Table 2). However, the addition of extra *sspA* or *B* genes did not increase the heat or UV resistance of wild-type spores (Table 2). Similarly, the *sspD* gene, which codes for a minor *B. subtilis* SASP, as well as the SASP-C gene, which codes for a major SASP of *B. megaterium*, were able to cure, at least in part, the heat- and UV-sensitive phenotypes of spores of the α^- and $\alpha^-\beta^-$ strains (Table 2, Fig. 1). Strikingly, the UV killing curve of spores of the $\alpha^-\beta^-$ strain carrying pJM1 was very biphasic (Fig. 1). Analysis of the SASP produced in spores of strains transformed with pHV33 carrying the *sspA*, *sspB*, or *sspD* gene showed that these plasmid-carried genes were expressed (data not shown; see below), as was the plasmid-carried SASP-C gene, as was shown previously (9) (Fig. 2, lanes 2 and 4).

The experiments cited above strongly suggested that different α/β-type SASP play similar roles in spore heat and UV resistance and that differences in the resistance properties of

TABLE 2. UV and heat resistance of spores of *B. subtilis* strains transformed with pHV33 derivatives carrying SASP genes[a]

SASP gene carried by transforming plasmid	Level of resistance[b]					
	Wild type		α^-		$\alpha^-\beta^-$	
	UV	Heat	UV	Heat	UV	Heat
None	++	++	—	—	—	—
sspA	++	++	++	++	++	++
sspB	++	++	++	++	++	++
sspD	++	++	−	++	−	−
SASP-C	++	++	+	++[c]	±	++[c]

[a] Spores were purified, and heat and UV resistance was measured at least twice as described in Materials and Methods. All survival was determined by plating on L broth plates plus chloramphenicol (5 μg/ml).
[b] The designations for level of resistance are as follows. UV: ++, 45 to 60% survival after 7 min; +, 4 to 10% survival after 7 min; ±, 4 to 10% survival after 1 min; −, 0.2 to 2% survival after 1 min; —, <0.2% survival after 1 min. Heat: ++, 15 to 30% survival after 30 min; −, 4 to 10% survival after 15 min; —, 0.3 to 1% survival after 15 min.
[c] See Fig. 1.

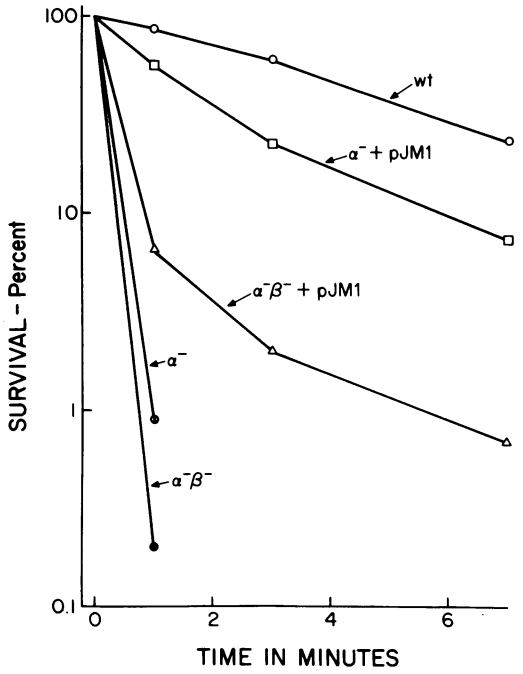

FIG. 1. UV resistance of spores of various strains with and without pJM1. Spores were prepared and purified and UV resistance was measured by plating on L broth plates with chloramphenicol (5 μg/ml) as described in Materials and Methods. The untransformed α⁻ and α⁻ β⁻ strains analyzed were the chloramphenicol-resistant strains. The wild-type (wt) strain was plated on drug-free plates.

spores of different SASP gene mutant strains reflected differences only in the amount of total spore SASP. However, definitive analysis of SASP levels in spores of strains transformed with pHV33-derived plasmids was difficult, because the partition of pHV33 and its derivatives into spores was

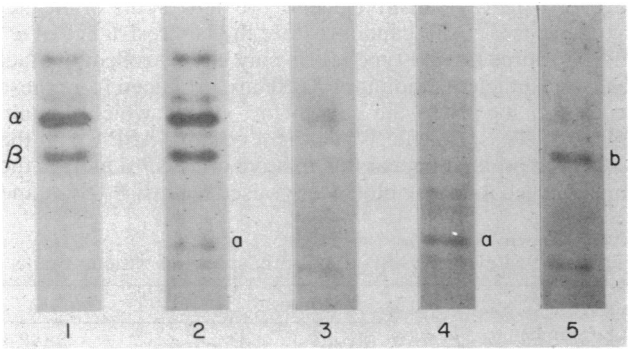

FIG. 2. Immunoblot of SASP from various strains. Strains were sporulated, samples were harvested, SASP were extracted, and equivalent amounts of spores were run by acrylamide gel electrophoresis and transferred to nitrocellulose. α- and β-type SASP were then detected as described in Materials and Methods. All strains sporulated equally well as determined by synthesis of dipicolonic acid and SASP-γ. Lanes: 1, wild type; 2, wild type plus pJM1; 3, α⁻ β⁻; 4, α⁻ β⁻ plus pJM1; 5, α⁻ β⁻ plus JD_{50}. The letters a and b denote the bands corresponding to *B. megaterium* SASP-C (9) and the *sspD* gene product, respectively. The bands which ran slower than SASP-α are probably breakdown products of SASP-α or SASP-β (data not shown), while the faint unlabeled bands running with or below SASP-α (see, for example, lane 3) are probably minor proteins produced from other SASP-α/β-type genes.

TABLE 3. Partition of pHV33 and its derivatives into spores of various *B. subtilis* strains[a]

Plasmid (size in kb)	% of spores that grew with chloramphenicol[b]		
	Wild type	α⁻	α⁻ β⁻
pHV33 (7.3)	56	—[c]	—
pHVA (8.3)	12	13	18
pHVB (9.3)	39	39	34
pHVD (8.5)	55	—	35
pJM1 (8)	54	42	54

[a] Strains were sporulated as described in Materials and Methods with chloramphenicol (5 μg/ml). Spores were plated on L broth plates with or without chloramphenicol and scored after 24 h at 37°C. The results were identical after an additional 24 h of growth.

[b] The values given are the percentage of spores which gave colonies on the chloramphenicol plates.

[c] —, Not tested.

significantly less than 100% and varied significantly for different pHV33 derivatives (Table 3). Thus, only ~50% of the spores from the wild-type strain transformed with pHV33 were chloramphenicol resistant, and this value was even lower for strains transformed by pHVA (Table 3).

Given the poor partition of pHV33-derived plasmids during sporulation, it clearly would be difficult to obtain meaningful values for plasmid copy number in spores to relate gene dosage, SASP gene expression, and spore resistance. Consequently, we turned to use of plasmid pJH101 for further experiments. This plasmid provides stable chloramphenicol resistance in *B. subtilis* only if it integrates into the chromosome by homologous recombination between the chromosome and a plasmid-carried insert (5). Thus, if a *B. subtilis* SASP gene is carried on this plasmid, the SASP gene will also be integrated into the chromosome. In addition, the copy number of the integrated SASP gene, along with the chloramphenicol resistance gene of pJH101, can be amplified by growing cells in higher and higher chloramphenicol concentrations (16), and this copy number should be uniform throughout the spore population. Indeed, >95% of the spores of strains carrying integrated pJH101 were chloramphenicol resistant and exhibited the same level of chloramphenicol resistance as the corresponding vegetative cells (data not shown).

As found with spores of strains transformed with pHV33 derivatives containing SASP genes, the heat and UV resistance of wild-type spores was not increased by extra copies of the *sspA*, *B*, or *D* genes integrated into the chromosome (Table 4). However, analysis of the SASP from spores of the wild-type strain carrying 0, 2, or 7 extra *sspB* genes revealed that the increase in *sspB* gene dosage caused a dramatic shift

TABLE 4. UV and heat resistance of spores of *B. subtilis* strains carrying extra integrated copies of SASP genes

SASP gene integrated (copy no.)	Level of resistance[a]					
	Wild type		α⁻		α⁻ β⁻	
	UV	Heat	UV	Heat	UV	Heat
None	++	++	−	—	—	—
sspA (1)	++	++	++	++	++	++
sspB (2)	++	++	++	++	+	++
sspB (7)	++	++	++	++	NT[b]	NT
sspD (1)	++	++	−	++	−	−
sspD (7)	++	++	+	++	−	++

[a] See Table 2, footnote *b*, for explanation of symbols.

[b] NT, Not tested.

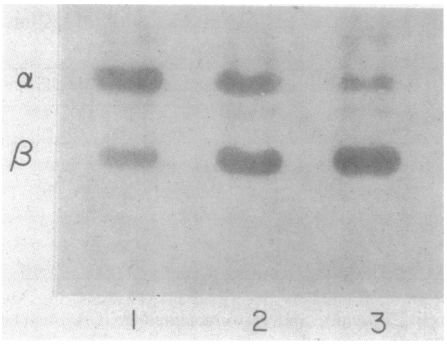

FIG. 3. Immunoblot of SASP from spores of the wild-type strain carrying increasing *sspB* gene copy number. Lanes: 1, wild type; 2, wild type transformed with pJB and containing a total of 3 *sspB* genes (Table 4); 3, wild type transformed with pJB and then amplified and containing 8 *sspB* genes (Table 4). These strains were grown, sporulated, and harvested, and SASP from equivalent amounts of spores were extracted, separated, and visualized as described in Materials and Methods. All three strains sporulated equally well as measured by production of dipicolonic acid and SASP-γ.

in the relative level of SASP-α and -β (Fig. 3). While the total amount of SASP-α plus -β did not change, the increase in *sspB* gene dosage resulted in a large increase in SASP-β, with a corresponding decrease in SASP-α (Fig. 3, Table 5).

The heat- and UV-sensitive phenotype of spores of the α^- or α^- β^- strains was restored to wild-type levels by introduction of a single *sspA* gene, as was expected given the wild-type resistance levels of α^- spores (Table 4). Similarly, the heat resistance of α^- and α^- β^- spores was restored to wild-type levels by two extra *sspB* genes (Table 4), while two extra *sspB* genes restored full UV resistance to α^- spores. However, two *sspB* genes did not restore the α^- β^- spores to wild-type UV resistance (Table 4). Unfortunately, we were unable to test the effect of a high *sspB* gene copy number on the UV resistance of α^- β^- spores because we were unable to amplify the *sspB* gene in the α^- β^- strain by growing it in high levels of chloramphenicol. Analysis of the levels of SASP-β in spores of these strains indicated that increasing levels of SASP-β were produced as the *sspB* copy number increased (Table 5). However, the total level of SASP-β did not rise above the wild-type spore level of SASP-α plus -β (Table 5).

As found with the *sspD* gene carried on pHV33, extra *sspD* genes integrated into the chromosome also cured the heat-sensitive phenotype of α^- as well as α^- β^- spores (Table 4). In addition, multiple *sspD* genes had a significant effect in curing the UV-sensitive phenotype of spores of these strains, although the effect was much more dramatic in the α^- strain (Table 4). Comparison of immunoblot analyses of SASP in spores of the α^- β^- strain (Fig. 2, lane 3) with those in the α^- β^- strain carrying multiple *sspD* genes also allowed identification of the product of the *sspD* gene as a protein which comigrated with SASP-β (Fig. 2, lane 5, band b).

DISCUSSION

The results reported in this communication lead to a number of conclusions. The first is that plasmid pHV33 and its derivatives do not partition efficiently into spores and presumably are not incorporated into the majority of developing forespores. Unfortunately, why this is the case, and why pHV33 derivatives carrying different SASP genes partition with different efficiencies, is not clear. However, since pHV33 was constructed from plasmids isolated from nonsporulating bacteria (11), it presumably does not have the ability to partition efficiently at the unequal cell division which divides the sporulating cell into the large mother cell and smaller prespore cell. The result of this low-efficiency partitioning is that it is difficult to quantitatively relate SASP gene copy number, SASP level, and heat and UV resistance in spores of strains transformed with pHV33 derivatives, especially since plasmid copy number may vary widely in those forespores which incorporate the plasmid. Indeed, the extremely biphasic nature of the UV resistance curve of spores of the α^- β^- plus pJM1 strain (Fig. 1) could reflect a heterogeneous spore population in which different spores have different plasmid copy numbers and thus very different levels of SASP-C.

A second major conclusion from this work is that the two major α/β-type SASP of *B. subtilis* appear to have interchangeable roles in spore heat and UV resistance. Thus, a large increase in the ratio of SASP-β to SASP-α had no effect on the UV or heat resistance of wild-type spores, and the UV- and heat-sensitive phenotypes of α^- and α^- β^- spores could be completely cured by introduction of *sspB* genes alone. However, in the absence of a wild-type *sspA* gene, at least three *sspB* genes were necessary to produce sufficient SASP-β to provide wild-type levels of UV resistance, since the *sspB* gene is expressed at a lower level than the *sspA* gene (see below). Quantitation of the total amount of SASP-α plus -β in various strains suggests that some minimum level of SASP-α plus -β is required for spore UV resistance but that it is immaterial whether this protein is SASP-α or SASP-β.

The third conclusion which can be drawn is analogous to the one given above and suggests that not just SASP-α and -β, but any α/β-type SASP can play a major role in spore resistance. Thus, the minor SASP produced by the *sspD* gene, as well as *B. megaterium* SASP-C, can cure the heat-sensitive phenotype of α^- β^- spores and can, in large part, cure the UV-sensitive phenotype of α^- spores. The fact that these two SASP cannot restore the UV resistance of α^- and β^- spores to wild-type levels may simply reflect the fact that an insufficient amount of SASP are produced from these genes—analogous to the case of α^- spores which contain only a single *sspB* gene. While the levels of SASP-C and the *sspD* gene product appear low relative to SASP-α and -β, the antisera used for these blots were raised against SASP-α and

TABLE 5. Level of SASP-α and -β in spores of various strains

Strain	Level (U)[a]		
	SASP-α	SASP-β	SASP-α plus -β
Wild type	100 (1)	40 (1)	140
Wild type plus JB	50 (1)	100 (3)	150
Wild type plus JB_{50}	15 (1)	135 (8)	150
α^-	0 (0)	70 (1)	70
α^- plus JB	0 (0)	135 (3)	135
α^- plus JB_{50}	0 (0)	145 (8)	145
α^- β^-	0 (0)	0 (0)	0
α^- β^- plus JB	0 (0)	100 (2)	100

[a] All values are in arbitrary units expressed relative to the value for the level of SASP-α in wild-type spores, which was set at 100. The values in parentheses are the copy numbers of the genes coding for the individual SASP.

-β. Consequently, accurate quantitation of these two SASP in this experiment was difficult.

A fourth conclusion which can be drawn from these data is that less α/β-type SASP are needed for spore heat resistance than are needed for UV resistance. pHVD and pJM1 restored full heat resistance to α^- and α^- β^- spores, respectively, while providing only a small increase in UV resistance (Table 2). Similarly, α^- β^- spores carrying two integrated *sspB* genes were fully heat resistant but only partially UV resistant. While the reason for the requirement of different levels of α/β-type SASP for spore heat and UV resistance is not clear, studies on the mechanism(s) by which SASP cause spore resistance (13) may shed some light on this question.

A final conclusion drawn from this work concerns the regulation of *B. subtilis* α/β-type SASP gene expression, in particular *sspA* and *sspB*. Previous work had suggested that there was some autoregulation of the *sspA* and *sspB* genes (10). Data in this communication on levels of SASP-α and -β in wild-type strains containing increasing numbers of *sspB* genes are consistent with this idea, as the total amount of SASP-α plus -β did not change, only the ratio of SASP-β to -α. Further analysis of these strains, with determination of levels of SASP gene mRNAs, should provide further information on how these genes are regulated.

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