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Genes for *Bacillus megaterium* Small, Acid-Soluble Spore Proteins: Cloning and Nucleotide Sequence of Three Additional Genes from This Multigene Family

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Three genes coding for small, acid-soluble spore proteins (SASP) were cloned from *Bacillus megaterium*, using previously cloned *B. megaterium* SASP genes (SASP-C and -C-3) as DNA-DNA hybridization probes. One gene (SASP-A) codes for the A protein, a previously identified major SASP. The other two (termed genes for SASP-C-4 and -C-5) are extremely similar in much of their nucleotide sequence to the previously cloned *B. megaterium* SASP-C-2 gene. The proteins coded for by all these SASP genes had extensive sequence homology with each other and with those coded for by the *B. megaterium* SASP-C, -C-1, -C-2, and -C-3 genes. Their coding sequences are preceded by strong ribosome-binding sites and are followed by regions of dyad symmetry which presumably are transcription stop sites. The SASP-A, -C-4, and -C-5 genes are expressed in parallel during sporulation, and their transcription start points were localized by the size of the mRNAs produced. The sequences localized 10 and 35 base pairs upstream from the transcription start points show significant homology with the analogous regions of the SASP-C, -C-1, -C-2, and -C-3 genes. The identification of seven closely related SASP genes in *B. megaterium* indicates that the SASP are the products of a very extensive multigene family.

Approximately 20% of the protein of dormant spores of *Bacillus megaterium* is degraded in the first minutes of spore germination, thus providing amino acids for both metabolism and protein synthesis (14). Three small, acid-soluble spore proteins (SASP) make up ~85% of the protein degraded in this process, with a number of other SASP contributing the remaining 15% (14). Two of the major SASP (A and C) are extremely similar in primary sequence, with the third major SASP (B) being more distantly related to SASP-A and -C (16–18). Recent studies of this system at the level of SASP genes have indicated that the situation is much more complex than suggested by studies of the SASP themselves (5). Thus, genes for four different SASP closely related to SASP-A have been cloned and sequenced to date. One gene is that for SASP-C (3, 6), one is for SASP-C-3, a minor spore protein immunologically related to SASP-A and -C (2, 7), and the other two (SASP-C-1 and -C-2 genes) are almost identical in their coding sequence and code for proteins extremely similar in sequence to SASP-A and -C (8). All these genes (as well as the SASP-A gene) are expressed in parallel during sporulation but only within the developing forespore, and SASP synthesis is regulated at the transcriptional level (5, 8).

The SASP genes clearly represent an extensive, coregulated, multigene family, the first such identified in a procaryote. Because of the novelty of this finding, and because a gene for a predominant SASP (SASP-A) had not yet been isolated, we felt it would be valuable to isolate all possible members of the family of genes related to SASP-C and -A. In this communication we report the cloning and sequencing of the SASP-A gene as well as two additional members of this multigene family.

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MATERIALS AND METHODS

Plasmids and bacterial strains and isolation of DNA. The sources of all bacterial and phage strains and plasmids have been described previously (1–8). *B. megaterium* chromosomal DNA was isolated as previously described (3), as was phage DNA (1). Clones containing most plasmids were grown overnight in L broth plus 0.5% glucose with ampicillin (50 μg/ml), chloramphenicol (10 μg/ml), and tetracycline (10 μg/ml) as needed. Clones containing pUC-derived plasmids were grown overnight in 2× YT medium with ampicillin (100 μg/ml) (1). Plasmids were isolated as previously described (3) and purified by CsCl density gradient centrifugation if necessary. *B. megaterium* QMB1551 was grown and sporulated in supplemented nutrient broth, and samples were harvested and RNA was extracted and purified as previously described (4).

Analytical procedures. DNA fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose paper as described by Southern (19). These blots were hybridized under nonrestrictive conditions (55°C) as described previously (2). The probes used were: (i) an SASP-C gene probe, a 0.7-kilobase (kb) SASP-C gene fragment described previously (3, 6) which contains the SASP-C gene coding sequence; (ii) an SASP-C-3 gene fragment, a 0.6-kb *Msp*I-*Eco*RI fragment containing the SASP-C-3 gene coding sequence (7); and (iii) a partial SASP-A gene probe, a 0.9-kb *Eco*RI-*Pvu*II fragment containing part of the SASP-A gene coding sequence (fragment 7; see Fig. 1 and Table 1). Phage DNA in plaques was transferred to nitrocellulose paper, treated, and hybridized to the SASP-C gene probe as described previously (1).

RNA samples were treated with glyoxal and run on 2%

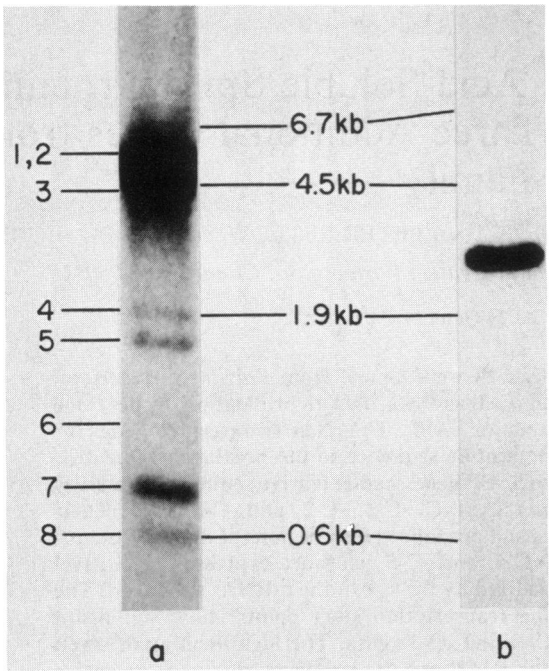

FIG. 1. Southern blot analysis of *B. megaterium* DNA digested with *Eco*RI plus *Pvu*II and hybridized with the SASP-C gene probe (a) or *Cla*I and hybridized with the cloned fragment 7 probe (b). *B. megaterium* DNA was digested to completion with *Eco*RI plus *Pvu*II (a) or *Cla*I (b), samples (2 μg) were run on a 1.2% agarose gel, and the DNA was transferred to nitrocellulose. The blots were hybridized under either nonrestrictive hybridization conditions (55°C [2]) with the SASP-C gene probe (a) or restrictive hybridization conditions (73°C [2]) with the fragment 7 probe (b) and then washed and autoradiographed. The numbered arrows adjacent to lane a designate the fragments tabulated in Table 1; the sizes of marker DNAs run in parallel are given between lanes a and b. In lane a the presence of the 5-kb fragment containing the SASP-C gene (3) obscures the presence of the 4.9-kb fragment containing the SASP-C-1 gene (2) and partially obscures the presence of the 4.5-kb fragment with the SASP-C-2 gene (2). However, their presence was established in other experiments.

agarose gels, RNA was transferred to nitrocellulose, and these Northern blots were hybridized under restrictive conditions, i.e., pH 6.5 and 45°C in 50% formamide–0.75 M NaCl–0.075 M sodium citrate–50 mM sodium phosphate–10 μg of sonicated denatured salmon sperm DNA per ml–0.02% bovine serum albumin–0.02% Ficoll–0.02% polyvinylpyrrolidone as previously described (8). Previous work has shown that under these conditions the SASP-C gene probe does not hybridize to the SASP-C-3 or -C-1 and C-2 mRNAs even though the latter have up to 78% base sequence identity with the SASP-C gene in the 171 base pairs (bp) between the sequences coding for the two most distant conserved amino acids (8). Consequently, under our hybridization conditions SASP gene probes appear to hybridize only with extremely homologous mRNAs. The probes used for these hybridizations were: (i) an SASP-A gene probe, a 0.5-kb *Cla*I-*Hin*dIII fragment containing the SASP-A gene coding sequence (see Fig. 2); (ii) an SASP-C-4 gene probe, the 0.52-kb fragment 8 containing the SASP-C-4 gene coding sequence (see Fig. 3); and (iii) an SASP-C-5 gene probe, a 0.5-kb *Hha*I fragment containing 80% of the SASP-C-5 gene coding sequence (see Fig. 4). These fragments were isolated as previously described (2) and labeled by nick translation (11).

DNA sequence analysis was carried out by either the chain termination method (13) or the chemical cleavage method (10). All restriction sites used in subcloning for sequencing were overlapped, and all sequences reported were determined completely in both directions.

Cloning of SASP genes. *B. megaterium* DNA (50 μg) was digested with *Eco*RI and *Pvu*II and run on a 1.2% agarose gel, and DNA in regions of the gel containing fragments hybridizing to the SASP-C and -C-3 gene probes (0.5 to 1.0 kb; 1.0 to 1.5 kb; and 1.5 to 2.5 kb) was isolated as previously described (2). *Eco*RI linkers were added to the purified DNA, the samples were redigested with *Eco*RI, and linker fragments were removed by spermine precipitation (9). The purified DNA was then ligated with *Eco*RI-cut DNA from λgt10, and the ligation mix was packaged and used to infect *Escherichia coli* POP138. Plaques hybridizing to the SASP-C gene probe were detected, purified, phage grown, and DNA isolated as previously described (1). The cloned *Eco*RI fragments were then recloned in either M13 phage DNA or a pUC plasmid.

The *Cla*I fragment containing the complete SASP-A gene was cloned in plasmid pBR325. *B. megaterium* DNA was cut with *Cla*I, fragments were resolved by agarose gel electrophoresis, and DNA of the appropriate size range (2.2 to 3.5 kb) was isolated (2). This DNA was ligated with *Cla*I-cut pBR325, and the mix was used to transform *E. coli* RR101 to ampicillin and chloramphenicol resistance, but tetracycline sensitivity. Approximately 400 colonies carrying inserts were screened by colony hybridization (2) with the partial SASP-A gene probe; four positive clones were obtained, all of which contained a single 2.7-kb *Cla*I fragment.

RESULTS

Cloning of SASP-A, -C-4, and -C-5 genes. In previous work from this laboratory we reported the cloning of four closely related SASP genes from *B. megaterium* (2, 3). One of these genes coded for SASP-C, a predominant SASP in *B. megaterium*. However, the gene for SASP-A, the most predominant SASP and one very closely related to SASP-C, has not yet been isolated. Southern blot analysis of *Eco*RI-*Pvu*II digests of *B. megaterium* DNA hybridized with the SASP-C gene probe revealed eight hybridizing fragments,

TABLE 1. Sizes of *B. megaterium* DNA fragments containing SASP genes[a]

Gene	Enzyme(s) used to generate fragment	Fragment no.	Fragment size (kb)
SASP-C[b]	*Eco*RI, *Pvu*II	1	5
SASP-C-1[c]	*Eco*RI, *Pvu*II	2	4.9
SASP-C-2[c]	*Eco*RI, *Pvu*II	3	4.5
SASP-A[d]	*Eco*RI, *Pvu*II	4	1.9
SASP-C-5	*Eco*RI, *Pvu*II	5	1.6
SASP-C-3	*Eco*RI, *Pvu*II	6	1.2
SASP-A[d]	*Eco*RI, *Pvu*II	7	0.9
SASP-C-4	*Eco*RI, *Pvu*II	8	0.6
SASP-A[e]	*Cla*I		2.7

[a] The identity of the SASP genes carried by various fragments was established by restriction maps published previously (SASP-C, -C-1, -C-2, and -C-3 genes) (2, 3) or by nucleotide sequence analyses reported in this work. The sizes of the fragments were taken from data in Fig. 1, lane a.

[b] Previous work has shown that this fragment is actually a *Pvu*II-*Pvu*II fragment (3).

[c] Previous work has shown that this fragment is actually an *Eco*RI-*Eco*RI fragment (2).

[d] DNA sequence analysis of these cloned fragments showed that they contained only part of a SASP gene.

[e] This fragment contained the complete SASP-A gene.

FIG. 2. Nucleotide sequence of the SASP-A gene coding and flanking sequences and the predicted amino acid sequence of the SASP-A protein. The underlined bases from positions 198 to 207 show complementarity to the 3' end of the 16S rRNA of *B. megaterium*. The dyad symmetry regions (433 to 444 and 449 to 460) are doubly underlined. Dots below nucleotides are positioned every 10 bases. The vertical arrows labeled 1, 2, 3, and 4 denote cleavage sites for the restriction enzymes *Cla*I, *Pvu*II, *Eco*RI, and *Hin*dIII, respectively. The horizontal arrow (residue 99) gives the postulated transcription start point.

four of which corresponded to the previously cloned SASP-C, -C-1, -C-2, and -C-3 genes (Fig. 1, lane a; Table 1). The other four hybridizing bands appeared likely to represent additional SASP genes, because they also hybridized with the SASP-C-3 gene probe (data not shown). Consequently, all four new fragments were cloned in the phage vector λgt10 and then subcloned in DNA sequencing vectors. DNA sequence analysis of fragments 5 and 8 showed that they contained complete SASP genes but that fragment 7 contained only a part of an SASP gene. Consequently, this fragment was used as a probe against Southern blots of *B. megaterium* DNA digested with various restriction enzymes. The *Cla*I digest (Fig. 1, lane b) was chosen for isolation of the complete SASP gene carried by fragment 7, and the appropriate 2.7-kb *Cla*I fragment was cloned in pBR325 and isolated by colony hybridization (see above). Subsequent sequence analysis of fragment 4 showed that it was the other part of the SASP-A gene carried on fragment 7.

Nucleotide sequence analysis of SASP-A, -C-4, and -C-5

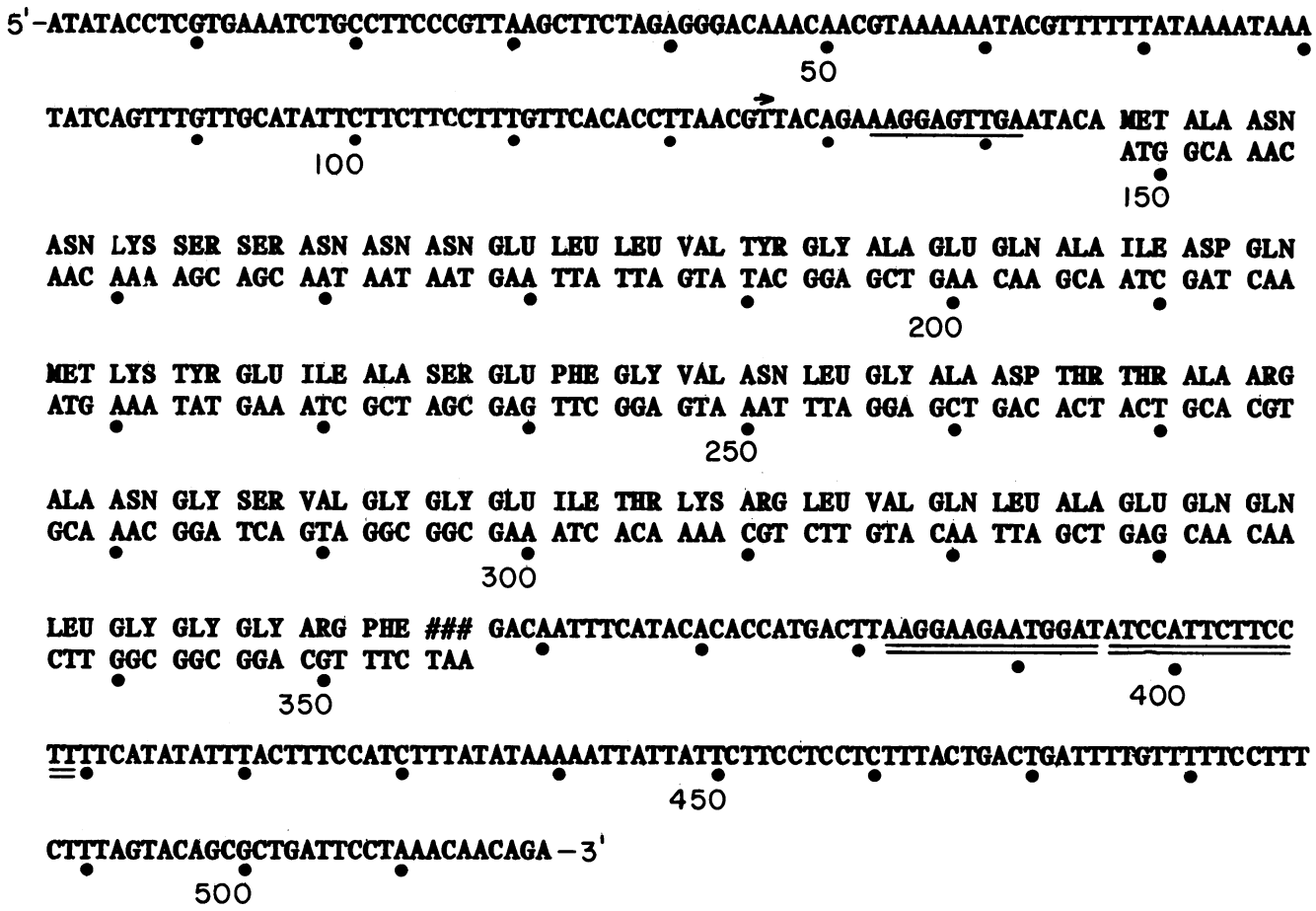

FIG. 3. Nucleotide sequence of the SASP-C-4 gene coding and flanking sequences and the predicted amino acid sequence of the SASP-C-4 protein. The underlined bases from positions 133 to 142 show complementarity to the 3' end of the 16S rRNA of *B. megaterium*. The dyad symmetry regions (382 to 395 and 396 to 409) are doubly underlined. Dots below nucleotides are positioned every 10 bases. The horizontal arrow at residue 126 gives the postulated transcription start point.

genes. With the three new SASP genes in hand, their complete nucleotide sequence was determined (Fig. 2, 3, and 4). The *Cla*I fragment from which fragment 7 was derived was found to code for an SASP whose amino acid sequence is identical to that previously reported for SASP-A (16), except for the amino-terminal methionine which is presumably removed posttranslationally. The other two fragments coded for SASP which were related to SASP-A but differed significantly. Indeed, the SASP-C-4 and -C-5 genes were most similar to the previously cloned SASP-C-2 gene, with only 15 and 34 bp differences in the coding sequences, respectively (8) (data not shown). As has been observed previously with the *B. megaterium* SASP-C, -C-1, -C-2, and -C-3 genes, the proteins coded for showed a high degree of sequence conservation, with the amino acid residues previously found to be conserved in this group of SASP being conserved in SASP-A, -C-4, and -C-5 as well (Fig. 5).

As has been found previously (2), the coding sequences of the three newly cloned SASP genes are preceded by a strong ribosome-binding site (Fig. 2, 3, and 4). In addition, the coding sequences are followed by a region of dyad symmetry which presumably is a transcription stop signal (12).

Expression of SASP-A, -C-4, and -C-5 genes during sporulation. Previous work has shown that the SASP-C and -C-3 and -C-1 and -C-2 genes are transcribed in parallel only during sporulation and generate only monocistronic transcripts (6–8). Studies measuring levels of SASP-A mRNA by in vitro translation of SASP-A have shown that SASP-A mRNA is also synthesized in parallel with SASP-C mRNA (4). This was confirmed by measuring SASP-A mRNA levels by Northern blot analysis of RNAs extracted at various times in sporulation; the size of the SASP-A mRNA was found to be 355 bases (data not shown). Similar analysis of Northern blots with the SASP-C-4 and -C-5 probes indicated that mRNAs for these genes of 290 and 285 bases in size, respectively, were also synthesized in parallel with the SASP-A mRNA (data not shown). While this suggests that both the SASP-C-4 and -C-5 genes are expressed, we cannot conclusively rule out the possibility that the SASP-C-4 and -C-5 probes are hybridizing to the mRNAs for the SASP-C-1 and C-2 genes, both of which are thought to be 290 to 295 bases long, unlike SASP-C and -C-3 mRNAs which are 390 and 335 bases long, respectively (8). However, the SASP-C-5 gene probe does have a minimum of 50 bp mismatches with the putative SASP-C-1 and -C-2 mRNAs (Fig. 4) (8). Since the hybridization of Northern blots was done under restrictive conditions, it certainly seems possible that the SASP-C-5 probe is detecting only SASP-C-5 mRNA.

DISCUSSION

With the cloning of the SASP-A, -C-4, and -C-5 genes, a total of seven closely related SASP genes have been isolated

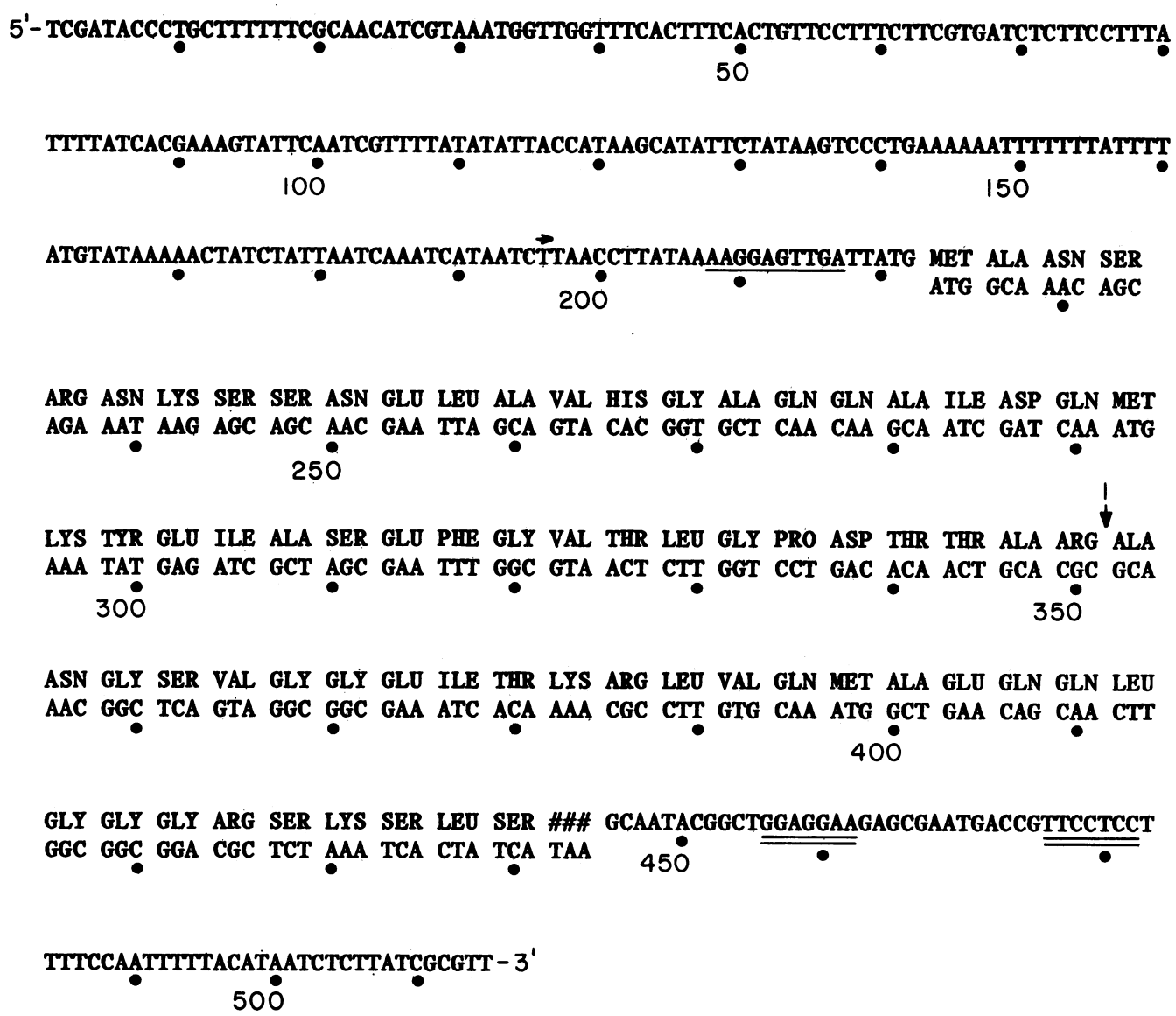

FIG. 4. Nucleotide sequence of the SASP-C-5 gene coding and flanking sequences and the predicted amino acid sequence of the SASP-C-5 protein. The underlined bases from positions 208 to 217 show complementarity to the 3' end of the 16S rRNA of *B. megaterium*. The dyad symmetry regions (456 to 462 and 476 to 482) are doubly underlined. Dots below nucleotides are positioned every 10 bases. The arrow labeled 1 denotes an *Hha*I cleavage site. The fragment used for the SASP-C-5 gene probe encompasses 0.5 kb upstream from this *Hha*I site. The horizontal arrow at residue 196 gives the postulated transcription start point.

from *B. megaterium*. It appears likely that this represents the great majority (if not all) of the members of this SASP gene subfamily, since all *Eco*RI-*Pvu*II DNA fragments hybridizing reasonably well to SASP-C and -C-3 gene probes have now been cloned. However, this group of seven SASP genes is by no means the total SASP gene family, as there are a number of other SASP (B, D, E, F, and G) whose genes have not yet been cloned (14). If each of these proteins is only the predominant representative of a multigene subfamily (as was the case with SASP-A and -C), then the SASP gene superfamily may be extremely large and complex, rivaling the multigene families found in higher organisms.

Strikingly, all of these SASP genes appear to be expressed, at least at the transcriptional level, and all are transcribed in parallel. However, it is clear that different SASP genes are expressed at the translational level in very different amounts, since SASP-A and -C are much more abundant than SASP-C-3, while the protein products of the SASP-C-1, -C-2, -C-4, and -C-5 genes have not yet been detected. While the reason for the difference in level of expression of these genes is not clear, a possible reason for their parallel transcription during sporulation can be adduced when the regions upstream from the putative transcription start sites of these genes are compared. While the transcription start site of the SASP-C gene has been determined precisely by S1 nuclease mapping (6), S1 mapping has not been successful with the other six SASP genes. However, the regions in which transcription of the other six SASP genes begins have been localized from the sizes of the mRNAs produced and assigning the transcription stop points

AMINO ACID SEQUENCES

SASP

A: NH_2-MANT K VAP SAAA S N P AT A M EQQ G K-COOH

C: NH_2-MANYQNASNRNSS K VAP AQAA S N P AT A L EQN G KY-COOH

C-1: NH_2-MANNNSSNN E LVY AEQA S N A TT A L EQQ G GRF-COOH

C-2: NH_2-MANNKSSNN E LVY AEQA S N A TT A L EQQ G GRSKTTL-COOH

Conserved Residues: N L G $\begin{matrix} I \\ L \end{matrix}$ DQ $\begin{matrix} M \\ Y \end{matrix}$ K $\begin{matrix} Y \\ F \end{matrix}$ EIA EFGV LG $\begin{matrix} D \\ E \end{matrix}$ AR NGSVGGEITKRLVQ A L G

C-3: NH_2-MART K LTP VEQF Q T S TA S Q QAH S STQK-COOH

C-4: NH_2-MANNKSSNN E LVY AEQA S N A TT A L EQQ G GRF-COOH

C-5: NH_2-MANSRNKSS E AVH AQQA S T P TT A M EQQ G SKSLS-COOH

FIG. 5. Comparison of primary sequences of SASP-A, -C, -C-1, -C-2, -C-3, -C-4, and -C-5. The data are taken from references 6, 7, and 8 and Fig. 2, 3, and 4, and sequences have been aligned to give maximum homology. Residues in the center are conserved in all seven of these SASP. The spore protease cleavage site (15) is given by the arrow.

SASP Gene

	-50	-40	-30	-20	-10	+1
A:	GCGGTAAATAATGTAGAAACGCTTGAGATGACAGCTTCTTCGGCTACAAATACCG					
C-4:	GTTGCATATTCTTCTTCCTTTGCATATTCTTCTTCCTTTGTTCACACCTTAACGT					
C-5:	CTGAAAAATTTTTTTATTTTATGTATAAAAACTATCTATTAATCAAATCATAATCT					
C:		TAGTAA			GCAAAC	
C-1:		ACGAAT			GGAAAC	
C-2:		ATGTAT			GGAAAC	
C-3:		TTGAAA			GCAAAC	

FIG. 6. Comparison of upstream flanking sequences of SASP-A, -C, -C-1, -C-2, -C-3, -C-4, and -C-5 genes. The sequences are numbered starting with the transcription start point which is designated as +1. Data are taken from references 6, 7, and 8, or in this work the transcription start points (Fig. 2, 3, and 4) were localized from the size of the SASP gene mRNA and assigning the transcription stop point as five bases past the end of the dyad symmetry region (7, 12). With the transcription start point thus localized, the sequences were aligned to give maximum homology in the regions 10 and 35 bases upstream from the transcription start points as previously described (7, 8). These alignments predicted mRNA sizes of 367, 290, and 292 bases for the SASP-A, -C-4, and -C-5 genes, respectively, extremely close to the measured mRNA sizes. Homologous regions 10 and 35 bases upstream from the transcription start points are noted by double and single underlining, respectively.

as 5 bp beyond the regions of dyad symmetry downstream from the coding sequences (7, 8) (Fig. 6). When the DNA sequences upstream from these localized regions are compared with that upstream from the precisely determined transcription start point (SASP-C gene), the sequences can be aligned such that: (i) the predictions of SASP gene mRNA sizes are in excellent agreement with the experimental findings (7, 8) (Fig. 6); and (ii) there are significant homologies among all seven genes (7, 8) (Fig. 6). These homologies are particularly pronounced in the region centered 10 bp upstream (−10 region) from the known (SASP-C) or putative (the other six SASP genes) transcription start sites, with a consensus sequence of $G^{G}_{C}AAAC$. There is less homology in regions centered approximately 35 bp upstream (−35 region) from the transcription start sites, with a possible consensus sequence of $^{TT}_{AA}G^{T}_{A}A^{T}_{A}$. However, the A+T region found to start ~50 bp upstream from the transcription start of the SASP-C, -C-1, -C-2, and -C-3 genes (6–8) is not present in the SASP-A, -C-4, and C-5 genes (Fig. 6). As noted previously, these tentative consensus sequences in the −10 and −35 regions do not match those for other known *Bacillus* RNA polymerases (5, 7). However, their presence is consistent with our suggestion of the coordinate transcription of this multigene family by an as yet unidentified form of RNA polymerase containing a new σ subunit (5).

The identification of seven closely related SASP genes in *B. megaterium*, all of which are coregulated, indicates that this procaryotic organism contains at least one (and perhaps many) extensive divergent multigene families. The presence of such multigene families had been thought to be unique to eucaryotes, but clearly it is not. With this procaryotic system, it should now be possible to analyze the evolution, function, and regulation of this multigene family. This analysis may also provide insight into questions about multigene families in eucaryotic cells.

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