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New Small, Acid-Soluble Proteins Unique to Spores of *Bacillus subtilis*: Identification of the Coding Genes and Regulation and Function of Two of These Genes

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Eleven small, acid-soluble proteins (SASP) which are present in spores but not in growing cells of *Bacillus subtilis* were identified by sequence analysis of proteins separated by acrylamide gel electrophoresis of acid extracts from spores which lack the three major SASP (α, β, and γ). Six of these proteins are encoded by open reading frames identified previously or by analysis of the complete sequence of the *B. subtilis* genome, including two minor α/β-type SASP (SspC and SspD) and a putative spore coat protein (CotK). Five proteins are encoded by short open reading frames that were not identified as coding regions in the analysis of the complete *B. subtilis* genomic sequence. Studies of the regulation of two of the latter genes, termed *sspG* and *sspJ*, showed that both are expressed only in sporulation. The *sspG* gene is transcribed in the mother cell compartment by RNA polymerase with the mother cell-specific sigma factor for RNA polymerase, σ^K, and is cotranscribed with a downstream gene, *yurS*; *sspG* transcription also requires the DNA binding protein GerE. In contrast, *sspJ* is transcribed in the forespore compartment by RNA polymerase with the forespore-specific σ^G and appears to give a monocistronic transcript. A mutation eliminating SspG had no effect on sporulation or spore properties, while loss of SspJ caused a slight decrease in the rate of spore outgrowth in an otherwise wild-type background.

Dormant spores of *Bacillus subtilis* contain a number of proteins which are not present in growing cells, including spore coat proteins, components of the spore germination apparatus, a few unique spore enzymes, and a group of small, acid-soluble spore proteins (SASP) (38, 39). Among the latter proteins are the multiple α/β-type SASP and the single γ-type SASP; three of these proteins (SASP α, β, and γ) make up the great majority of all SASP in spores (38, 40). However, *B. subtilis* spores also contain a number of minor SASP, and similar minor proteins are present in spores of other *Bacillus* species (16, 37, 38, 46). While one of the minor SASP in *B. subtilis* is a minor α/β-type SASP termed SspC (46), the identity of the other minor proteins is not known.

Identification and analysis of these additional minor SASP and study of the regulation of their coding genes may be of interest for a number of reasons. First, since the minor SASP are undoubtedly very small, it is possible that their coding regions were not identified as open reading frames (ORFs) in the recently completed *B. subtilis* genomic sequence (18). Identification of any new ORFs will thus assist in completion of the analysis of the genomic sequence. Second, if the new minor SASP are indeed spore-specific proteins, then their coding genes should exhibit sporulation-specific expression. Study of the regulation of expression of these new genes, and in particular of their dependence on sporulation-specific sigma factors for RNA polymerase and their promoter sequences, would thus expand our knowledge of regulation of sporulation-specific genes. Finally and most importantly, several SASP, particularly the major α/β-type SASP, have major functions in the dormant spore in (i) providing resistance to spore DNA against damage caused by heat and oxidizing agents (6, 40); (ii) altering spore DNA photochemistry, thus providing a major element of spore UV resistance (25, 36, 40); and (iii) generating free amino acids for protein synthesis by their degradation early in spore germination (38). It is certainly possible that the new minor SASP has redundant functions in the spore, and thus loss of only one may have no phenotypic effect or at most a minor one, as is the case for the two major α/β-type SASP (25, 40). However, the essential role of the latter proteins in several of the properties unique to or characteristic of bacterial spores suggests that the new minor SASP might also have some specific function in sporulation, spores, or spore germination. Consequently, mutagenesis of the genes encoding these new minor SASP, alone or in various combinations, might give new insight into mechanisms determining various aspects of sporulation, spore properties, and spore germination. Given these reasons, we have determined the N-terminal amino acid sequences of minor *B. subtilis* SASP and have identified the genes encoding 11 of these proteins; five of these genes were not identified as ORFs in the *B. subtilis* genomic sequence. We also report detailed studies on the regulation of expression and function of two of the latter genes, both of which are new sporulation-specific genes.

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MATERIALS AND METHODS

Bacterial strains and spore preparation. *Escherichia coli* TG1 (33) and DH5α (11) were used for cloning; the *B. subtilis* strains used in this study are listed in Table 1. *B. subtilis* PS482 was used for identification of minor SASP, as this strain carries deletions of the *sspA*, *-B*, and *-E* genes, which code for the three major *B. subtilis* SASP, α, β, and γ, respectively (9). This strain (termed $\alpha^-\beta^-\gamma^-$) was sporulated at 37°C in 2× SG medium (8), and the spores were purified and stored as described previously (29). Growing cells of strain PS482 were prepared in the same medium but harvested in the late log phase of growth (optical density at 600 nm [OD_{600}] ≅ 1) and washed once with 0.15 M NaCl, and the cell pellet fraction was frozen and lyophilized. *B. subtilis* strains with the PS832 background were used to study *sspG* and *sspJ* expression and for analysis of the phenotypes of the *sspG* and *sspJ* mutants; *B. subtilis* strains with a PY79 genetic background were used for studies of the genetic dependence of *sspG* and *sspJ* expression. PS832 and PY79 are very similar wild-type strains of *B. subtilis*, but PS832 sporulates slightly more efficiently, while a number of mutations in genes for

TABLE 1. *B. subtilis* strains used in this study

Strain	Relevant genotype or phenotype	Source or reference
PS482[a]	Δ*sspA* Δ*sspB* Δ*sspE* Cm^{r}[b] ($\alpha^{-}\beta^{-}\gamma^{-}$)	9
PS832[a]	Wild-type derivative of strain 168	Laboratory stock
PY79[c]	Wild type	47
IB464[a]	*sspG*::*sspG-lacZ* Cm^{r}	pIB454→PS832
IB465[a]	*sspJ*::*sspJ-lacZ* Cm^{r}	pIB452→PS832
IB488[a]	Δ*sspG-yurS*::*spc* Sp^{r}[d]	This work
IB490[a]	Δ*sspJ*::*spc* Sp^{r}	This work
IB466[c]	*spoIIAC sspG*::*sspG-lacZ* Cm^{r}	IB464→SC1159
IB467[c]	*spoIIGB sspG*::*sspG-lacZ* Cm^{r} Em^{r}[e]	IB464→SC137
IB468[c]	*spoIIIG sspG*::*sspG-lacZ* Cm^{r}	IB464→SC500
IB469[c]	*spoIVCB sspG*::*sspG-lacZ* Cm^{r} Em^{r}	IB464→SC64
IB470[c]	*sspG*::*sspG-lacZ* Cm^{r}	IB464→PY79
IB471[c]	*spoIIAC sspJ*::*sspJ-lacZ* Cm^{r}	IB465→SC1159
IB472[c]	*spoIIGB sspJ*::*sspJ-lacZ* Cm^{r} Em^{r}	IB465→SC137
IB473[c]	*spoIIIG sspJ*::*sspJ-lacZ* Cm^{r}	IB465→SC500
IB474[c]	*spoIVCB sspJ*::*sspJ-lacZ* Cm^{r} Em^{r}	IB465→SC64
IB475[c]	*sspJ*::*sspJ-lacZ* Cm^{r}	IB465→PY79
IB502[c]	*sspG*::*sspG-lacZ* P*spac-sigK sigK*Δ*19* Cm^{r} Sp^{r}	IB470→BZ536
IB480[c]	*sspJ*::*sspJ-lacZ spoIIIG* [pSDA4] Cm^{r} Km^{r}[f]	pSDA4→IB473
IB481[c]	*sspJ*::*sspJ-lacZ spoIIIG* [pDG298] Cm^{r} Km^{r}	pDG298→IB473
IB492[g]	*sspG*::*sspG-lacZ trpC2* Cm^{r}	IB464→SG38
IB494[g]	*gerE36 sspG*::*sspG-lacZ trpC2* Cm^{r}	IB464→522.2
IB498[g]	SPβ*cotA-lacZ trpC2* Cm^{r} Em^{r}	SPβ::*cotA-lacZ*→SG38
IB511[a]	Δ*sspG-yurS*::*spc*Δ *sspA* Δ*sspB* Δ*sspE* Cm^{r} Sp^{r}	IB488→PS482
IB515[a]	Δ*sspJ*::*spc* Δ*sspA* Δ*sspB* Δ*sspE* Cm^{r} Sp^{r}	IB490→PS482
SC64[c]	*spoIVCB* Em^{r}	S. Cutting
SC137[c]	*spoIIGB* Em^{r}	S. Cutting
SC500[c]	*spoIIIG*Δ*1*	S. Cutting
SC1159[c]	*spoIIAC1*	S. Cutting
BZ536[c]	P*spac*-P*sigK sigK*Δ*19* Sp^{r}	L. Kroos
522.2[g]	*gerE36 trpC2*	L. Kroos
SG38[g]	*trpC2*	J. Errington

[a] Genetic background is PS832.
[b] Cm^{r}, chloramphenicol (5 μg/ml) resistance.
[c] Genetic background is PY79.
[d] Sp^{r}, spectinomycin (100 μg/ml) resistance.
[e] Em^{r}, erythromycin (1 μg/ml) resistance.
[f] Km^{r}, kanamycin (10 μg/ml) resistance.
[g] Derivatives of *B. subtilis* strain CU267 (originally obtained from S. A. Zahler).

sporulation sigma factors are available in the PY79 background. All transformations of *B. subtilis* strains were carried out as described previously (1).

Identification and analysis of minor SASP. Lyophilized dormant spores (100 mg [dry weight]) or late-log-phase cells were broken in a dental amalgamator (Wig-L-Bug) with glass beads (100 mg) as the abrasive for either 10 min (spores) or 2 min (growing cells). The dry powder was extracted twice at 4°C for 30 min with 5 ml of 3% acetic acid, and the supernatant fluids were combined and dialyzed against 1 liter of 1% acetic acid in Spectrapor 3 tubing (molecular weight cutoff, 3,500) for 16 h at 6°C with two changes. The pellet from the acetic acid extract was further extracted twice with 5 ml of 0.3 N HCl at 4°C, and the supernatant fluids were pooled and dialyzed as described above. The dialyzed material was lyophilized, and the dry residue was dissolved in 200 μl of 8 M urea plus 100 μl of acid gel diluent (31). Aliquots of the redissolved material were subjected to polyacrylamide gel electrophoresis (PAGE) at low pH (31), proteins were electrophoretically transferred to polyvinylidine difluoride paper (Immobilon) in 10% methanol–0.7% acetic acid for 45 min at 200 mA (24), and the proteins on the paper were stained lightly with Coomassie blue, destained, and air dried. Selected protein bands were cut from the paper with a clean razor blade and subjected to protein sequence analysis as described previously (46).

For analysis of minor SASP in decoated spores, 55 mg (dry weight) of spores of strain PS482 were decoated in 8 M urea–1% sodium dodecyl sulfate (SDS)–50 mM dithiothreitol–10 mM EDTA–50 mM Tris-HCl (pH 8.0) for 90 min at 37°C, and the spores were washed as described previously (29). The decoated spores were lyophilized, and SASP were extracted as described above. In other experiments, wild-type and mutant spores were boiled in SDS-PAGE loading buffer as described previously (13), and low-molecular-weight soluble proteins were analyzed by SDS-PAGE (35). For analysis of minor SASP in germinated spores, 50 mg (dry weight) of spores of strain PS482 in 2 ml of water was heat shocked for 30 min at 70°C and then cooled on ice. The spores were germinated for 45 min in 200 ml of prewarmed (37°C) 2× YT medium ([per liter] tryptone, 16 g; yeast extract, 10 g; NaCl, 5 g) containing 4 mM L-alanine to stimulate the initiation of spore germination, harvested by centrifugation, washed once with 100 ml of 0.15 M NaCl, lyophilized and broken with 8 min of dry rupture, extracted with both acetic acid and HCl, dialyzed, lyophilized, redissolved, and analyzed as described above.

For analysis of minor SASP in strains with mutations in new *ssp* genes, spores were prepared as described above, and 75 OD_{600} U of cleaned spores were lyophilized, disrupted, extracted twice with 3% acetic acid (1 ml), dialyzed, lyophilized, and redissolved as described above, and aliquots were analyzed by PAGE at low pH (31).

Construction of *B. subtilis* strains containing translational *sspG*- and *sspJ-lacZ* fusions. Fragments encompassing 220 bp upstream of the *sspG* ORF as well as 22 bp of the coding region or 201 bp upstream of the *sspJ* ORF and 20 bp of the coding region were amplified by PCR. The primers used for *sspG* were prot2TXN5′ (5′-GGAATTCGAGATGATAAGCCGTCG-3′) and prot2TXN3′ (5′-CGGGATCCTTTTCATGACGATTTTCGCTC-3′). The primers used for *sspJ* were prot3-5′ (5′-GGAATTCGCGATGCCTCCCATGATG-3′) and prot3-3′ (5′-CGGGATCCTCTTTATTAAAGAAACCCATTC-3′). In all cases, the primers had extra residues at the 5′ end, including an *Eco*RI or a *Bam*HI site (underlined). The PCR fragments were cut with *Eco*RI and *Bam*HI and cloned between the *Eco*RI and *Bam*HI sites of pJF751, a vector for construction of translational *lacZ* fusions (7). The resulting plasmids, termed pIB454 (*sspG*) and pIB452 (*sspJ*), were integrated into the PS832 chromosome by a single crossover event with selection for Cm^{r}. Transformants containing a single copy of the translational *sspG*- or *sspJ-lacZ* fusion at the *sspG* or *sspJ* locus as shown by Southern blot analysis were called strains IB464 and IB465, respectively. Chromosomal DNA was isolated from these strains and used to transform *B. subtilis* strains containing different *spo* mutations in the PY79 background or strain 522.2 to Cm^{r}.

Analysis of β-galactosidase activity in sporulating cells, spores, and vegetative cells. Sporulation of *B. subtilis* was induced at 37°C by the resuspension method (43) or by the nutrient exhaustion method in 2× SG medium (8, 20), and samples (1 ml each) were harvested by centrifugation and stored frozen. Strains carrying genes encoding σ^{F}, σ^{G}, or σ^{K} under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *spac* promoter (P*spac*) were grown at 37°C in 2× YT medium to an OD_{600} of 0.25. The culture was then divided in half, IPTG was added to 2 mM to one-half, incubation was continued, and samples were taken and stored frozen as described above. β-Galactosidase activity was determined with *o*-nitrophenyl-β-D-galactopyranoside as the substrate as described previously (29); lysozyme (200 μg/ml) was used for cell permeabilization prior to enzyme assay. To analyze β-galactosidase activity in spores, the spores were first decoated and then treated with lysozyme prior to enzyme assays as described previously (29). All β-galactosidase specific activities are expressed in Miller units (27).

Determination of the *sspG-yurS* and *sspJ* transcription start sites. Total RNA was extracted from cells of strains IB464 and IB465 sporulating in 2× SG medium as described previously (28). The RNA was used in primer extension reactions at 47°C with avian myeloblastosis virus reverse transcriptase (28). The primers used were prot2-55 (5′-CTTTTGCTAATCCGCTGTTTTGG-3′), which anneals to *sspG* mRNA; yurS-140 (5′-GTTAAGAGGAATGATGTTTTCGTTC-3′), which anneals to *yurS* mRNA; prot3-50 (5′-TCTTCAAGAGCTCCTTGGATTAC-3′), which anneals to *sspJ* mRNA; and lacZ-70 (5′-AAGGCGATTAAGTTGGGTAACG-3′), which anneals to the *lacZ* portion of *sspG-lacZ* or *sspJ-lacZ* mRNAs. Size standards for analysis of the primer extension products were produced with the same four primers in DNA sequencing reactions. The prot2-55 and yurS-140 primers were used with plasmid pIB517, which carries a 1,055-bp fragment encompassing the *sspG* and *yurS* region (see below); the prot3-50 primer was used with plasmid pIB460, which carries a 1,190-bp fragment encompassing the *sspJ* region (see below); and the lacZ-70 primer was used with plasmids pIB454 and pIB452, which carry the translational *sspG-lacZ* and *sspJ-lacZ* fusions, respectively, in plasmid pJF751.

Cloning of a fragment encompassing the *sspG-yurS* and *sspJ* regions. A fragment encompassing 1,055 bp of the *sspG-yurS* region was amplified by PCR. The primers used were prot2TXN5′ (see above) and prot2mut4 (5′-GCTCTAGATCAAGACATGGCACTGG-3′). The PCR product was cloned in the TA-cloning vector pCR2.1 (Invitrogen) according to the manufacturer's instructions, and the resulting plasmid was called pIB517.

A fragment encompassing 1,190 bp of the *sspJ* region was also amplified by PCR. The primers used were prot3mut5′ (5′-GGAATTCGCGGATCGTGGAAGGG-3′) and prot3mut3′ (5′-CGGGATCCACGGACTCGCAATTGAAGC-3′); the primers had extra residues at the 5′ end, including an *Eco*RI or a *Bam*HI site (underlined). The PCR product was cut with *Eco*RI and *Bam*HI and cloned between the *Eco*RI and *Bam*HI sites of pSGMU2 (30), giving plasmid pIB460.

Construction of an *sspG-yurS* null mutant. A 490-bp fragment containing the region directly upstream of the *sspG* ORF was amplified by PCR. The primers used were prot2mut1 (5′-GGAATTCTTATACGCCCTTTCCTCC-3′) and prot2mut2 (5′-AACTGCAGGTATCATCCTTTCTCTATTG-3′), each containing extra residues, including an *Eco*RI or *Pst*I site at their 5′ ends (underlined). The PCR fragment was cut with *Eco*RI and *Pst*I and cloned between the *Eco*RI and *Pst*I sites of plasmid pJL74 (19), which contains an Sp^{r} cassette, giving plasmid pIB458. A 539-bp fragment containing the region starting 149 bp downstream of the *sspG* ORF and encompassing the second half of the *yurS* ORF was amplified by PCR. The primers used were prot2mut3 (5′-CGGGATCCGCCGCAAAGCAGATGAC-3′) and prot2mut4new (5′-ATAAGAATGCGGCCGCATCAAGACATGGCACTGG-3′), each containing extra residues, including a

*Bam*HI or *Not*I site at their 5′ ends (underlined). The PCR fragment was cut with *Bam*HI and *Not*I and cloned between the *Bam*HI and *Not*I sites of plasmid pIB458. The resulting plasmid (pIB479) contains the *sspG* flanking regions, with the Sp^r cassette replacing the *sspG* ORF and the first half of the *yurS* ORF. pIB479 was linearized with *Xho*I and used to transform *B. subtilis* PS832 to Sp^r (100 μg/ml). In this transformation the Sp^r cassette was integrated into the *B. subtilis* chromosome by a double crossover event removing the *sspG* ORF and the first half of the *yurS* ORF. One Sp^r transformant whose expected chromosomal structure was confirmed by Southern blot analysis (data not shown) was termed IB488. To construct an *sspG-yurS* null mutant that did not produce SASP α, β, and γ, we transformed strain PS482 to Sp^r with chromosomal DNA from strain IB488. The resulting Cm^r Sp^r strain was called IB511.

Construction of the *sspJ* null mutant. A 521-bp fragment containing the region immediately downstream of the *sspJ* ORF was amplified by PCR. The primers used were prot3mut1 (5′-GGAATTCGCTCCAAACGGACTCGC-3′) and prot3mut2 (5′-AACTGCAGCCACATGCGGATAGGGC-3′), each containing extra residues, including an *Eco*RI or *Pst*I site at their 5′ ends (underlined). The PCR fragment was cut with *Eco*RI and *Pst*I and cloned between the *Eco*RI and *Pst*I sites of plasmid pJL74 (19), giving plasmid pIB476. A 513-bp fragment containing the first 7 bp of the *sspJ* ORF and 506 upstream bp was amplified by PCR. The primers used were prot3mut3 (5′-CGGGATCCAACCCATTCGTATCACCTC-3′) and prot3mut4 (5′-TCCCCGCGGTGATTCCGTTCACCGTCC-3′), each containing extra residues, including a *Bam*HI or *Sac*II site at their 5′ ends (underlined). The PCR fragment was cut with *Bam*HI and *Sac*II and cloned between the *Bam*HI and *Sac*II sites of plasmid pIB476. The resulting plasmid (pIB482) contains the *sspJ* flanking regions, with the Sp^r cassette replacing the *sspJ* ORF. pIB482 was linearized with *Xho*I and used to transform *B. subtilis* PS832 to Sp^r. In this transformation the Sp^r cassette was integrated into the *B. subtilis* chromosome by a double crossover event, removing the *sspJ* ORF. One Sp^r transformant whose chromosomal structure was confirmed by Southern blot analysis (data not shown) was termed IB490. To construct an *sspJ* null mutant which does not produce SASP α, β, and γ, we transformed strain PS482 to Sp^r with chromosomal DNA from strain IB490. The resulting Cm^r Sp^r strain was called IB515.

Analysis of resistance, germination, and outgrowth of *B. subtilis* spores. Spores were harvested from cultures grown for 48 h at 37°C in 2× SG medium and purified as described previously (25, 29). Spores in water were heat treated (85°C) or UV irradiated with 254-nm light, and survival was measured as described previously (6, 25). For analyses of spore lysozyme and chloroform resistance, spores were diluted to an OD_{600} of 1 in 10 mM potassium phosphate buffer (pH 7.4) containing 50 mM KCl and 1 mM $MgSO_4$ and treated with lysozyme at 1.5 mg per ml for 30 min at 37°C or with chloroform as described previously (29).

For spore germination and outgrowth, purified spores in water were heat activated for 30 min at 65°C (for spores lacking SASP α, β, and γ) or 70°C, cooled on ice, and then diluted to an OD_{600} of 0.4 to 0.5 in 2× YT medium containing 4 mM L-alanine or an OD_{600} of 0.7 to 0.9 in Spizizen's minimal medium (42) without Casamino Acids but containing 4 mM L-alanine and 50 μg of L-tryptophan/ml. Cultures were incubated at 37°C with good aeration, and the OD_{600}s of the cultures were monitored.

RESULTS

Identification of new SASP and their coding genes. PAGE at low pH of acetic acid extracts of *B. subtilis* spores (termed $\alpha^-\beta^-\gamma^-$) lacking the three major SASP gave a series of protein bands, none of which were present in acetic acid extracts of growing cells (Fig. 1, lanes a and b). The levels of at least some of these acetic acid-soluble proteins unique to spores were similar in both $\alpha^-\beta^-\gamma^-$ and wild-type spores (data not shown); however, the presence of SASP α, β, and γ precluded the observation of several of the minor protein bands in extracts of wild-type spores. Consequently, all routine analysis of these proteins was carried out with $\alpha^-\beta^-\gamma^-$ spores. Subsequent extraction of $\alpha^-\beta^-\gamma^-$ spores with HCl dissolved more of the proteins that were present in the acetic acid extract as well as a number of additional proteins (Fig. 1, lane c). However, many of these latter additional proteins appeared to be present in the HCl extract of growing cells (Fig. 1, lane d). Consequently, we focused on the acetic acid-soluble proteins from $\alpha^-\beta^-\gamma^-$ spores and identified 10 distinct protein bands which were present in significant amounts in acetic acid extracts from spores but not in HCl extracts of growing cells (Fig. 1, lane e). Automated protein sequence analysis showed that band 8 was the product of the *cotK* gene, while band 5 has been shown previously to be the minor α/β-type SASP SspC (Table 2) (46).

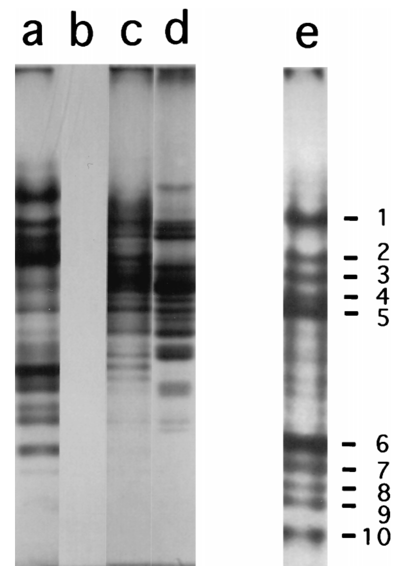

FIG. 1. Low-pH PAGE analysis of minor SASP from *B. subtilis*. Acetic acid and HCl extracts from dormant spores and growing cells of strain PS482 were prepared and redissolved as described in Materials and Methods, aliquots were run on PAGE at low pH, either the gel was stained with Coomassie blue (lanes a to d) or proteins were transferred to polyvinylidene difluoride paper, and the paper was stained with Coomassie blue (lane e). The samples (and amounts) of redissolved extract run in the various lanes were as follows: a and e, acetic acid extract of spores (20 μl); b, acetic acid extract of growing cells (20 μl); c, HCl extract of spores (20 μl); and d, HCl extract of growing cells (5 μl). The numbers adjacent to lane e denote protein bands present in spores but not in growing cells and are the numbers of the bands analyzed in Table 2.

Band 4 gave two different protein sequences, one identical to the product of an additional gene, *sspD*, coding for a minor α/β-type SASP (SspD), with the other sequence derived from an ORF encoded by a gene of unknown function termed *ysfA*. All other bands gave single unambiguous amino acid sequences. Two were products of additional ORFs of unknown function which had been identified in the *B. subtilis* genomic sequencing project, while five were products of small ORFs in intergenic regions which had not previously been identified as coding genes (Table 2). None of these five new proteins exhibits any significant sequence similarity to known proteins (data not shown). Since the new proteins identified are small, acid-soluble proteins that appear unique to spores, with two exceptions we have termed them SASP and their coding genes *ssp* (Table 2). The exceptions are (i) the gene encoding band 2, which had been termed *tlp*, based on the encoded protein's homology to thioredoxin; and (ii) the gene encoding band 8, termed *cotK*, for which there is some evidence that the encoded protein is a spore coat protein (12).

All of the proteins identified in this study are small, 34 to 83 amino acids in length (Table 2), as is expected given their acid solubility. Analysis of the levels of the new minor SASP in spores from which the majority of coat proteins were removed, as described in Materials and Methods, showed that none, including CotK and SspG (data not shown), were removed by the extraction procedure used. However, for a number of the new proteins, the great majority (>80%) disappeared after 45 min of spore germination (see Materials and Methods); the proteins that disappeared included SspC, SspD, SspH, SspI, SspK, SspL, SspM, and Tlp, while CotK, SspG, and SspJ did not (<25%) disappear during spore germination (data not shown).

Properties of *sspG* and *sspJ*. While the new SASP described above appeared unique to the spore, we decided to investigate this in more detail by examining the regulation of expression of

TABLE 2. Characterization of minor SASP in *B. subtilis* spores[a]

Protein band[b]	N-terminal sequence	Gene identified in *B. subtilis* genome[c]	No. of amino acid residues[d]	New gene designation
1	SENRHENEENRRDA-	3353125 to 3353268	47	*sspG*
2	TKNQNQYQQPN-	*tlp*[e]	82	*tlp*
3	MNIQRAKEIVES-	*yfjU*	59	*sspH*
4	ASRNKLVVPGVE- and MDLNLRHAVIAN-	*sspD* *ysfA*	63 71	*sspD* *sspI*
5	AQQSRSRSNNNN-[f]	*sspC*	71	*sspC*
6	GFFNKDKGKRSE-	3420667 to 3420530	45	*sspJ*
7	VRNKEKGFPYEN-	927771 to 927622	49	*sspK*
8	VKRKANHVINGM-	*cotK*	47	*cotK*
9	MKKKDKGRLTGG-	2310101 to 2310226	42	*sspL*
10	MKTRPKKAGQQK-	2338912 to 2339013	34	*sspM*

[a] Protein bands were generated and sequenced as described in Materials and Methods.
[b] As labeled in Fig. 1, lane e.
[c] Gene names are as in the *B. subtilis* genomic sequence available on the World Wide Web at www.pasteur.fr/Bio/SubtiList.html. Numbers given are the locations in the genome of regions coding for proteins not previously identified as ORFs. The first and second numbers are those of the first nucleotide of the start codon and the last nucleotide of the coding sequence, respectively.
[d] Residues in the intact protein; the N-terminal methionine of a number of the proteins is removed posttranslationally.
[e] This was the major sequence observed; however, there were also significant amounts of the same sequence but with an N-terminal methionine (~20% of the total) or beginning at the first lysine residue (~30% of the total).
[f] Sequence from reference 46.

two of the genes encoding these proteins. The two we chose for this detailed analysis were *sspG* and *sspJ*.

The *sspG* ORF has 48 codons and is located in the intergenic region between two divergently oriented ORFs encoded by *yurR* and *yurS* (Fig. 2A). The *sspG* gene is preceded by a good ribosome binding site, but there is no obvious transcription terminator after the gene. Since the last nucleotide of the *sspG* stop codon is the first nucleotide of the *yurS* start codon and there is a transcription terminator immediately downstream of *yurS* (18), *sspG* and *yurS* may constitute an operon in which the two genes are translationally coupled. There are 158 bp between the *sspG* start codon and the start codon of the upstream divergently oriented gene *yurR*, which is more than enough space to accommodate a prokaryotic promoter.

The *sspJ* ORF has 46 codons and is located between two divergently oriented ORFs, *yvsG* upstream and *yvsH* downstream (Fig. 2B). The coding region of *sspJ* is preceded by a good ribosome binding site and is followed by a strong potential transcriptional terminator. There are 166 bp between the start codon of *sspJ* and the upstream divergently oriented *yvsG* start codon and 369 bp between the *sspJ*'s terminator and the *yvsH* start codon. Consequently, *sspJ* appears likely to give only a monocistronic transcript.

Expression and regulation of *sspG* and *sspJ* during sporulation. To examine *sspG* and *sspJ* expression, we integrated translational *sspG*- and *sspJ-lacZ* fusions at the *sspG* and *sspJ* loci, respectively, and measured β-galactosidase activity during vegetative growth and sporulation and in dormant spores. No significant expression of either fusion was observed in vegetatively growing cells (data not shown and see below). However, the *sspG-lacZ* fusion was expressed beginning ~4 h after the induction of sporulation, with maximum β-galactosidase specific activity attained ~6 h after induction of sporulation (Fig. 3A and data not shown); the *sspJ-lacZ* fusion was expressed beginning at ~2.5 h after induction of sporulation, with maximum specific activity attained ~4 h after induction of sporulation (Fig. 4A). No detectable β-galactosidase activity was found in purified spores of the *sspG-lacZ* fusion, but ~100% of *sspJ*-driven β-galactosidase was found in purified spores (data not shown). These data indicate that both *sspG* and *sspJ* are sporulation-specific genes which are likely expressed in the mother cell and the forespore compartment, respectively, of the sporulating cell.

To analyze the genetic dependence of *sspG* expression further, we examined the expression of the *sspG-lacZ* fusion in different *spo* mutant backgrounds. Mutations in all genes coding for sporulation-specific RNA polymerase sigma factors (*spoIIAC* [σ^F], *spoIIGB* [σ^E], *spoIIIG* [σ^G], and *spoIVCB* [σ^K]) abolished expression of the *sspG-lacZ* fusion (Fig. 3A and data not shown). These data indicate that *sspG* expression likely depends on the sigma factor which is last in the sporulation regulatory cascade, the late mother cell-specific sigma factor σ^K, encoded in part by *spoIVCB*. Some σ^K-dependent genes require the transcriptional regulator GerE for their transcription (10, 32); consequently, we also introduced the *sspG-lacZ* fusion into a *gerE* mutant and again measured β-galactosidase activity during sporulation (Fig. 3B). Since the expression of the *sspG-lacZ* fusion was abolished in the *gerE* mutant, this indicates that GerE is necessary to activate *sspG* expression, thus placing *sspG* in the last temporal class of mother cell-specific genes, those dependent on both σ^K and GerE. Indeed, *sspG* expression is switched on 1 h later in sporulation than is the expression of *cotA*, a gene requiring only σ^K for its expression (Fig. 3B) (10, 34).

In contrast to the results with *sspG*, a mutation in *spoIVCB* did not block *sspJ-lacZ* expression during sporulation (Fig. 4A). However, a mutation in *spoIIIG*, which codes for the late forespore-specific sigma factor σ^G, decreased the level of *sspJ*-

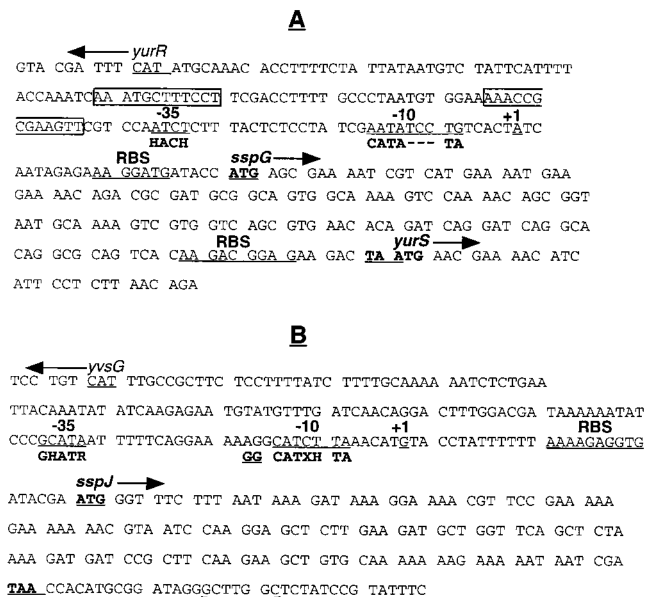

FIG. 2. Nucleotide sequence of the *sspG-yurS* (A) and *sspJ* (B) regions. (A) The sequence shown includes nucleotides 3352955 to 3353300 in the *B. subtilis* genome. The *sspG* start and stop codons are in bold and underlined. The start codon for *yurR* is underlined, and that for *yurS* is in bold. Sequences corresponding to −10 and −35 promoter elements and the *sspG* and *yurS* ribosome binding sites (RBS) as well as those corresponding to the start of transcription of *sspG-yurS* (+1) are underlined. The consensus −35 and −10 sequences for σ^K-dependent promoters (10, 48) are shown in bold below these elements in the *sspG* sequence; the abbreviation used in the −35 consensus sequence is H for A or C. The boxed residues denote putative GerE binding sites (32); one is from positions 3353061 to 3353050 on the strand transcribed to give *sspG*, and the other is from positions 3353013 to 3353024 on the nontranscribed strand. (B) The sequence shown includes nucleotides 3420842 to 3420491 in the *B. subtilis* genome (note that the direction of transcription of *sspJ* is counterclockwise). The *sspJ* start and stop codons are in bold and underlined; the start codon for *yvsG* is underlined. Sequences corresponding to −10 and −35 promoter elements and the *sspJ* ribosome binding site (RBS) as well as those corresponding to the start of transcription (+1) are underlined. The consensus −10 and −35 sequences for σ^G-dependent promoters (10) are shown in bold below these elements in the *sspJ* sequence. The designations in the consensus sequence are H for A or C, R for A or G, and X for A or T. The two underlined G residues upstream of the −10 consensus sequence show the position of the two G residues in promoters recognized primarily by σ^F (44). The apposed arrows denote the putative transcriptional terminator of *sspJ*.

driven *lacZ* expression to only ~5% of that of the wild-type level (Fig. 4B), and a mutation in the *spoIIAC* gene, which codes for the early forespore-specific sigma factor σ^F, essentially abolished *sspJ-lacZ* expression (Fig. 4B). Since σ^F is required for synthesis of σ^G (10), these data indicate that *sspJ* is a forespore-specific gene which is transcribed primarily by $E\sigma^G$ and to a very small extent by $E\sigma^F$. The level of expression of the *sspJ-lacZ* fusion in a *spoIIGB* mutant lacking the mother cell-specific σ^E was higher than in a σ^G mutant (Fig. 4B), as was observed previously for some other genes dependent at least in part on σ^F (17, 21–23).

To provide additional evidence that σ^K is able to direct the transcription of *sspG*, we used *B. subtilis* IB502, which contains the *sspG-lacZ* fusion and a chromosomal copy of the structural gene for the mature form of σ^K under the control of the IPTG-inducible P*spac*. While vegetatively growing cells of this strain had no β-galactosidase activity, upon IPTG induction of σ^K synthesis, a significant increase in β-galactosidase activity was observed starting ~1 h after addition of IPTG (Fig. 5A). In a parallel experiment, with a similar strain containing a *cotA-lacZ* fusion, β-galactosidase activity increased immediately after addition of IPTG (data not shown). This difference is likely due to the requirement for both σ^K and GerE for *sspG* expression, in contrast to *cotA*, which needs only σ^K. The 1-h delay in β-galactosidase synthesis from the *sspG-lacZ* fusion after the addition of IPTG is presumably the time needed for the σ^K-dependent *gerE* gene to be expressed and the GerE protein to accumulate to a level sufficient to stimulate *sspG* expression.

To prove conclusively that σ^G and, to a lesser extent, σ^F are able to direct the transcription of *sspJ*, we introduced plasmid pSDA4 (41), which contains the structural gene for σ^F under P*spac* control into the strain containing the *sspJ-lacZ* fusion as well as a mutation in *spoIIIG*. While vegetatively growing cells of this strain had no β-galactosidase activity, upon induction of σ^F synthesis with IPTG, a significant increase in β-galactosidase activity was observed (Fig. 5B), showing that σ^F was able to direct some expression of *sspJ-lacZ*. However, vegetative cells containing plasmid pDG298 (45) carrying *spoIIIG* under P*spac* control gave more than 10-fold-higher expression of the *sspJ-lacZ* fusion upon induction of σ^G synthesis (Fig. 5C). Therefore, we conclude that *sspJ* is transcribed primarily by

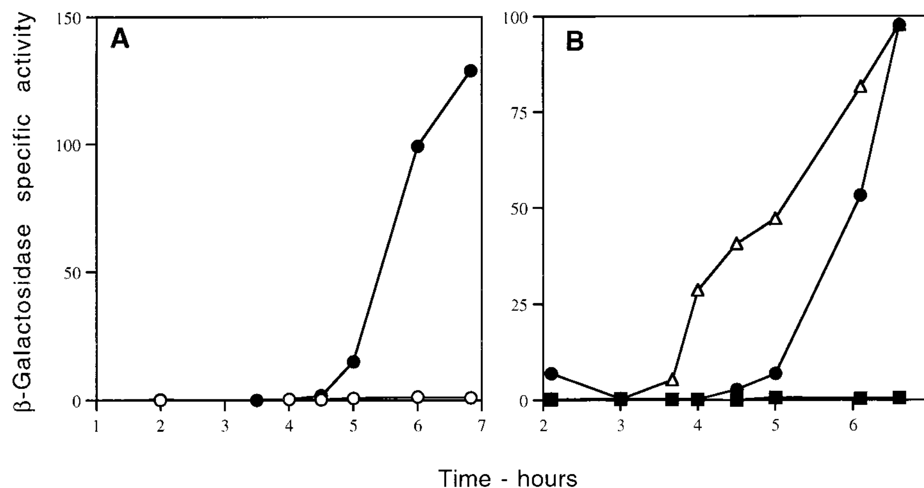

FIG. 3. Expression of the translational *sspG-lacZ* fusion in various *spo* mutants. Strains with a PY79 background (A) and a CU267 background (B) were sporulated by the resuspension method, and β-galactosidase was assayed as described in Materials and Methods. Time 0 is when sporulation was initiated. The symbols used for the various strains are as follows. (A) ●, IB470 (spo⁺); ○, IB469 (*spoIVCB*), (B) ●, IB492 (spo⁺); ■, IB494 (*gerE36*); △, IB498 (this strain does not contain an *sspG-lacZ* fusion but rather has a *cotA-lacZ* fusion in a spo⁺ background).

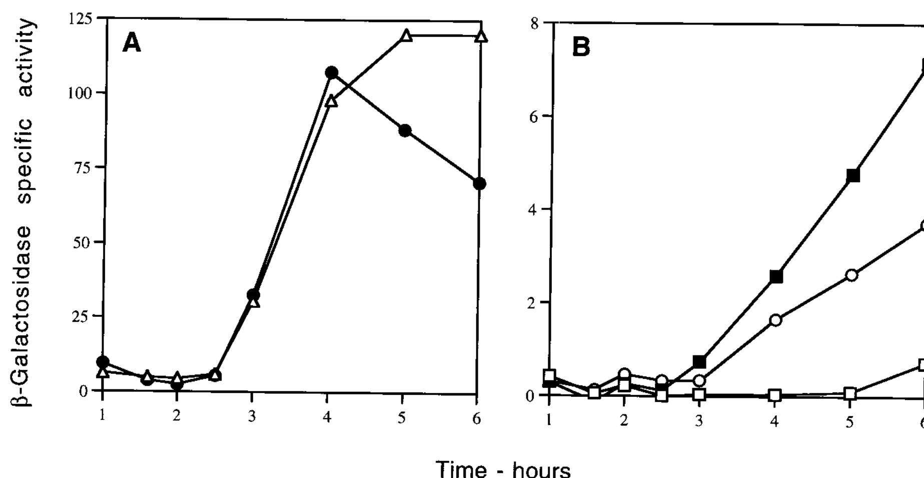

FIG. 4. Expression of the translational *sspJ-lacZ* fusion in various *spo* mutants. Strains with a PY79 background were sporulated by the resuspension method, and β-galactosidase was assayed as described in Materials and Methods. Time 0 is when sporulation was initiated. The symbols used for the various strains are as follows. (A) ●, IB475 (spo$^+$); △, IB474 (*spoIVCB*), (B) □, IB471 (*spoIIAC*); ■, IB472 (*spoIIGB*); ○, IB473 (*spoIIIG*).

Eσ^G but can also be recognized to a small extent by σ^F. However, it is unclear whether this σ^F-dependent expression is of any functional significance.

Localization of the *sspG-yurS* and *sspJ* promoters. To localize the *sspG* promoter and to determine if *yurS* is also transcribed from the same promoter, we carried out primer extension analysis with RNA from sporulating cells of strain IB464 containing the translational *sspG-lacZ* fusion at the *sspG* locus. Three different primers were used for this analysis; one was annealed to the *lacZ* portion of *sspG-lacZ* mRNA, another annealed only to the *sspG* mRNA, and the third was complementary to RNA transcribed from *yurS*. All three primers gave the same transcription start site, indicating that *sspG* and *yurS* are indeed transcribed from the same promoter, with transcription initiating 23 nt upstream of the *sspG* AUG codon, at an A residue (Fig. 2 and 6 and data not shown). Additionally, sequences centered approximately 10 and 35 nt upstream of the transcription start site show good similarity to the −10 and −35 consensus sequences recognized by σ^K, and upstream of the −35 element are several putative GerE binding sites (see Discussion).

To localize the *sspJ* promoter by primer extension analysis, we used RNA from sporulating cells of strain IB465 containing the translational *sspJ-lacZ* fusion at the *sspJ* locus. Two different primers were used for this analysis; one annealed to the *lacZ* portion of *sspJ-lacZ* mRNA, and the other annealed only to the *sspJ* mRNA. Both primers gave the same start site for transcription, as transcription initiates 29 nt upstream of the *sspJ* AUG codon, at a G residue (Fig. 2 and 7 and data not shown). Sequences centered approximately 10 and 35 nucleotides upstream of the transcription start site also show good similarity to the −10 and −35 consensus sequences recognized by both σ^G and σ^F (see Discussion).

Characterization of *sspG-yurS* and *sspJ* null mutants. While it was clear that both SspG and SspJ were spore-specific gene products, it was not clear if these proteins had any function in the spore or any role in sporulation or spore germination. Consequently, we generated and analyzed *sspG* and *sspJ* null

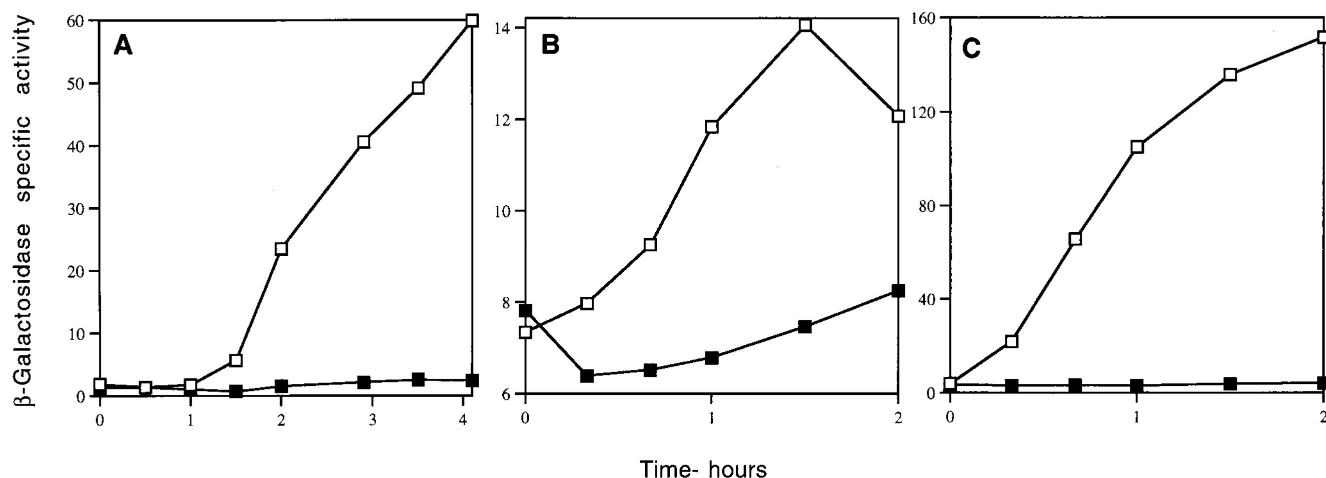

FIG. 5. Induction of expression in vegetative growing cells of *sspG-lacZ* in cells expressing σ^K (A) or *sspJ-lacZ* in cells expressing σ^F (B) or σ^G (C). Cells of strains IB502 (*sspG-lacZ* P*spac*-σ^K) (A), IB480 (*sspJ-lacZ* P*spac*-σ^F) (B), or IB481 (*sspJ-lacZ* P*spac*-σ^G) (C) were grown at 37°C in 2× YT medium. An OD_{600} of 0.25 (time 0 in the figure), the cultures were divided in half, one-half was made 2 mM in IPTG, incubation was continued, and samples were taken from both cultures for assay of β-galactosidase. ■, without IPTG; □, with IPTG. Note the different scales in panels B and C.

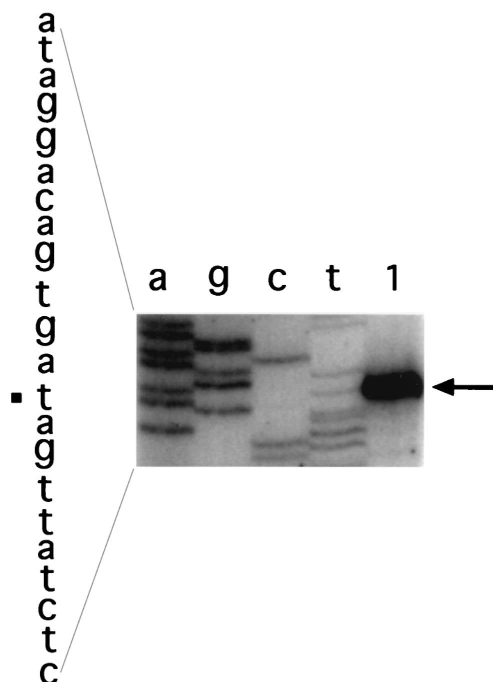

FIG. 6. Primer extension analysis of the start site for transcription of *sspG* and *yurS*. RNA from cells of strain IB464 was isolated 7.5 h into sporulation, and the primer extension product was obtained and analyzed as described in Materials and Methods. The primer used is yurS-140, which anneals only to *yurS*. Lanes a, g, c, and t are DNA sequencing reactions with the same primer and plasmid pIB517; lane 1 is a primer extension reaction with sporulating-cell RNA. The primer extension product is marked with an arrow, and the transcription start site on the *sspG* upstream sequence to the left of the figure is marked with a square. Note that the sequence shown is the complement of the mRNA sequence.

mutants. In the *sspG-yurS* mutant, the entire *sspG* ORF and the first half of the *yurS* ORF were removed, and a Sp^r cassette was inserted; in the *sspJ* mutant the Sp^r cassette was substituted for the *sspJ* ORF. To confirm that the mutations eliminated synthesis of SspG and SspJ, we transferred each of the mutations into a strain lacking the three major SASP, purified spores of the resultant strains, and extracted and analyzed SASP from these spores by PAGE at low pH. As expected, the bands corresponding to SspG and SspJ were absent from the extracts of the *sspG-yurS* and *sspJ* mutant spores, respectively (data not shown). In an attempt to localize SspG, and possibly YurS, to spore coats, we also extracted coat proteins from wild-type and *sspG yurS* spores by boiling and analyzed the extracts by SDS-PAGE as described in Materials and Methods. However, the coat proteins revealed by this analysis had molecular weights that did not even approximate those of the proteins that were absent from the mutant spores (data not shown).

Despite the absence of SspG (and presumably YurS) or SspJ, mutant strains lacking these proteins sporulated normally, with the kinetics and yield of phase-bright spores being identical to those of the wild-type strain, and the mutant spores had the same resistance to heat and UV radiation as did the wild-type spores (data not shown). The mutant spores exhibited no defect in the initiation of spore germination, as measured by the initial fall in OD of a spore culture following mixing of spores with germinant (data not shown). However, *sspJ* spores did have a slight defect in spore outgrowth in a minimal medium, as they returned to vegetative growth slightly more slowly than did wild-type spores (data not shown); this was observed with two different spore preparations (data not shown). However, *sspG-yurS* spores had no such outgrowth defect (data not shown). We also compared the resistance and germination properties of spores of the *sspG-yurS* $\alpha^-\beta^-\gamma^-$ and *sspJ* $\alpha^-\beta^-\gamma^-$ strains and the parental $\alpha^-\beta^-\gamma^-$ strain. Again, there were no differences in the resistance properties or germination properties of spores of these strains (data not shown). In addition, the outgrowth kinetics of *sspJ* $\alpha^-\beta^-\gamma^-$ spores were identical to those of $\alpha^-\beta^-\gamma^-$ spores (data not shown), although the rate of outgrowth of the latter spores is significantly slower than that of wild-type spores in a minimal medium (9).

DISCUSSION

The identification of 10 new proteins in spores from their amino-terminal sequences confirms that four ORFs identified by sequence analysis of the *B. subtilis* genome (*tlpA*, *yfjU*, *ysfA*, and *cotK*) do indeed code for proteins and also identifies five new coding genes which were not identified as such in the analysis of the genome's sequence. All five of the latter code for quite small proteins, which is presumably why the genes were not initially identified as coding regions. However, the translational initiation codon for all five is ATG, the preferred initiation codon in *B. subtilis*, and these are preceded by sequences with good to reasonable homology to the 3′ end of 16s rRNA, which is presumably a ribosome binding site; all coding sequences terminate with TAA, which is also the preferred termination codon in *B. subtilis* (18). The new proteins, however, appear present in spores at rather low levels. Previous work has shown that the level of SspC, the most prominent minor SASP, is only ~35% of that of SASP-β, indicating that

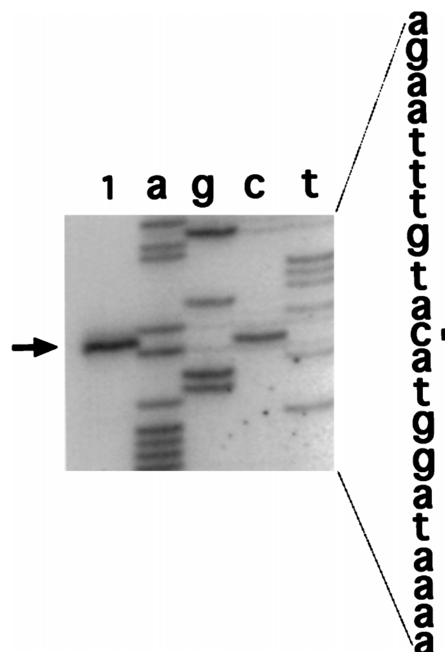

FIG. 7. Primer extension analysis of the *sspJ* transcription start site. RNA from cells of strain IB465 was isolated 5 h into sporulation, and primer extension products were obtained and analyzed as described in Materials and Methods. The primer used is prot3-50, which anneals only to *sspJ* mRNA. Lanes a, g, c, and t are DNA sequencing reactions with the same primer and plasmid pIB460; lane 1 is the primer extension reaction with sporulating-cell RNA. The primer extension product is marked with an arrow, and the transcription start site on the *sspJ* upstream sequence to the right of the figure is marked with a square. Note that the sequence shown is the complement of the mRNA sequence.

SspC is ~0.5% of total spore protein, since SASP-β is ~1.5% (38, 46). Comparison of the intensities of the other minor SASP bands with those of SspC indicates that each, including the minor α/β-type SASP SspD, comprises only 0.05 to 0.2% of total spore protein, assuming that all bands stain equally. Even though these latter values are rather low, given the small size of these proteins, there are clearly a large number of molecules of each of them per spore.

All SASP analyzed previously in *B. subtilis* have been shown to be located in the spore core, and the presence of the β-galactosidase expressed from an *sspJ-lacZ* fusion in the dormant spore, specifically the spore core, suggests that SspJ is also present in the spore core. The situation with SspG, however, is different, as *sspG* is clearly expressed in the mother cell and β-galactosidase from an *sspG-lacZ* fusion is not found in the spore. These data are consistent with SspG being a spore coat protein, but we were unable to detect SspG in spore coat extracts. While SspG might be in the spore cortex, it is not obvious why it should move there. Clearly, further work, possibly using direct protein localization techniques such as immunofluorescence or immunoelectron microscopy, will be needed to definitively establish the location of SspG in the spore.

The identification of SspD in spores brings to four the number of α/β-type SASP identified in *B. subtilis* spores, which is the number of genes encoding α/β-type SASP present in the *B. subtilis* genome (18). Interestingly, one *Bacillus* species, *B. megaterium*, has at least seven genes encoding α/β-type SASP (38, 40), with the additional genes encoding minor proteins. The reason(s) for the loss of multiple genes encoding α/β-type SASP in *B. subtilis* (or their gain in *B. megaterium*) is not clear, but it is known that deletion of genes encoding at least one minor α/β-type SASP has no obvious phenotypic effect in *B. subtilis* (38, 40).

Information available to date indicates that all the minor SASP identified in this work are sporulation-specific proteins. This was shown previously for SspC and SspD, whose coding genes are expressed only in the developing forespore during sporulation under the control of σ^G (26). The two genes studied in this work, *sspG* and *sspJ*, are also sporulation specific, and preliminary work in our laboratory has shown that expression of *tlp*, *sspH*, and *sspL* is also sporulation specific (4). If, as seems likely, given the absence of obvious vegetative cell protein bands comigrating with CotK, SspI, SspK, and SspM, all of these new genes are indeed sporulation specific, a question to ask is what function, if any, is served by these genes or their products. As shown in this work, deletion of *sspG* (and also *yurS*) has no obvious phenotypic effect, while deletion of *sspJ* has only a minor effect on spore outgrowth in a wild-type genetic background. In previous work, we found that loss of another minor small spore-specific protein (but one which is not acid soluble) termed YhcN also causes a slight spore outgrowth defect (3). However, the reason(s) for these outgrowth defects is not clear. The combination of mutations deleting *yhcN*, *sspJ*, and other minor SASP may be responsible for a major phenotype. However, it also seems possible that the situation will be similar to that observed with deletions removing many of the spore coat proteins, as these mutations often have no discernible effect beyond the loss of an individual protein from spores (39). Perhaps these new minor SASP have no function individually, have a function that requires special conditions to become obvious, or have functions that are redundant with one or more other minor SASP. Indeed, the latter is the case for the minor α/β-type SASP SspC and SspF, as loss of these proteins has no noticeable effect on spore resistance (24, 38, 40). However, in a strain lacking the major α/β-type SASP α and β, overexpression of SspC (or SspF) can restore some to much of the resistance to $\alpha^-\beta^-$ spores (24, 38, 40). Perhaps overexpression of some of the new minor SASP in spores (wild type or $\alpha^-\beta^-$) may assist in elucidating their function.

The detailed analysis of the regulation of the two new *ssp* genes analyzed in this work indicates that transcription of the *sspG* operon is directed by $E\sigma^K$ and also requires GerE, while transcription of *sspJ* depends primarily on $E\sigma^G$. Examination of the sequence upstream of the transcription start site of *sspG* reveals sequences with good matches to the consensus −10 and −35 sequences recognized by $E\sigma^K$ (Fig. 2A). Slightly further upstream but still in the intergenic region between *yurR* and *sspG* are also two sequences with a reasonable match to the consensus sequence recognized and bound by GerE (Fig. 2A). Preliminary in vitro transcription of *sspG* templates with partially purified $E\sigma^K$ has also given transcripts of the size expected based on the 5′ end of *sspG* mRNA determined in vivo, and this in vitro transcription by $E\sigma^K$ was strongly stimulated by GerE (14). While the precise location of the GerE binding site(s) in the *sspG* promoter has not yet been determined, the analysis of *sspG* transcription has added another promoter sequence to define the σ^K consensus recognition sequence. Upstream of the *sspJ* transcription start site are also −10 and −35 sequences, which match rather well the consensus −10 and −35 sequences recognized by $E\sigma^G$ (Fig. 2B). The *sspJ* promoter was recognized not only by $E\sigma^G$ but also to a slight degree by $E\sigma^F$, both during sporulation and upon induction of σ^F synthesis in growing cells. The −10 and −35 promoter sequences recognized by σ^G and σ^F are quite similar, with a major difference being the presence of G residues at both positions −14 and −15 in good σ^F promoters (2, 44). The presence of one G residue in these positions in the *sspJ* promoter is consistent with the poor but significant transcription of *sspJ* by $E\sigma^F$ compared to that of $E\sigma^G$ (44).

The transcription of the *sspG yurS* operon by $E\sigma^K$ plus GerE suggests that SspG and YurS may well be spore coat proteins. However, SspG was not removed from spores by solubilization of much of the spore coat, and neither SspG nor YurS was detected in spore coat extracts. Since certainly SspG should be soluble in the solutions used for preparing spore coat extracts, the precise location of this protein in the spore is presently unclear. The product of the *cotK* gene was also previously suggested to be a coat protein (12), but again CotK was not removed from spores by an extraction procedure removing most spore coat proteins. Consequently, the identity of CotK as a coat protein is also problematic at present. However, neither CotK nor SspG was lost upon spore germination, in contrast to SspC, -D, -H, -I, -K, -L, and -M and Tlp, which were lost, presumably by their degradation. However, with the exception of SspC and -D, none of these latter proteins have sequences similar to the somewhat-loose recognition sequence of the spore-specific protease that initiates degradation of both α/β- and γ-type SASP during spore germination (5, 15, 38). Consequently, the identity of the protease that initiates degradation of Tlp and SspH, -I, -K, -L, and M during spore germination is not evident. Clearly, there is much yet to be learned about the metabolism and function of these minor spore proteins.

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