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Cloning of a Small, Acid-Soluble Spore Protein Gene from *Bacillus subtilis* and Determination of Its Complete Nucleotide Sequence

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The first *Bacillus subtilis* small, acid-soluble spore protein (SASP) gene has been cloned by using previously cloned *B. megaterium* SASP genes as DNA-DNA hybridization probes. Determination of the DNA sequence of the *B. subtilis* SASP gene showed that it codes for a 72-residue protein (termed SASP-1) containing a single spore protease cleavage site as well as other sequences conserved in *Bacillus megaterium* SASPs A, C, C-1, C-2, and C-3. The *B. subtilis* SASP-1 genes's coding sequence is preceded by a potential *Bacillus* ribosome-binding site, and is followed by a sequence that could form a stem-and-loop structure characteristic of transcription termination sites. Upstream from the coding sequence there are no obvious homologies with other *B. subtilis* sporulation genes, but similarities with *B. megaterium* SASP genes are evident. SASP-1 mRNA (290 bases long) is absent from vegetative cells, but appears midway in sporulation and then disappears. The cloned SASP-1 gene hybridizes to three bands other than the SASP-1 gene itself in *Eco*RI or *Hin*dIII digests of *B. subtilis* DNA. Presumably these other bands represent SASP genes related to the SASP-1 gene, and we have been able to detect at least three such proteins in *B. subtilis* spores.

During the first minutes of germination of spores of various *Bacillus* species, 10 to 20% of the dormant spore's protein is degraded to amino acids (20). The proteins degraded in this process are a group of small, acid-soluble spore proteins (SASP) synthesized only during sporulation under transcriptional control (22). In *Bacillus megaterium*, eight different SASP have been identified, and genes for two additional SASP have been isolated and sequenced, giving a minimum number of 10 (3, 20–22; E. R. Fliss and P. Setlow, submitted for publication) SASP genes in *B. megaterium*. This SASP multigene family appears to be divided into a number of subfamilies of very closely related species. Thus, 5 of the 10 *B. megaterium* SASP genes (A, C, C-1, C-2, and C-3) exhibit at least 65% amino acid sequence identity (22; E. R. Fliss and P. Setlow, Gene, in press), and for some pairs there is greater than 95% identity (Fliss and Setlow, submitted for publication). In contrast, the other five SASP (B, D, E, F, and G) are more distantly related to the latter group, based on immunological and primary sequence analysis (20, 23). Since this is the first divergent multigene family identified in procaryotes, it is of obvious interest to determine the organization of SASP genes on the *Bacillus* chromosome. Such knowledge would further allow selected mutagenesis or elimination (or both) of individual SASP genes and thus assessment of their function. Although limited chromosome mapping studies have been carried out in *B. megaterium* (28), the obvious *Bacillus* species in which to map genes is *Bacillus subtilis*. In addition, analysis of SASP genes in a *Bacillus* species other than *B. megaterium* may prove fruitful, since it has been suggested that SASP genes evolve rapidly (29). Consequently in this communication we report the cloning and complete nucleotide sequence of an SASP gene from *B. subtilis*.

MATERIALS AND METHODS

Bacterial and phage strains and isolation of nucleic acid. The bacterial strains used in this work were as follows: *B. subtilis* 168 (trpC2) (obtained from D. J. Tipper), *Escherichia coli* AD5230 (F^- *his*$^-$ *ilv*$^-$ *galK*$^+$*T*$^+$*E*$^+$*OP*::IS2 p_R *c*I857 *N7 N53 int6*) containing plasmid p81/16, which contains the *B. megaterium* protein C gene (2); *E. coli* JM103 [Δ(*lac-pro*) *thi*$^-$ *rpsL supE endA sbcB hsdR*$^-$ F′ *traD36 proAB lacI*^{q} *ZΔM15*], obtained from Bethesda Research Laboratories (13); *E. coli* DP50 [F^- *tonA53 dapD8 lacY1 glnV44* Δ(*gal-uvrB*) *47* λ$^-$ *tyrT58 gyrA29* Δ(*thyA57*) *hsd-53*], obtained from J. A. Hoch (6); and *E. coli* AD7068 (F^- *his ilv*$^-$ *nadA*::Tn*10* *galK*$^-$*T*$^+$*E*$^+$*OP*::IS*1* o_L p_L ΔBam N^+ *c*I857 ΔH1 *bio*$^-$), obtained from A. Das (3). The latter strain carries a defective λ prophage containing the t^s*c*I857 gene; when shifted to 42°C, it produces λ N protein. The plasmid used was pMC13(3), which carries an ampicillin resistance gene and contains a *lac* promoter adjacent to a *nut* site with a single *Eco*RI site downstream from the *lac* promoter. DNA cloned into the *Eco*RI site of pMC13 will be transcribed from the *lac* promoter, and in an N^+ host (AD7068 at 42°C) transcription termination is suppressed. In addition, further downstream from the *Eco*RI site there is a promoterless *galK* gene. Consequently, in a *galK*$^-$ host (AD7068) transcription of *galK* from the lac promoter can readily be measured. The M13 phage used in this work were Mp8, Mp9, Mp10, and Mp11 (13) obtained from Bethesda Research Laboratories, and phage Mp8 with the 600-base-pair *Eco*RI-*Msp*I fragment containing the *B. megaterium* protein C-3 gene's coding sequence inserted in the *Hin*cII site (Fliss and Setlow, in press). The library of *Eco*RI fragments of *B. subtilis* DNA in the λ phage Charon 4A was obtained from J. A. Hoch (6).

For DNA isolation, *B. subtilis* was grown in L-broth plus 0.5% glucose to the late-log phase, and cells were harvested and DNA was extracted and purified as described previously (2). *B. subtilis* DNA was further purified by CsCl gradient centrifugation. Plasmid-containing strains were grown overnight at 30°C in L-broth plus glucose and ampicillin (50 μg/ml) for plasmid DNA isolation and purification as previously described (2). *E. coli* JM103 was grown and transfected with M13 phage derivatives, phage were grown, phage-containing inserts were identified, and single-stranded phage DNA for DNA sequencing as well as phage replicative-form

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DNA were isolated and purified as described by Messing (13). Charon 4A derivatives were grown on *E. coli* DP50, and phage were grown and purified as described by Davis et al. (4).

DNA fragments were separated by agarose gel electrophoresis, and individual fragments were isolated as previously described (2, 3). The 0.7-kilobase (kb) *Bgl*II-*Hae*III fragment of plasmid p81/16 containing the *B. megaterium* protein C gene's coding sequence was isolated as described previously (3). Peroxidase-conjugated goat anti-rabbit γ-globulin was obtained from Cappel Laboratories, and normal goat serum was from GIBCO Laboratories.

B. subtilis was grown and sporulated in 2× SG medium, samples (30 μl) were harvested, lyophilized, and disrupted, and RNA was extracted as previously described (5, 9). Parallel samples were also taken for analysis of dipicolinic acid (5).

Enzymes and reagents. Restriction enzymes were obtained from either New England Biolabs or Bethesda Research Laboratories and were used according to the directions of the suppliers. Phage T4 DNA ligase was obtained from New England Biolabs. Deoxynucleotide triphosphates and dideoxynucleotide triphosphates were obtained from New England Biolabs; all other enzymes and reagents for DNA sequencing by the chain termination method (18) were obtained from Bethesda Research Laboratories. [α-^{32}P]dATP (600 Ci/mmol) was obtained from New England Nuclear Corp.

B. subtilis spores were prepared by growth in 2× SG medium, and total SASP was isolated by dry rupture followed by HCl extraction as previously described (12). SASPs from 1 liter of culture were further purified by column chromotography on carboxymethyl-cellulose as described by Johnson and Tipper (11), and the peak containing SASP α plus β (*B. subtilis* SASP immunologically related to *B. megaterium* SASP A, C, and C-3 [3]) was isolated. The α plus β proteins were cross-linked with glutaraldehyde, and antiserum against these proteins was raised in rabbits by using the injection regimen described previously for preparation of antisera against *B. megaterium* protein A (19). Eight days after the second booster injection, the rabbits were bled by cardiac puncture, and the γ-globulin fraction was isolated as previously described (19).

Analytical methods. Transfer of DNA fragments from agarose gels to nitrocellulose paper was carried out as described by Southern (24). Detection of fragments by DNA-DNA hybridization was carried out under nonrestrictive hybridization conditions (55°C) as described previously (3). Transfer of phage from plaques to nitrocellulose was carried out as described by Benton and Davis (1), and DNA-DNA hybridization was carried out as described above. The following three probes were used in hybridization experiments: (i) a *B. megaterium* C gene probe, a 0.7-kb *Bgl*II-*Hae*III fragment containing the coding sequence of the *B. megaterium* protein C gene (8); (ii) a *B. subtilis* SASP-1 gene probe, a 0.6-kb *Eco*RI-*Hin*dIII fragment containing the coding sequence of the *B. subtilis* SASP-1 gene (see Fig. 5); and (iii) a *B. megaterium* C-3 gene probe, single stranded DNA from an Mp8 phage that contained the 0.6-kb *Eco*RI-*Msp*I fragment containing the coding sequence of the *B. megaterium* protein C-3 gene (Fliss and Setlow, in press). The *B. megaterium* C gene and *B. subtilis* SASP-1 gene probes were labeled by nick translation (16) and the *B. megaterium* protein C-3 gene probe was labeled by carrying out a DNA sequencing reaction without any dideoxynucleotide triphosphate.

B. subtilis RNA was treated with glyoxal, samples (6 μg) were run on a 2% agarose gel with glyoxal-treated, *Hae*III-cut φX174 DNA as markers, the nucleic acid was transferred to nitrocellulose paper, and the paper was baked as described by Thomas (25). Hybridization of these Northern blots to the SASP-1 gene probe was carried out as previously described (25; Fliss and Setlow, submitted for publication). DNA sequencing by the chain termination method (18) was carried out essentially as described by Messing (13).

Acrylamide slab gel electrophoresis at low pH was carried out as described by Reisfield et al. (15), proteins were transferred to nitrocellulose paper in 1% acetic acid as described by Towbin et al. (27), and the paper was baked at 80°C for 1 h as described previously (9). *B. subtilis* α and β protein antigens were detected by using anti-α/β-protein γ-globulin (1/400 dilution) and horseradish peroxidase-coupled goat anti-rabbit γ-globulin as described previously (9).

For analysis of SASP production, *E. coli* clones were grown at 30°C in tryptone (50 ml) plus ampicillin (50 μg/ml) as described previously (3). When cultures reached an optical density at 600 nm of 0.5, the culture was shifted to 42°C for 2 h. The culture was then harvested by centrifugation (5 min; 10,000 × *g*), and the pellet was frozen and lyophilized. The dry cells were ruptured and extracted with 2 N HCl, and the supernatant fluid was dialyzed and lyophilized as described previously (3). The dry residue was dissolved in a small volume of 6 M urea, and samples were run on acrylamide slab gels as described above; either the gels were stained with Coomassie brilliant blue, or the proteins on the gel were transferred to nitrocellulose and the nitrocellulose was probed with anti-α/β-SASP antiserum as described above.

RESULTS

Detection and cloning of *B. subtilis* SASP-1 gene. Previous work from our laboratory showed that the *B. megaterium* protein C gene probe hybridized strongly to four *Eco*RI fragments of *B. megaterium* DNA (3). One of these fragments was the protein C gene itself, whereas the other three were subsequently shown to be other, closely related SASP genes (3; Fliss and Setlow, submitted for publication). Given this result, it seemed possible that *B. subtilis* SASP genes also might hybridize significantly with a *B. megaterium* SASP gene probe. Indeed, the *B. megaterium* protein C gene probe hybridized significantly to four *Eco*RI fragments of *B. subtilis* DNA (Fig. 1, lanes 1a and 1b). Screening of a library of *Eco*RI fragments of *B. subtilis* DNA in the λ vector Charon 4A also gave a number of hybridizing plaques (data not shown). DNA was isolated from 100 hybridizing plaques and digested with *Eco*RI. All phage examined gave an identical restriction pattern (Fig. 1, lane 2a) or a subset of this pattern, and the only band hybridizing to the *B. megaterium* protein C gene probe was at 3.2 kb (Fig. 1, lane 2b). The inability to find the other hybridizing bands in the Charon 4A λ library was disappointing; however, the absence of some genes from this library has been previously reported (6).

Subcloning of *B. subtilis* SASP-1 gene. The 3.2-kb *B. subtilis* *Eco*RI fragment was isolated and ligated with the expression vector pMC13 cut with *Eco*RI, and the ligation mix was used to transform *E. coli* AD7068 to ampicillin resistance. Colonies were screened for plasmids carrying inserts, and plasmids were identified carrying the *B. subtilis* 3.2-kb fragment in both orientations relative to the plasmid's *lac* promoter. However, under the conditions where the *lac*

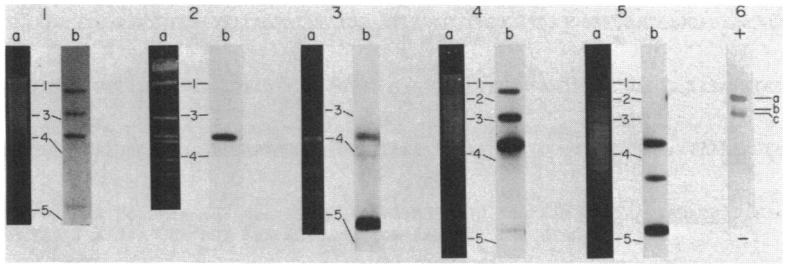

FIG. 1. Southern blots of chromosomal, phage, and plasmid DNAs and Western blot of *B. subtilis* spore SASPs. DNA samples were isolated, digested, electrophoresed on agarose gels, transferred to nitrocellulose, and hybridized as described in the text. *B. subtilis* spore SASPs were run on a polyacrylamide gel, proteins were transferred to nitrocellulose, and α/β-like SASPs were detected as described in the text. Lanes: 1a and b, *Eco*RI digest of *B. subtilis* chromosomal DNA (3 μg)—(a) stained gel, (b) Southern blot; 2a and b, *Eco*RI digest of phage DNA (1 μg) from a plaque that hybridized to the *B. megaterium* C gene probe—(a) stained gel, (b) Southern blot; 3a and b, *Msp*I digest of purified 3.2-kb fragment (0.4 μg)—(a) stained gel, (b) Southern blot; 4a and b, *Eco*RI digest of *B. subtilis* chromosomal DNA (3 μg)—(a) stained gel, (b) Southern blot; 5a and b, *Hin*dIII digest of *B. subtilis* chromosomal DNA (3 μg)—(a) stained gel, (b) Southern blot; 6, immunoblot of *B. subtilis* SASP (1 μg). Southern blots in lanes 1b, 2b, and 3b were hybridized with the *B. megaterium* C gene probe; blots in lanes 4b and 5b were hybridized with the *B. subtilis* SASP-1 gene probe.

promoter was induced and termination of transcription was suppressed (growth in tryptone and shift to 42°C; see above), no detectable SASP (<0.005% of total protein) was synthesized by strains carrying the 3.2-kb fragment in either orientation, even though the downstream *galK* gene was transcribed and translated (data not shown). However, the *galK* gene was not expressed at 30°C with plasmids carrying the 3.2-kb insert in either orientation, suggesting that at 42°C the transcription of the plasmid's *galK* gene has initiated at the *lac* promoter adjacent to the *nut* site, and thus that the 3.2-kb insert is transcribed at 42°C. Whereas several other *Bacillus* SASP genes (*B. megaterium* C and C-3) have been expressed in this system (2, 3), several others (*B. megaterium* C-1 and C-2) have not (3; Fliss and Setlow, submitted for publication). Consequently, the negative result with the *B. subtilis* SASP-1 gene was not conclusive. Indeed, that the 3.2-kb fragment did contain an SASP gene was strongly suggested by restriction mapping of the 3.2-kb fragment and hybridization of digests to *B. megaterium* protein C and protein C-3 gene probes. The 3.2-kb fragment contained a single *Msp*I site, at which cleavage yielded a 2.4-kb fragment and a 0.8-kb fragment (Fig. 1, lane 3a). Only the 0.8-kb fragment hybridized with the *B. megaterium* protein C gene probe (Fig. 1, lane 3b), and this 0.8-kb fragment also hybridized strongly with the *B. megaterium* protein C-3 gene probe (data not shown). Since the *B. megaterium* protein C and C-3 genes are homologous only in their coding sequences (Fliss and Setlow, in press), this finding strongly suggested that the 0.8-kb region contained a *B. subtilis* SASP coding sequence.

Determination of the nucleotide sequence of the *B. subtilis* protein SASP-1 gene. That the hybridizing region in the 3.2-kb *B. subtilis* *Eco*RI fragment did contain an SASP gene was confirmed by DNA sequence analysis (Fig. 2). Determination of the complete sequence of the SASP-1 gene's coding sequence plus ~600 base pairs of flanking sequence showed that this gene codes for a 72-residue protein, with the coding sequence preceded by a sequence with significant complementarity to the 3′ end of *B. subtilis* 16S rRNA and thus a potential ribosome binding site (Fig. 3; see below). The amino acid sequence of *B. subtilis* SASP-1 predicted from the gene sequence exhibits a number of the features found to be conserved in the related SASP of *B. megaterium*. In particular the amino acid sequence from residues 22 to 41 is very similar to that in five *B. megaterium* SASPs (Fig. 4a). This region encompasses the site of *B. megaterium* SASP cleavage by the *B. megaterium* sequence-specific spore protease, which initiates SASP hydrolysis during spore germination (20). Since the *B. subtilis* SASPs α and β are cleaved by the *B. megaterium* spore protease (11), it is perhaps not surprising that this region is conserved. In addition, residues 48 to 60 in *B. subtilis* SASP-1 are conserved exactly in all five related *B. megaterium* SASPs (Fig. 4b).

Expression of *B. subtilis* SASP-1 gene during sporulation. Comparison of the amino acid sequence and composition of SASP-1 with the data available for the major *B. subtilis* SASPs α, β, and γ (11, 29) indicates that SASP-1 is not one of these species. An obvious question then becomes, is the SASP-1 gene expressed and, if so, when? To answer this question, we hybridized an SASP-1 gene probe to Northern blots of total *B. subtilis* RNA isolated at various times during growth and sporulation (Fig. 5 and 6). Only a single hybridizing RNA band was detected, which appeared midway in sporulation and then disappeared (Fig. 5 and 6); the size of this RNA was 290 bases. Since the hybridization conditions used in this experiment have been shown to give little, if any, cross-hybridization between SASP gene probes and mRNA from different SASP genes (Fliss and Setlow, submitted for publication), these data indicate that the SASP-1 gene is expressed, and in the time period when other SASP genes have been found to be expressed (5, 9, 11; Fliss and Setlow, submitted for publication).

Presence of multiple SASP genes in *B. subtilis*. With the proof that the 3.2-kb fragment we had cloned did, in fact, contain an SASP gene, we could now use this gene as a

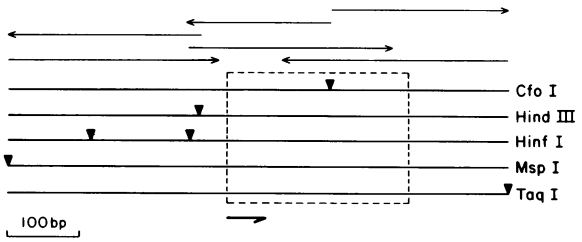

FIG. 2. Sequencing strategy for the *B. subtilis* SASP-1 gene. The region enclosed in the dashed lines is the SASP-1 gene's coding sequence, and the arrow below it gives the direction of transcription. Restriction sites are noted by inverted solid triangles; horizontal arrows represent the areas sequenced. The *Taq*I site at the right hand end of the fragment is within 150 base pairs of an *Eco*RI site at the end of the cloned 3.2-kb *Eco*RI fragment.

FIG. 3. Nucleotide sequence of the *B. subtilis* SASP-1 gene's coding and flanking sequence and the predicted amino acid sequence of SASP-1. The singly underlined bases from positions 243 to 253 show good complementarity to the 3' end of the 16S rRNA of *B. subtilis*. The two regions that are doubly underlined can base pair with each other and may be a transcription termination signal. Dots below nucleotides are positioned every 10 base pairs.

a. Spore Protease Cleavage Site

SASP	22						↓						41
B. subtilis-1	ser	ala	ile	glu	gln	met	lys	leu	glu	ile	ala	ser	glu phe gly val gln leu gly ala
B. megaterium-A	14 ala		asp			tyr					asn		pro
B. megaterium-C	23 ala		asp			phe					asn		pro
B. megaterium-C-1	19 gln		asp			tyr					asn		
B. megaterium-C-2	19 gln		asp			tyr					asn		
B. megaterium-C-3	14 gln	phe	leu	asp	tyr	tyr			gln		thr		ser

b. Second Conserved Peptide

	48										60
B. subtilis-1	asn	gly	ser	val	gly	gly	glu	ile	thr	lys	arg leu val

residues in B. megaterium SASP identical to residues 48 to 60 in B. subtilis SASP-1

B. megaterium-A residues 39-51

B. megaterium-C residues 49-61

B. megaterium-C-1 residues 45-57

B. megaterium-C-2 residues 45-57

B. megaterium-C-3 residues 40-52

FIG. 4. SASP conserved amino acid sequence in (a) the protease cleavage site region and (b) a second more carboxyl-terminal region. The data are taken from the following: references 19 and 23; Fliss and Setlow, in press; Fliss and Setlow, submitted for publication; and Fig. 3. Amino-terminal methionine residues have not been included in numbering the residues. The arrow in a denotes the protease cleavage site (20).

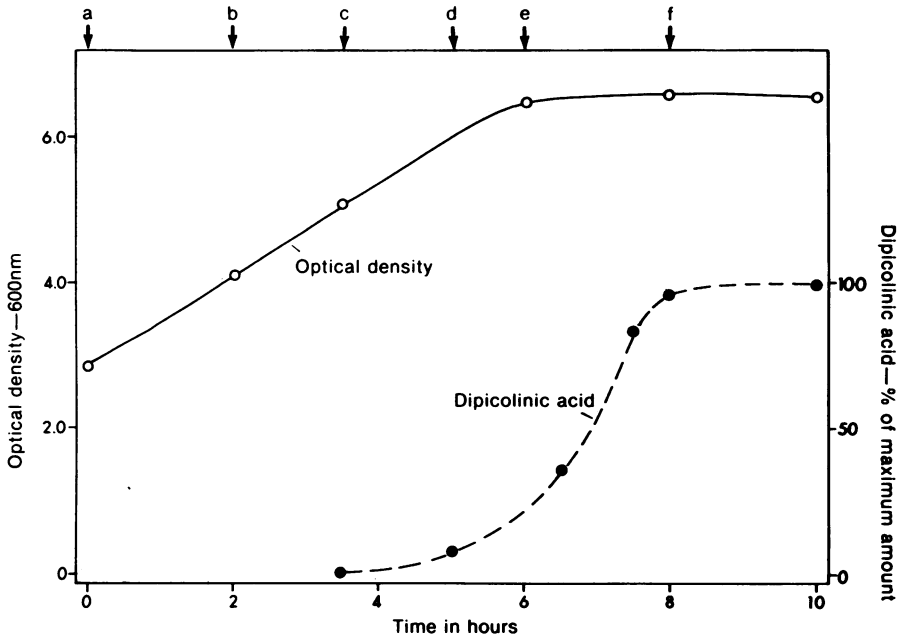

FIG. 5. Growth and sporulation of *B. subtilis*. *B. subtilis* was grown and sporulated as described in the text, and samples were analyzed for dipicolinic acid. At the times indicated by the lettered arrows, samples were harvested, and RNA was isolated.

hybridization probe to detect related *B. subtilis* SASP genes. The *B. subtilis* SASP-1 gene probe hybridized strongly to three fragments in either an *Eco*RI or *Hin*dIII digest of *B. subtilis* DNA (Fig. 1, lanes 4a 4b, 5a, and 5b) and more weakly to at least one band in each digest. The strongest band in the *Eco*RI digest was at 3.2 kb and was the SASP-1 gene itself; presumably the strongest band in the *Hin*dIII digest (0.6 kb) also contains the SASP-1 gene. Strikingly, the sizes of the additional *Eco*RI fragments hybridizing with the *B. subtilis* SASP-1 gene probe were the same as those that hybridized to the *B. megaterium* C gene probe (Fig. 1, lanes 1b and 4b). *B. subtilis* has been previously shown to contain only two SASPs related to *B. megaterium* SASPs A, C, and C-3 (11). However, extended gel electrophoresis of total *B. subtilis* SASP followed by immunoblotting with anti-α/β-protein serum showed the existence of at least three distinct α/β-like SASPs (Fig. 1, lane 6).

DISCUSSION

From the sequence homology between *B. subtilis* SASP-1 and *B. megaterium* SASPs A, C, C-1, C-2, and C-3, it appears clear that we have cloned a related SASP gene from *B. subtilis*. However, it is unclear which SASP gene we have cloned, since the *B. subtilis* SASPs to which the SASP-1 is related (α and β) have not been separated (11), and we have been unable to detect any expression of the SASP-1 gene in *E. coli*—even in an expression vector. However, the SASP-1 gene should be translatable, since the gene's coding sequence is preceded by a sequence with significant complementarity to the 3′ end of the 16S rRNA of *B. subtilis* (Fig. 3), and formation of an mRNA-rRNA duplex in this region is thought to be important for translation (14). When the calculated stability of the potential duplex formed between SASP-1 mRNA and *B. subtilis* 16S rRNA was calculated (26), it was found to be −14.4 kcal (ca. 60.3 kJ) per mol, not as great as with many other *Bacillus* mRNAs (14) and less stable than the potential duplex formed with SASP C-1 or C-2 mRNA (−25.4 kcal [ca. 106.3 kJ] per mol), but significantly more stable than the potential duplex formed with SASP C mRNA (−11.8 kcal [ca. 49.4 kJ] per mol). Since the latter mRNA is translated in *E. coli*, *B. megaterium*, and *B. subtilis* (2, 12), there is no obvious reason why the *B. subtilis* SASP-1 mRNA could not be translated in *E. coli* or *B. subtilis*. Although we have not been able to detect a protein

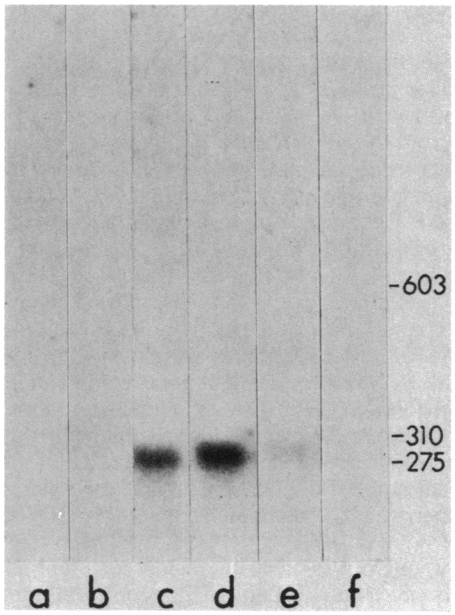

FIG. 6. Appearance of SASP-1 mRNA during sporulation. RNA samples were obtained, treated with glyoxal, run on agarose gel electrophoresis, and transferred to nitrocellulose paper; the paper was hybridized to the SASP-1 gene probe, washed, and autoradiographed. The letters below each lane indicate the time of RNA isolation as given in Fig. 5. The numbered arrows are the lengths, in bases, of glyoxal-denatured DNA markers.

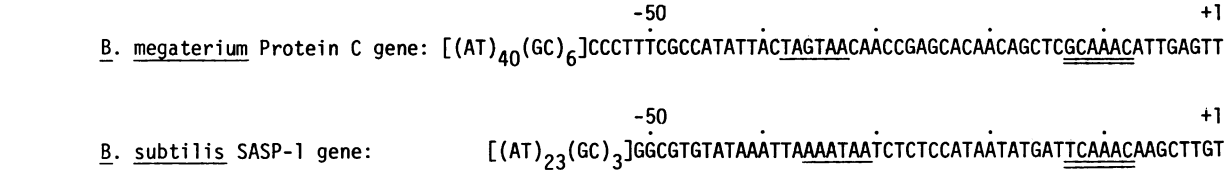

FIG. 7. Comparison of upstream flanking sequences of the *B. megaterium* C gene and *B. subtilis* SASP-1 gene. The sequence upstream from the transcription start point (labeled nucleotide +1) of the *B. megaterium* C gene was taken from reference 8, and the *B. subtilis* SASP-1 sequence from residues 158 to 235 (Fig. 3) was aligned with the *B. megaterium* C gene sequence. The A+T-rich regions are enclosed in brackets.

product from the SASP-1 gene, it does seem clear from Northern blot analysis that the SASP-1 gene is transcribed during sporulation. However, further analysis of individual *B. subtilis* SASP will be needed to prove that SASP-1 mRNA is translated.

Comparison of the amino acid sequence predicted for *B. subtilis* SASP-1 with those of related *B. megaterium* SASPs revealed that the proteins differ most in the amino-terminal and carboxy-terminal sequences, with two large conserved regions. Whereas one of the regions (the protease cleavage site) has a known function, and thus its conservation can be explained, the other sequence has as yet no known function. However, its perfect conservation both within and across species suggests it may have some important function.

As noted previously in analysis of *B. megaterium* SASP genes, the *B. subtilis* SASP-1 gene exhibits no large homology with other SASP genes outside the coding sequence. However, the SASP-1 gene does share one structural feature of other SASP genes—a region just downstream from the coding sequence that could form a stem-loop structure followed by a T-rich region (Fig. 3). As suggested previously, this may represent the site of termination of transcription of this gene (8, 7; Fliss and Setlow, in press).

The *B. subtilis* SASP-1 gene also exhibits no large homology upstream from the coding sequence with other sporulation-specific genes of *B. subtilis* that have been analyzed, but does show a marked homology with conserved features upstream from *B. megaterium* SASP genes—in particular with the regions upstream from the transcription start site of the *B. megaterium* protein C gene (Fig. 7). Although the *B. subtilis* SASP-1 gene's promoter has not yet been localized, if the −10 region of the *B. megaterium* C gene's promoter is aligned with the sequence CAAAC (positions 213 to 217) in the SASP-1 gene, this gives similar (4 of 6) sequences in the −35 regions and large A+T-rich regions (23 of 26 in the *B. subtilis* SASP-1 gene) beginning at about −50. This would predict that *B. subtilis* SASP-1 gene transcription would begin at nucleotide 235. The size measured for SASP-1 mRNA (290 bases) is certainly consistent with this assignment of a transcription start point, since if transcription stops at nucleotide 511 (5 base pairs beyond the region of dyad symmetry [17]), this would give an mRNA 277 bases long.

Certainly, one of the more significant accomplishments in this work is the cloning of an SASP gene from an organism that is readily amenable to genetic analysis. Techniques for mapping cloned *B. subtilis* genes have been developed and applied (7, 10) and should allow mapping of the *B. subtilis* SASP-1 gene. The mapping of this gene, as well as the other related SASP genes of *B. subtilis* identified in this work by Southern blotting, may provide some insight into the organization and evolution of this multigene family.

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