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Cloning of a New Low-Molecular-Weight Spore-Specific Protein Gene from *Bacillus megaterium*

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Three *Eco*RI fragments of *Bacillus megaterium* DNA hybridized only under nonrestrictive conditions on Southern blots to a probe containing the previously cloned gene for protein C, a small, acid-soluble spore protein (SASP) from *B. megaterium*. All three fragments were cloned in *Escherichia coli* cells in plasmid pBR325, and after being transferred to an *E. coli* expression vector, one of the fragments (C-3) directed the synthesis of a new small, acid-soluble spore protein (termed C-3) immunologically related to protein C. As previously observed with the protein C gene, protein C-3 gene expression in *E. coli* required an external promoter and suppression of termination of transcription. Protein C-3 was purified from induced *E. coli* cells, and its immunological properties, electrophoretic mobility, amino acid composition, and amino-terminal sequence were determined. These data indicated that protein C-3 was related, but not identical, to either protein C or the closely related protein A—two of the major small, acid-soluble spore proteins of *B. megaterium*. Detailed examination of acid extracts of *B. megaterium* spores showed that they contained a minor protein which comigrated with C-3 on acrylamide gel electrophoresis at low pH and reacted immunologically like C-3.

The first minutes of spore germination in *Bacillus* species are accompanied by the degradation of a significant percentage (10 to 20%) of dormant spore protein to free amino acids; the amino acids produced are reutilized by the developing spore for protein synthesis and energy metabolism (reviewed in reference 15). The proteins degraded in this process are a group of small, acid-soluble spore proteins (SASPs). The SASPs are synthesized only during sporulation, and their synthesis is regulated at the transcriptional level—very possibly by a mechanism involving positive control (6, 15).

The SASPs have been purified and characterized from a number of *Bacillus* species; the most information is available on the SASPs of *Bacillus megaterium*. Three major SASPs (termed proteins A, B, and C) are found in *B. megaterium* spores, as are a number of minor SASPs (15). The primary sequence of proteins A, B, and C is known; proteins A and C exhibit 85% sequence identity and are closely related immunologically (11, 17, 18), whereas protein B is more distantly related (19). Recently, the structural gene for *B. megaterium* protein C was cloned in *Escherichia coli* by measuring antigen expression with an expression vector, a procedure which should have yielded the protein A gene (4). However, the protein A gene was not detected in this initial work nor in subsequent screening done by a similar approach (B. Setlow and P. Setlow, unpublished data). Consequently, we decided to use the cloned protein C gene as a probe to detect, by DNA-DNA hybridization, what we thought would be a closely related DNA sequence, i.e., that of the protein A gene. In this work we report that although this screening procedure has not yet yielded the protein A gene, it has resulted in the cloning of a new member of the multigene SASP family which is closely related to the protein C and A genes.

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MATERIALS AND METHODS

Bacterial strains, plasmids, proteins, enzymes, and antisera. The bacterial strains and plasmids used in this work are listed in Table 1. All have been described previously (4, 6) except pMC13. This plasmid was derived from pNTK5 by removing its *Eco*RI site by cutting with *Eco*RI, filling in the ends, and religation, followed by conversion of the *Bam*HI site into an *Eco*RI site by filling in the ends of *Bam*HI-cut plasmid, adding *Eco*RI linkers, and religation.

B. megaterium spores, *B. megaterium* proteins A, B, and C, *B. megaterium* spore protease, anti-A protein serum, and anti-B protein serum were prepared as previously described (11–13, 16). Restriction enzymes, T4 DNA ligase, and *E. coli* DNA polymerase were obtained from New England Biolabs or Bethesda Research Laboratories and used according to the directions of the suppliers. Total SASPs of *B. megaterium* were obtained by dry rupture of spores (100 mg) as described previously (12), extraction with 3 ml of cold 2 N HCl (6), dialysis in Spectrapor 3 tubing (molecular weight cut-off, 3,500) against 1% acetic acid, and lyophilization. The dry residue was dissolved in a small volume (100 μl) of 8 M urea before analysis by acrylamide gel electrophoresis.

Analytical procedures. The radioimmunoassay for protein A or C antigen was carried out as previously described, using ^{125}I-labeled protein A as the labeled antigen (11). Proteins were analyzed by acrylamide gel electrophoresis on slab gels at low pH as described by Reisfield et al. (9) and stained with Coomassie brilliant blue. Alternatively, proteins on gels were transferred to nitrocellulose paper, the paper was baked at 80°C for at least 1 h, and the antigens on the paper were detected as described previously (6, 21). DNA fragments were separated by agarose gel electrophoresis, and the fragments were isolated by the method of Dretzen et al. (5). Alternatively, DNA fragments were transferred to nitrocellulose paper (20) and detected by hybridization to a radioactive probe as previously described, but this was done at 55°C to make hybridization less restric-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype	Source or reference
B. megaterium QMB1551	Wild type	H. S. Levinson
E. coli RR101	F⁻ *hsdS1 proA2 leu-6 ara-14 galK2 lacY1 xtl-5 mtl-1 str-20 thi-1 supE44*	M. Holland
E. coli AD4830	F⁻ *his ilv galK⁺T⁺E⁺OP*::IS*1* O_LP_L ΔBam N⁺ *c*I857ΔH1 *bio*	A. Das (4)
E. coli AD7068	F⁻ *his ilv nadA*::Tn*10 galK⁻T⁺E⁺OP*::IS*1* O_LP_L ΔBam N⁺ *c*I857ΔH1 *bio*	A. Das (6)
E. coli AD5230	F⁻ *his ilv galK⁺T⁺E⁺OP*::IS*2* P_R *c*I857 N_7 N_{53} *int-6*	A. Das (4)
p284	Amp^r λ pL *nutL* N⁺	A. Das (4)
pNTK5	Amp^r p*lac* λ *nutL galK⁺*	A. Das (6)
pMC13	Amp^r p*lac* λ *nutL galK⁺*	Derived from pNTK5
p81/16	Amp^r λ pL *nutL* protein C gene	4
pBR325	Amp^r Tet^r Cam^r	Bethesda Research Laboratories (3)

tive (1, 4). Unless otherwise noted, the 0.7-kilobase (kb) probe was used for all hybridizations (see below).

Amino acid compositions were determined on acid hydrolysates with a Beckman 121 automatic amino acid analyzer. Primary sequence determinations were carried out on a Beckman 890C sequencer, using the 0.1 M Quadrol program 102474. The sequencer was also coupled with a Sequemat P-6 autoconverter. The phenylthiohydantoin derivatives were identified and quantitated by high-performance liquid chromatography as described previously (19). In some cases the identification or quantitation or both was also carried out by amino acid analysis after back-hydrolysis of the phenylthiohydantoin derivatives with hydroiodic acid (19).

Spore protease cleavage of protein C-3 was monitored by acrylamide gel electrophoresis and by digestion of the cleavage products with aminopeptidase as previously described (13).

Growth and analysis of clones. Strains with p284-containing plasmids were grown at 30°C in L-broth plus ampicillin (50 μg/ml). Strains with pBR325-containing plasmids were grown at 37°C in L-broth plus ampicillin (50 μg/ml) and tetracycline (10 μg/ml), with or without chloramphenicol (10 μg/ml). Strains with pNTK5-containing plasmids were grown at 30°C in L-broth plus ampicillin (50 μg/ml) for plasmid isolation, but in tryptone plus ampicillin when expression of the *Bacillus* DNA inserted in the plasmid was to be monitored. For large-scale plasmid isolation, cells were grown overnight in 600 ml of medium in a 6-liter flask, and plasmids were isolated and purified by two cycles of CsCl-ethidium bromide gradient centrifugation (4). Miniplasmid isolations were made from overnight 1-ml cultures, and plasmids were isolated by alkaline lysis (2).

For analysis of the presence of a hybridizing plasmid in a pool of recombinants, plasmids from five 1-ml cultures were pooled, cut with *Eco*RI to maximize subsequent hybridization, denatured, and dotted on nitrocellulose paper as described by Kafatos et al. (7). The papers were then hybridized under nonrestrictive conditions as described above, washed, and autoradiographed. Individual members of positive pools were then grown and retested.

Preparation of probes. All hybridization probes were prepared from the protein C gene region in plasmid p81/16 (4). Two probes were used: a 1.5-kb fragment from the *Xba*I site to the *Hae*III site in *Hae*III fragment B of p81/16, and a 0.7-kb fragment from the *Bgl*II site to the *Hae*III site in *Hae*III fragment B of p81/16 (4). The 0.7-kb fragment is contained completely within the 1.5-kb fragment and contains the complete protein C coding sequence plus 88 nucleotides upstream from the translation initiation codon; the 1.5-kb fragment contains about 900 extra base pairs upstream from the translation initiation codon (4; E. R. Fliss and P. Setlow, unpublished data). Both fragments were isolated after gel electrophoresis and labeled by nick-translation (5, 10).

Cloning of *Eco*RI fragments of *B. megaterium* DNA. *B. megaterium* DNA (200 μg) was digested to completion with *Eco*RI, phenol extracted, and precipitated, and the digest was resolved by agarose gel electrophoresis. Regions of the gel containing appropriate-size fragments were excised, and the DNA was isolated (5). The sized DNA fragments (10 to 15 μg) were ligated with *Eco*RI-cut plasmid pBR325 (3 to 4 μg) in a total volume of about 200 μl. The ligation mix was used to transform *E. coli* RR101 to ampicillin and tetracycline resistance (4), and about 300 colonies per μg of total DNA were obtained. Colonies were replica-plated or tooth-picked on L-broth-ampicillin-tetracycline plates with or without chloramphenicol, and 150 to 200 Amp^r Tet^r Cam^s colonies were picked. The percent insertion ranged from 7 to 18%. Clones containing plasmids hybridizing to a protein C gene probe were identified as described above.

Analysis and purification of SASP produced by *E. coli* clones. For analysis of SASP, cells (25 ml) were grown in appropriate medium at 30°C to an optical density of about 1.0 and shifted to 42°C for 90 min. The culture was harvested, and the cell pellet was frozen and lyophilized. The dry cells were broken by dry rupture, with glass beads used as the abrasive, extracted with 2 ml of cold 2 N HCl for 30 min, and centrifuged for 10 min at 15,000 × *g*. The supernatant fluid was dialyzed at 4°C against 1% acetic acid in Spectrapor 3 tubing (4) and lyophilized, and the dry residue was dissolved in 200 μl of water before analysis by gel electrophoresis or radioimmunoassay.

For preparation of large amounts of protein C-3, 1 liter of culture was grown as described above but shifted to 42°C for 120 min, reaching a final optical density of 2.4. The cells were harvested by centrifugation, lyophilized, and dry ruptured; the proteins were extracted with 40 ml of 2 N HCl for 30 min at 4°C; the extract was centrifuged for 10 min at 15,000 × *g*); and the supernatant fluid was dialyzed in Spectrapor 3 tubing as described above and lyophilized. The lyophilized powder was dissolved in 20 ml of 10 mM Tris-maleate, pH 6.0, and protein C-3 was purified by chromatography on carboxymethyl (CM) cellulose as previously described (12). Fractions were analyzed for protein (8) and by gel electrophoresis at low pH as described above. Protein C-

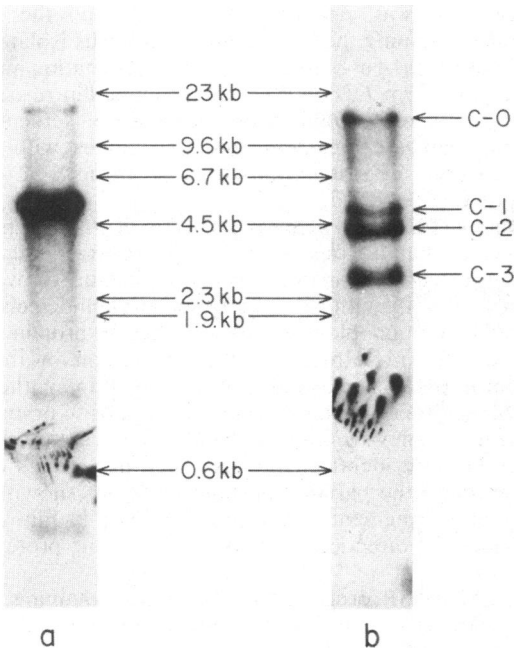

FIG. 1. Southern blot of *B. megaterium* DNA digested with (a) *Pvu*II or (b) *Eco*RI. Digested *B. megaterium* DNA (3 μg) was run on a small (0.5 by 6 by 10 cm) 0.8% agarose gel along with molecular weight markers. DNA on the gel was denatured, transferred to nitrocellulose paper, and hybridized to the 0.7-kb probe.

3-containing fractions were pooled, dialyzed against 1% acetic acid as described above, lyophilized, and dissolved in 1 ml of distilled water.

RESULTS

Detection of DNA fragments hybridizing to the protein C-3 gene. Southern blots of *Eco*RI and *Pvu*II digests of *B. megaterium* DNA gave a number of bands when hybridized under nonrestrictive conditions to the 0.7-kb probe (Fig. 1). The strongest band (5 kb) hybridizing in the *Pvu*II digest is the protein C gene-containing fragment (4), but a number of other bands were evident, including a strong band at 17 kb. The *Eco*RI digest gave four distinct bands: C-0 (15 kb), C-1 (4.9 kb), C-2 (4.4 kb), and C-3 (3.2 kb). The C-0 fragment contained the protein C gene itself because (i) the 5-kb *Pvu*II fragment carrying the protein C gene contains no *Eco*RI site (4), and (ii) hybridization of Southern blots under more restrictive conditions (65°C) decreased the intensity of bands C-1, C-2, and C-3 but had little effect on C-0 (data not shown). A number of other restriction enzymes were used to generate Southern blots of genomic DNA, but with *Eco*RI the pattern of new bands was simplest and all the new bands were smaller than 5 kb (data not shown). Strikingly, use of the 1.5-kb probe gave the same band pattern as was obtained with the 0.7-kb probe, consistent with the C protein gene sequence's being the hybridizing element in the probe (data not shown).

Cloning of fragments C-1, C-2, and C-3. The three hybridizing *Eco*RI fragments of *B. megaterium* DNA were cloned in pBR325 as described above. One clone that contained the C-1 fragment and two identical clones that each contained the C-2 and C-3 fragments were isolated. Restriction maps of each fragment are shown in Fig. 2, along with the location of that region of the fragment responsible for the hybridization to the 0.7-kb probe.

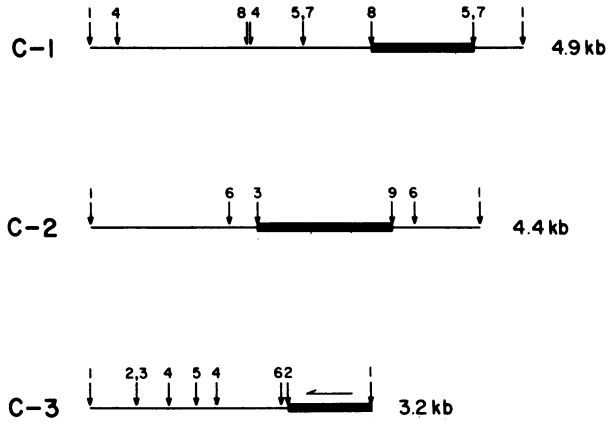

FIG. 2. Restriction maps of fragments C-1, C-2, and C-3. Individual fragments were isolated from appropriate clones in pBR325, and restriction maps were determined by agarose gel electrophoresis of appropriate single or double enzyme digests. The part of each fragment encompassing the region hybridizing to the 0.7-kb probe is indicated by heavy lines; the location was determined by hybridization of Southern blots of appropriate digests of each fragment to the 0.7-kb probe. The direction of transcription of the C-3 gene needed to give C-3 protein synthesis is shown by the horizontal arrow over fragment C-3. Numbered vertical arrows indicate restriction enzyme cleavage sites: 1, *Eco*RI; 2, *Pvu*II; 3, *Pst*I; 4, *Hae*III; 5, *Hin*cII; 6, *Xmn*I; 7, *Hpa*I; 8, *Eco*RV; and 9, *Xba*I.

Fragments C-2 and C-3 were ligated into the *Eco*RI sites of the expression vectors pMC13 and pNTK5, respectively, and transformed into *E. coli* AD7068, and a number of insert-containing clones were identified by *Eco*RI digestion of the plasmid from 1-ml cultures. Appropriate restriction enzyme analysis of plasmids containing either fragment C-2 or C-3 allowed the identification of clones containing a single fragment but in opposite orientations relative to the *lac* promoter of the plasmid.

Despite repeated attempts, fragment C-1 could not be inserted directly into an expression vector. Consequently,

TABLE 2. Amino acid composition of proteins A and C-3

Amino acid	Composition	
	C-3 protein[a]	A protein[b]
Lysine	6.1 (4)	6.6 (4)
Histidine	2.2 (1)	0 (0)
Arginine	4.9 (3)	3.3 (2)
Aspartate	7.3 (5)	8.2 (5)
Threonine	9.2 (7)	4.9 (3)
Serine	6.5 (5)	4.9 (3)
Glutamate	18.5 (13)	14.8 (9)
Proline	3.1 (2)	3.3 (2)
Glycine	10.2 (7)	13.3 (8)
Alanine	8.7 (6)	16.4 (10)
Valine	7.4 (5)	6.6 (4)
Methionine	0.1 (0)	3.3 (2)
Isoleucine	2.9 (2)	4.9 (3)
Leucine	8.8 (6)	6.6 (4)
Tyrosine	1.6 (1)	1.7 (1)
Phenylalanine	2.9 (2)	1.7 (1)

[a] Data are given as residues per 100 residues; values in parentheses are the nearest integer values, assuming 1.5 tyrosines per 100 residues and correcting for serine and threonine breakdown. Tryptophan and cysteine/cystine were not determined.

[b] Data are given as residues per 100 residues; values in parentheses are the residues per molecule. Values were calculated from the primary sequence (17).

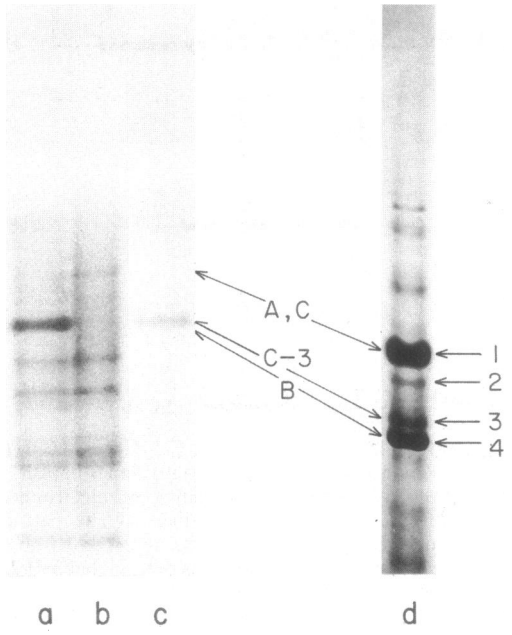

FIG. 3. Acrylamide gel electrophoresis of various extracts. Samples were run on 9-cm-long gels and the dye front was run to the bottom of the gel (lanes a, b, and c) or on 13-cm-long gels for twice the time needed to run the dye front off the bottom of the gel (lane d). Lanes: a and b, extract from 1 ml of *E. coli* AD7068 containing pEd (a) or pEe (b), shifted to 42°C for 90 min; c, 1 μg of protein C-3 purified by CM-cellulose chromatography; d, acid extract from 3 mg of *B. megaterium* spores. Positions of proteins A and C (which comigrate), B, and C-3 are shown. Numbered arrows: 1, proteins A and C; 4, protein B; 2 and 4, new minor SASPs. Protein C-3 comigrates with band 3.

this fragment was cleaved with *Hin*cII, and the 1.8-kb fragment containing the hybridizing region was isolated and ligated with *Hpa*I-cut plasmid p284 (4). This ligation mix was used to transform *E. coli* AD4830 to ampicillin resistance. The plasmids of a number of clones were screened by restriction enzyme digestion, and two clones with single inserts in opposite orientations relative to the λ pL of the plasmid were identified.

Synthesis of SASPs by clones. Analysis of SASP synthesis by clones containing fragment C-2 or the subfragment of C-1 in either orientation in an expression vector showed no detectable SASP synthesis even when acrylamide gels were analyzed by immunoblotting with anti-A or -B protein serum (data not shown). However, one orientation of the C-3 fragment in pNTK5 (plasmid pEd) did result in synthesis of an SASP not seen in the clone with the opposite orientation (pEe) (Fig. 3, lanes a and b). All bands seen in extracts of the pEe strain were identical to those seen in extracts of the strain carrying the parental plasmid (data not shown). The orientation of fragment C-3 in pEd is shown in Fig. 2, and this defines the direction of transcription of the protein C-3 gene.

The new SASP produced by the pEd-containing strain (termed protein C-3) migrated faster than proteins A and C (which comigrate) but slightly slower than protein B (Fig. 3). Immunoblots of unstained gels showed that protein C-3 reacted with antiserum to protein A but not protein B (data not shown).

Purification and analysis of protein C-3. Protein C-3 was prepared in large quantity from acid extracts of induced strains carrying pEd as described above. CM-cellulose chromatography of the extract gave a single major protein peak, which contained protein C-3 (Fig. 4). A total of 1.2 mg of protein C-3 was obtained per ml of culture, and the protein

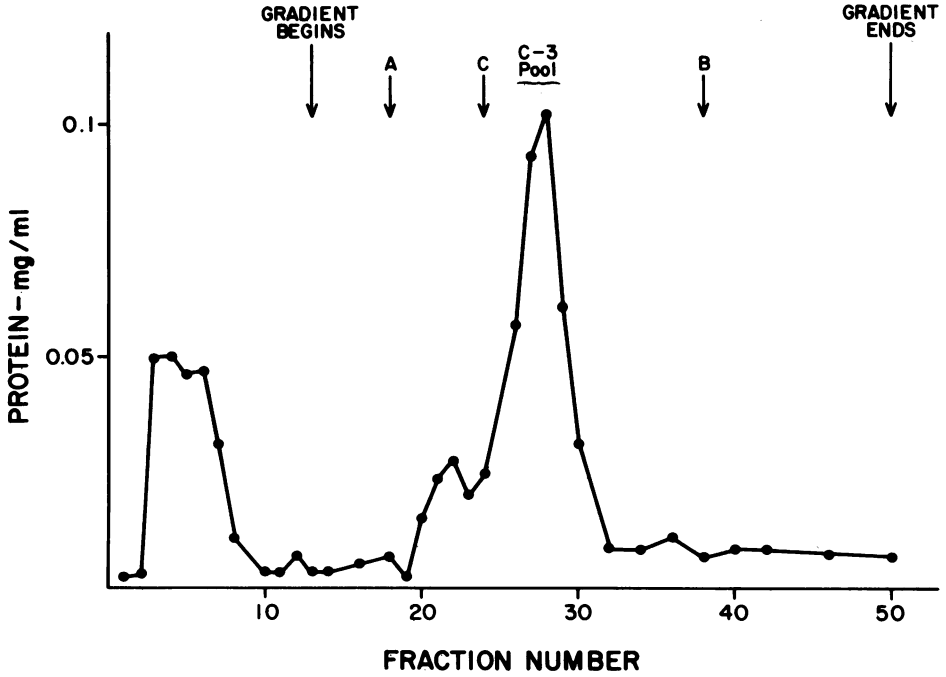

FIG. 4. CM-cellulose chromatography of acid extract of clone carrying pEd. The extract was applied to a CM-cellulose column (1.5 by 9 cm) in 10 mM Tris-maleate, pH 6.0 (buffer A), washed with 50 ml of buffer A, and protein eluted with a linear gradient of 100 ml each of buffer A to 250 mM NaCl in buffer A. Fractions (4 ml) were collected and analyzed. Fractions containing pure protein C-3 were pooled. Arrows show the positions of elution of proteins A, B, and C as determined by conductivity measurements of fractions from parallel columns.

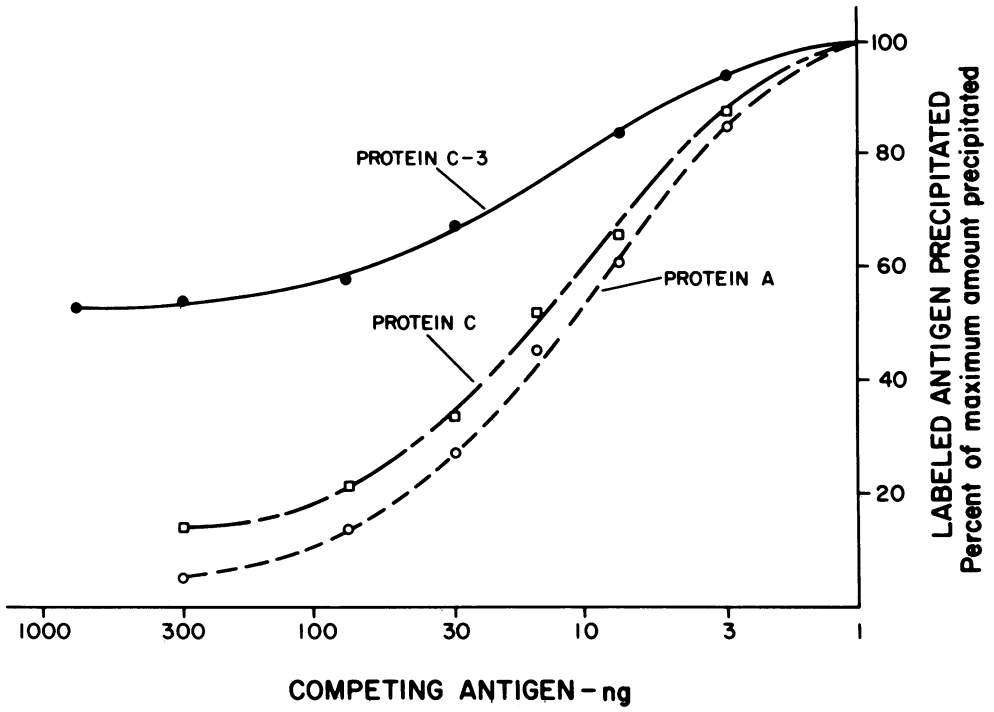

FIG. 5. Radioimmunoassay curves for proteins A, C, and C-3. The radioimmunoassay curves were determined as described in the text. Concentrations of proteins A, C, and C-3 were determined by amino acid analyses.

gave only a single band on acrylamide gel electrophoresis (Fig. 3, lane c).

Strikingly, protein C-3 eluted from the CM-cellulose column well after proteins A and C and just before protein B (Fig. 4). This finding, plus the different migration positions of proteins A, B, and C-3 on acrylamide gel electrophoresis (Fig. 3), strongly suggested that protein C-3 was a new member of the SASP family. This suggestion was substantiated by detailed analysis of purified protein C-3.

Radioimmunoassay of purified protein C-3 gave a curve similar (but not identical) to that for proteins A and C at low antigen levels, but at high antigen levels protein C-3 was a much less effective competitor (Fig. 5). These data indicated that some of the antigenic determinants on proteins A and C are not present on protein C-3. The amino acid composition of protein C-3 was again similar to that of protein A, but there were significant differences: protein C-3 lacked methionine and contained histidine, in contrast to protein A (Table 2). The amino-terminal sequence of protein C-3 was identical to that of protein A in five of six positions, but differed at position 2 (Table 3). The sequence of protein C-3 was also different from that of proteins B and C (Fig. 6). However, like proteins A, B, and C, protein C-3 was a substrate for the sequence-specific spore protease (15) and was cleaved into at least two oligopeptide fragments, as determined by gel electrophoresis of the cleavage products (data not shown).

TABLE 3. Automated sequenator analysis of protein C-3 (50 nmol)

Cycle	Amino acid (nmol)
1	Alanine (20)
2	Arginine (11)[a]
3	Threonine (6)[a]
4	Asparagine (11)
5	Lysine (9)[a]
6	Leucine (16)

[a] The quantitation (and identity) of these residues was determined by amino acid analysis after back hydrolysis of the phenylthiohydantoin derivatives with hydroiodic acid.

Level of protein C-3 in various *E. coli* strains. The data given above indicated that the C-3 fragment contained a gene which was closely related to that for protein C. Previous work has shown that the protein C gene is expressed in *E. coli* only from an external promoter and even then only if transcription termination is suppressed (4). Consequently, it was of obvious interest to analyze the requirements for protein C-3 gene expression in *E. coli*. Strikingly, there was no detectable synthesis of protein C-3 in strains carrying plasmid pEd that were grown at the nonpermissive temperature (Table 4), but a shift to high temperature, allowing synthesis of N protein and thus suppressing termination of transcription from the *lac* promoter of the plasmid (6), resulted in at least a 100-fold increase in protein C-3 synthesis (Table 4). However, even under inducing conditions there was no synthesis of protein C-3 in an N^- host nor in an N^+ host carrying the C-3 fragment in opposite orientation to that in pEd (Table 4). The maximum level of protein C-3 accumulated was 1.7 μg/ml of culture, which was only 1/5

A PROTEIN: NH_2-Ala-Asn-Thr-Asn-Lys-Leu

C-3 PROTEIN: NH_2-Ala-Arg-Thr-Asn-Lys-Leu

C PROTEIN: NH_2-Ala-Asn-Tyr-Gln-Asn-Ala

B PROTEIN: NH_2-Ala-Lys-Gln-Thr-Asn-Lys

FIG. 6. Amino-terminal sequences of proteins A, B, C, and C-3. The amino-terminal sequence of protein C-3 is taken from the data shown in Table 3. The sequences of proteins A, B, and C are taken from the literature (17–19).

TABLE 4. Levels of C-3 gene product in various strains

E. coli strain	Plasmid	Growth temp (°C)	C-3 protein (ng/ml of culture)[a]
AD7068 (N^+)	pEd	30	<5
AD7068 (N^+)	pEd	42	1,720
AD7068 (N^+)	pEe	42	<5
AD5230 (N^-)	pEd	42	<10

[a] C-3 protein was determined by radioimmunoassay, using the calibration curve with purified C-3 protein shown in Fig. 5.

that of protein C, when the C gene was carried in either plasmid p284 (4) or pNTK5 (data not shown).

Presence of protein C-3 in *B. megaterium* spores. The data given above strongly suggested that we had cloned a new SASP gene. However, the specific properties of protein C-3 differed from those of all other *B. megaterium* SASPs. Thus it was of obvious interest to reanalyze spores of *B. megaterium* for a C-3-like protein. When acid extracts of *B. megaterium* spores were run on long acrylamide gels and stained for protein, several minor bands migrating between the positions of proteins A and B were observed (Fig. 3, lane d). One of these bands (band 3) comigrated with protein C-3, and the protein in band 3 reacted in immunoblots with antisera to protein A but not B (Fig. 3 and data not shown). The level of this new protein was estimated at about 10% of that of protein B. The additional band (band 2) between proteins A and B also reacted with antiserum to the A protein.

DISCUSSION

It seems clear that, by using the *B. megaterium* protein C gene as a probe, we have isolated a new SASP gene, since protein C-3 is similar to the A and C proteins in its acid solubility, reaction with antiserum to protein A, amino acid composition, amino-terminal sequence, and susceptibility to spore protein cleavage. However, it seems equally clear that protein C-3 is not identical to any previously described SASP, in particular to protein A, since there are significant differences between the detailed properties of proteins C-3, A, B, and C. Thus, we have a new member of the SASP gene family. Although we have not rigorously proven that the C-3 protein gene is expressed in *B. megaterium*, the identification of a minor spore protein which comigrates with protein C-3 and reacts with anti-A protein serum strongly suggests that protein C-3 is expressed in *B. megaterium*. This brings to eight the number of different SASPs identified in *B. megaterium* spores (14), with three of these—A, C, and C-3—being closely related. It will be of obvious interest to determine the sequence of the C-3 protein gene and its corresponding protein sequence and to compare this with the sequences of the A and C proteins and genes.

Although the C-3 protein gene is clearly distinct from the C protein gene, it shares a number of its properties. In particular, both C-3 and C protein gene expression in *E. coli* required an external promoter and suppression of termination of transcription. This suggests that SASP genes have promoter sequences different from those recognized by *E. coli* RNA polymerase and is consistent with the SASP genes' being sporulation specific and regulated by positive control (4, 6). In this respect these SASP genes differ from two other sporulation genes, those for *Bacillus thuringiensis* crystal protein and *Bacillus subtilis* glucose dehydrogenase, which are expressed in *E. coli* without external promoters (22, 23). However, the crystal protein gene is transcribed in *E. coli* from a different promoter than that used in *B. thuringiensis* (23). The promoters utilized for glucose dehydrogenase gene expression in *E. coli* and *B. subtilis* have not yet been reported.

Use of the protein C gene as a probe to detect related SASP genes was undertaken with the expectation that this would lead to isolation of the protein A gene. However, it is clear that protein C-3 is a different gene product than protein A. Where then is the A protein gene? An obvious possibility is that it resides on fragment C-1 or C-2 but is not translated in *E. coli*. Translation of some SASP genes might be hindered in *E. coli* due to their transcription as large mRNAs with long leader sequences; in contrast, A and C protein mRNAs in *B. megaterium* are only 300 to 350 bases long (B. Setlow and P. Setlow, unpublished data), which provides for only 100 to 150 bases upstream from the coding sequence. Although there is significant expression of the C protein gene in *E. coli*, C-3 protein gene expression is fivefold lower with the same plasmid vector, a difference that seems likely to be due to translational differences between the two genes. Possibly the A protein gene is translated extremely poorly, if at all, in *E. coli*. However, if the A protein gene (or another related SASP gene) is on fragment C-1 or C-2, it should be possible to identify these genes by subcloning those regions which hybridize to the protein C gene probe and determining their nucleotide sequences.

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