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Acid-Soluble Spore Proteins of *Bacillus subtilis*

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Acid-soluble spore proteins (ASSPs) comprise about 5% of the total protein of mature spores of different *Bacillus subtilis* strains. They consist of three abundant species, α, β, and γ, four less abundant species, and several minor species. α, β, and γ make up about 18, 18, and 36%, respectively, of the total ASSPs of strain 168, have molecular weights of 5,900, 5,900, and 11,000, respectively, and resemble the major (A, C, and B) components of *Bacillus megaterium* ASSPs in several respects, including sensitivity to a specific *B. megaterium* spore endopeptidase. However, they have pI's of 6.58, 6.67, and 7.96, all lower than those of any of the *B. megaterium* ASSPs. Although strains varied in the proportions of different ASSPs, the overall patterns seen on gel electrophoresis are constant. ASSPs are located interior to the cortex, presumably in the spore cytoplasm, and are synthesized during sporulation and degraded during germination.

Setlow (13) observed that about 15% of the protein present in dormant spores of *Bacillus megaterium* is rapidly degraded to free amino acids during germination. These amino acids are excreted, and their re-utilization is essential for protein synthesis in simple media, since spores lack significant pools of most amino acids and germinating spores initially lack several key amino acid synthetic enzymes (20). Setlow (14) demonstrated that the major fraction of protein degraded during germination was a mixture of acid-soluble spore proteins (ASSPs), initially thought to contain two major components, A and B. A third component, C, related to A, and four minor species were found later (15, 17), and complete amino acid sequences have recently been published for A, B, and C (18, 19, 19a). An endopeptidase present in *B. megaterium* spores was purified and found to be specific for cleavage of the *B. megaterium* ASSPs (16). Its unique pentapeptide sequence specificity is most fully described in the accompanying paper (26), which also describes its sites of action on *Bacillus cereus*, *B. megaterium*, and *Bacillus subtilis* ASSPs.

In this paper, we describe the isolation, fractionation, and partial characterization of the major ASSPs of *B. subtilis*. They are similar to the *B. megaterium* proteins in size and amino acid composition, and the presence in them of pentapeptide sequences cleaved by the *B. megaterium* enzyme indicates a conserved mechanism for the degradation and utilization of ASSPs during germination. Sequences are compared in the accompanying paper (26). The *B. subtilis* ASSPs are also similar to the *B. megaterium* ASSPs in total complexity, but are lower in total concentration and are less basic.

This comparison substantiates the proposed functions of ASSPs as storage proteins, since this is dependent on common properties. Functions proposed for the major *B. megaterium* proteins which are related to their affinity for DNA do not seem to be of universal significance.

MATERIALS AND METHODS

Bacterial strains and media. *B. subtilis* strain 168 is the *trpC2* strain previously used in this laboratory in studies of spore coat structure (4) and erythromycin resistance (5, 23). *B. subtilis* strain BR151 (*trpC2 lys-3 metB10*) was obtained from Glenn Chambliss. *B. subtilis* strain SMY, a wild-type Marburg strain, was obtained from Richard Losick (Harvard University) and originated from P. Schaeffer's laboratory (6). *B. subtilis* strain 168 (*trp*$^+$), derived from transformation of strain 168 with DNA from strain SMY, was obtained from H. O. Halvorson, Brandeis University, Waltham, Mass.

Difco sporulation medium is Schaeffer nutrient broth-based sporulation medium (22). Modified Schaeffer medium (MSM) is the same, with 16 g of nutrient broth and 1 g of glucose per liter (23). Potato extract medium contained, per liter (adjusted to pH 7.2): 10 g of tryptone and 1 g of yeast extract (both from Difco Laboratories, Detroit, Mich.) and a filtered boiling water extract of 100 g of sliced potatoes. Unless otherwise stated, cultures (500 ml) were grown at 37°C in 2-liter Fernbach flasks rotating at 300 rpm on a New Brunswick G25 shaker.

Spore preparations. Cultures in flasks were shaken until T_{24} to T_{36}. T_n indicates the number of

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hours after the end of exponential turbidity increase (determined by Klett colorimeter). Cultures (10 liters) of strain 168 in MSM or potato extract medium, growing at 37°C in 14-liter fermentors (Fermentation Design), were harvested at T_{30}. Strain SMY was grown in Difco sporulation medium in a 180-liter fermentor at 37°C and also harvested at about T_{30}. In each case, the harvested cultures consisted of at least 80% free spores, as determined by phase-contrast microscopy, and the average yield was 0.5 g of clean, dry spores per liter. Spores were harvested by centrifugation and cleaned by lysozyme hydrolysis and washing with 1 M NaCl–0.1 M salt–0.1% sodium dodecyl sulfate (SDS)–water as previously described (4), followed by lyophilization and storage at −20°C.

Dry breakage of spores. Small quantities (10 to 100 mg) of lyophilized spores were broken (90% after 10 periods of 1 min), using a Wig-L-Bug dental amalgamator (model 5AR, Crescent Dental Manufacturing Co., Chicago, Ill.), essentially as described by Setlow (14). Larger quantities of spores (100 to 2,500 mg) were broken (95 to 99%) by shaking with 120-μm glass beads, using a Bronwill cell disrupter (model 2376; Bronwill Scientific Inc., Rochester, N.Y.). Dessication of the dry spore-bead mixture in vacuo immediately before breakage was essential for recovery of intact ASSPs.

The mixture of fragmented spores and glass beads was usually extracted twice with 3% (0.52 M) acetic acid (5 to 10 ml/100 mg of spores) for 20 min at 0°C, followed by centrifugation and lyophilization of the supernatant extracts. Subsequent extraction of the pellets at 0°C with 2 N HCl or reversal of this 2 N HCl, 3% acetic acid sequence was sometimes employed.

Cryo-impaction breakage (spores or cells). Smucker and Pfister (21) described a device for breaking microorganisms, frozen in liquid nitrogen, by shaking in a stainless steel vessel containing a loosely fitting stainless steel ball. We constructed a similar device (R. M. Pfister, personal communication) by attaching a similar chamber (0.5 by 3 in. [ca. 1.3 by 7.6 cm], inner dimensions; 3/8-in. [ca. 0.95-cm] steel ball) to the piston of a lawn mower engine whose drive shaft was rotated at 500 rpm by a 0.5-hp electric motor. Six 2-min periods of shaking, separated by cooling of the chamber by immersion in liquid N_2, resulted in at least 95% breakage of frozen pellets of sporulating cells, spores, or germinating spores (2 to 5 g).

The powdered, frozen products of cryo-impaction breakage were rapidly melted by pouring into an equal volume of 6% acetic acid or 4 N HCl. After 20 min at 0°C, centrifugation, and reextraction of the pellet, the combined supernatants were lyophilized.

Wet breakage. Spores, germinated spores, or sporulating cells (100 mg, dry weight) were suspended in 2 N HCl (3 ml) and shaken with glass beads (6 g) in a Bronwill cell disruptor, using the microchamber adapter. Breakage of 90 to 99% resulted from a total of 10 min of shaking, during which the temperature was maintained near 0°C. At this temperature, acid rupture of mature spores is slow.

Acid rupture of intact spores. Clean, dry spores were dispersed in 2 N HCl at 20°C at 2 to 10 mg/ml. After 30 min, the suspension was centrifuged (10 min at 10,000 × *g*), the pellets were extracted again with 2 N HCl, and the combined supernatants were lyophilized. The final pellet was extracted twice with 3% acetic acid (half of the original volume of 2 N HCl) for 20 min at 20°C, and these extracts were also pooled and lyophilized.

Alternatively, clean, dry spores were dispersed in 2 N HNO_3 at 0°C at 10 to 100 mg/ml. After 25 min, the suspension was centrifuged and the pellets were extracted with half of the original volume of 2 N HNO_3 for 5 min at 0°C. The combined supernatants were made slightly basic by the addition of 4 N NH_4OH and then lyophilized.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was as follows. Samples of lyophilized extracts or column fractions were dissolved in SDS gel sample buffer and fractionated and stained with Coomassie brilliant blue R-250 as previously described (5). Linear 7.5 to 15% acrylamide gradient gels were employed, except where otherwise indicated.

pH 4.7 PAGE. Samples dissolved in 1% acetic acid were applied to tube gels of 7.5% acrylamide and run in the β-alanine-acetic acid pH 4.7 gel buffer of Reisfeld et al. (9) for 2 h at 25 mA per tube. Staining and destaining reagents were as for SDS gels.

Determination of molecular weights. Molecular weights were estimated by migration rates of SDS-PAGE, using urea-phosphate buffers, by comparison with polypeptides of known size. Because small proteins may migrate anomalously under any particular set of conditions (25), mobilities were determined on 7.5 to 15% and 5 to 20% linear gradient gels as well as on uniform 10 and 15% gels.

Isoelectric points. The procedure followed was as described by Ames and Nikaido (1). Gels were prepared on the day of use. Protein samples were precipitated with acetone and redissolved in sample buffer (1) before fractionation for 14 h at 300 V.

Analytical techniques. Neutral hexoses were determined by the anthrone procedure (10). Glycoproteins were stained after SDS-PAGE with periodate-Schiff reagent (12). Dipicolinic acid was assayed by the procedure of Rotman and Fields (11). Samples for amino acid analysis were hydrolyzed in vacuo with constant-boiling-point HCl (Pierce Chemical Co.) at 110°C for 23 h. The hydrolysates were lyophilized before quantitation on a Durrum model D-500 amino acid analyzer. Cys and Met were determined as cysteic acid and methionine sulfone by performic acid oxidation before hydrolysis. For the estimation of trp, adsorption spectra and fluorescence emission spectra were determined in 0.1 M NaOH with excitation at 290 nm, using an Aminco-Bowman model 23 spectrofluorimeter.

Protein was routinely assayed by the procedure of Lowry et al. (7), using bovine serum albumin as a standard. Protein was also assayed by the binding of Coomassie brilliant blue G-250 (Sigma Chemical Co., St. Louis, Mo.) by the procedure of Bradford (3) and by fluorescamine binding by the procedure of Bohlen et al (2).

RESULTS

Isolation of the major *B. subtilis* ASSPs, α, β, and γ. *B. subtilis* strain 168 ASSPs were first isolated by dry breakage of mature spores

(10 g) which had been uniformly labeled with L-[U-^{14}C]Leu (100 μCi, total) during growth and sporulation in MSM. The yield of ASSPs was about 7% of the spore dry weight (10% of the spore protein), and ASSPs contained 11% of the incorporated Leu. Fractionation on Sephadex G-50 in 1% acetic acid (Fig. 1) gave a high-molecular-weight peak (fractions 15 to 30) containing 60% of the protein and radioactivity, a low-molecular-weight peak (fractions 31 to 48) containing the residual protein and radioactivity, and also large amounts of material adsorbing at 260 nm (dipicolinic acid and RNA fragments). Fractionation of the high-molecular-weight peak by pH 4.7 PAGE showed the presence of three components, called α, β, and γ, in order of increasing migration rate (basicity; Fig. 2, lane 1). All were considerably less basic than the major *B. megaterium* ASSPs (Fig. 2, lane 4). The low-molecular-weight peak failed to give visible bands at pH 4.7 or by SDS-PAGE (data not shown). Because ASSPs were moderately soluble during staining and destaining procedures after pH 4.7 PAGE, and because this procedure does not give good stacking, bands were rather diffuse. Fortunately, elution from gels during staining after SDS-PAGE did not seem to be significant. For this reason and because ASSPs stain poorly with Coomassie dyes (see below), large protein samples (25 to 75 μg) were routinely used for PAGE analysis.

The high-molecular-weight peak was separated on carboxymethyl (CM) cellulose into a peak of unretarded protein (Fig. 3, fractions 9 to 17), and a fraction eluted at 0.15 M NaCl (Fig. 3, fractions 44 to 48). The former contained α + β (Fig. 2, lane 2), and the latter contained γ (Fig. 2, lane 3). Each fraction contained half of the total protein applied, although this procedure (7) underestimated α + β by 25% (see below). The Leu content of γ was only one-third that of α + β (see below), and the ratios of protein to label in the two peaks varied accordingly (Fig. 3). Densitometry of Coomassie-stained pH 4.7 gels (Fig. 2, lane 1) indicated that the ratio of α to β to γ in the fraction applied to CM cellulose was close to 1:1:2. All attempts at further fractionation of α from β were unsuccessful, leading to lack of recovery (preparative gel electrophoresis at pH 4.7) or coelution (chromatography on various ion-exchange media). α and β were, therefore, analyzed as an approximately equimolar mixture. Fractionation by SDS-PAGE showed that γ gave a single band with mobility close to that of lysozyme, whereas α + β mixture gave a single band of higher mobility (Fig. 4, lane S). Acid rupture (see below) extracted an additional ASSP, δ, that migrated on SDS-PAGE more slowly than γ (Fig. 4, lane 2).

Analyses of α + β and γ. Molecular weights

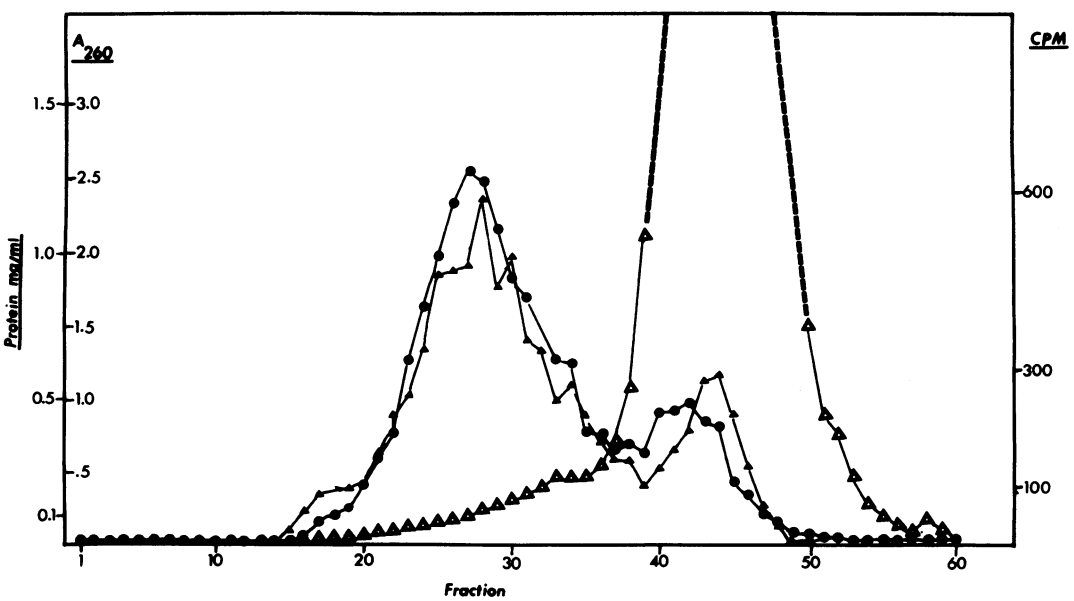

FIG. 1. *Fractionation of ASSPs from strain 168 on Sephadex G-50. ASSPs extracted from 10 g of spores labeled with* L-[U-^{14}C]*Leu were fractionated on a column (500 ml, bed volume) of Sephadex G-50 equilibrated at 4°C in 1% HOAc. Fractions of 10 ml were collected and analyzed for absorbance at 260 nm* (A_{260}) (△), *radioactivity* (▲), *and total protein (7)* (●).

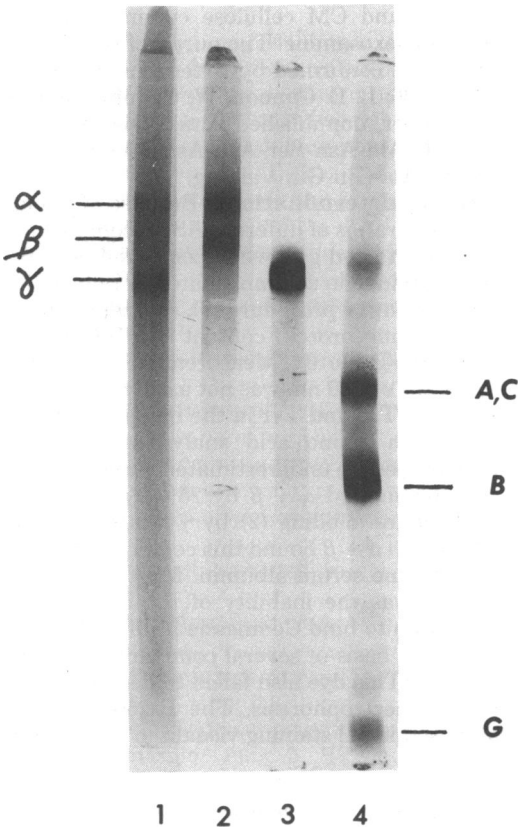

FIG. 2. *pH 4.7 PAGE analysis of samples from column separations. Samples (60 μg) of proteins were fractionated at pH 4.7. Gel 1: Sephadex G-50 column (Fig. 1): pooled fractions 15 to 30. Gel 2: CM cellulose (Fig. 3) flow-through (fractions 9 to 17). Gel 3: CM cellulose, NaCl eluate (fractions 44 to 48). Gel 4: B. megaterium ASSPs (40 μg).*

of the α, β, γ, and δ fractions were estimated from relative migration rates on SDS-PAGE in urea-phosphate buffers. The data derived from averaging of information from gels run under four different conditions of gel concentration and cross-linking are shown in Table 1. Values obtained under any single set of conditions varied by 10%. On all gel formulations, α and β migrated together. The rate of migration of these ASSPs on Tris-buffered SDS gels (Fig. 4 and 7) was anomalously slow. Under these conditions, estimated molecular weights of α + β, γ, and δ were 9,500, 18,000 and 19,000 respectively.

The isoelectric points of α, β, and γ were determined by isoelectric focusing, using several combinations of ampholines giving pH ranges from 5 to 7 and 7 to 9. After focusing, the pH gradient was determined, using a micro-surface electrode (Table 1). It was assumed that the component in the α + β mixture with the lowest pI was α, but this has not been confirmed.

Amino acid analyses (Table 2) revealed that neither the mixture of α + β nor γ contained His, Pro, Tyr or Cys. Only α + β contained Met, and the mixture contained only 1.3 mol% suggesting that either α or β (but not both) contain Met. However, autoradiography of the α + β fraction, purified from spores continuously labeled during sporulation with L-[^{35}S]Met and fractionated by pH 4.7 PAGE, showed equal incorporation into both α and β (data not shown). The γ + δ fraction from these same spores did not incorporate ^{35}S, confirming the absence of Met in γ and demonstrating its absence in δ. α + β and γ contain 29 and 42 mol% of Asx + Glx, respectively, and the neutral or basic pI's of these proteins indicate that most of these residues are amidated. α + β has a considerably higher frac-

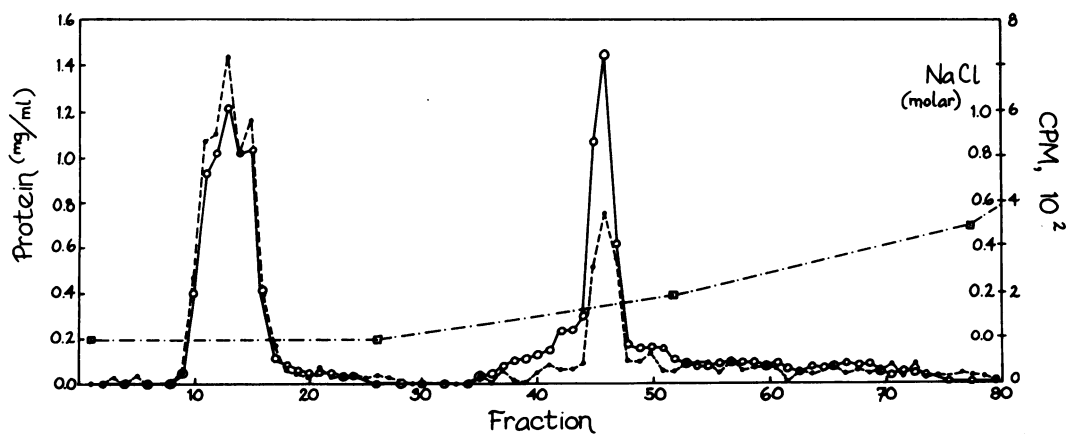

FIG. 3. *Fractionation of ASSPs on CM cellulose. Fractions 15 to 30 from Sephadex G-50 fractionation (150 mg of protein) were lyophilized, dissolved in 50 ml of 5 mM Tris-maleate buffer (pH 6.0), and applied at 4°C to a column of CM cellulose (110 ml, bed volume) equilibrated with the same buffer. After elution with buffer (300 ml), the column was eluted with a linear gradient of NaCl (0 to 0.5 M) in the same buffer (500 ml). Fractions (10 ml) were analyzed for protein* (○) *and radioactivity* (●).

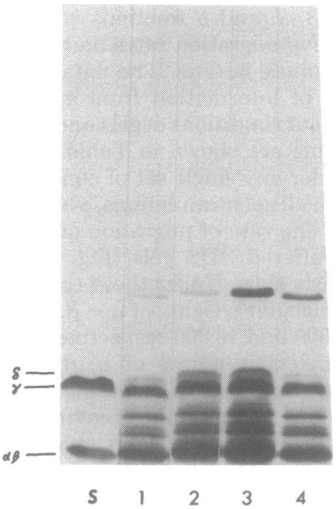

FIG. 4. *ASSPs produced from strains 168 by acid rupture. Lane S: mixture of purified α + β and γ. Lanes 1, 2, 3, and 4: spores of strain 168 were isolated at T_{36}, T_{48}, T_{10}, and T_{16}, respectively, washed, and ruptured in 2 N HCl. Each lane contained 40 μg of extracted protein.*

TABLE 1. *Molecular weights and isoelectric points*

ASSP component	Molecular wt	Isoelectric point
α	5,900	6.58
β	5,900	6.67
γ	11,000	7.96
δ	12,100	>8.5

tion (24%) of hydrophobic amino acids than does γ (13%). Fluorescence emission spectra (Fig. 5) demonstrated that neither α + β nor γ contain Trp, and the absence of Trp and Tyr was also evident from adsorption spectra (not shown). The fluorescence in α + β is probably due to dipicolinate since this fraction contained about 1.6 mol of dipicolinate per mole of α or β. Cofractionation on Sephadex G-50 is surprising, but may reflect tailing of the low-molecular-weight peak (Fig. 1). The γ fraction contained no dipicolinate.

Whereas γ also lacked significant amounts of carbohydrate, the α + β preparations contained 32 mg per 100 mg of protein. After fractionation of α + β (30 μg) by SDS-PAGE, staining with periodate-Schiff reagent (12) failed to detect any associated carbohydrate (data not shown), even though a band due to rabbit heavy-chain immunoglobulin G containing 0.5 μg of carbohydrate was clearly visible on the same gel. It was concluded that α + β contained less than 1% covalently bound carbohydrate and that the carbohydrate in this preparation had copurified on Sephadex and CM cellulose columns. It contained no hexosamine. The purity of the γ preparation was confirmed by N-terminal sequence analysis (26; L. E. Cannon, W. C. Johnson, and D. J. Tipper, unpublished data). The sequence is: NH_2-Ala-Asn-Ser-Asn-Asn-Phe-Ser-Lys-Thr-Asn-Ala-Gln-Gln-Val-Arg.

Protein determinations. Because of anomalies in the ratios of different ASSP components visible on stained gels when compared with ratios determined in column eluates by radioactivity or the Lowry procedure (7), several methods for estimating protein content of ASSPs were compared (Table 3). Measurement of absorbance at 260 or 280 nm was not usable because of the lack of Trp and Tyr in the major ASSPs.

Based on amino acid analytical data, the Lowry procedure underestimated γ slightly, and it underestimated α + β by 25%. Surprisingly, fluorescamine binding (2) by γ was inefficient, even though α + β bound this compound as well as did bovine serum albumin. Most surprising, however, was the inability of the α + β or γ preparation to bind Coomassie brilliant blue G-250 (3), the basis of several commercial protein assay kits. This dye also failed to detect ASSPs after gel electrophoresis. The R-250 dye normally used in gel staining visualized γ with only

TABLE 2. *Amino acid analysis*[a]

Amino acid	γ		α + β	
	Mol%	Residues/molecule	Mol%	Residues/molecule
Asx	14	14	14	8
Thr	6	6	6	3
Ser	8	8	8	4
Glx	28	28	15	8
Gly	9	9	12	6.5
Ala	11	11	15	8
Val	5	5	7	4
Met	0	0	1	0.65
Ile	1	1	7	4
Leu	2	2	6	3
Phe	5	5	3	2
Lys	7	7	4	2
Arg	4	4	3	2

[a] Acid hydrolysis and amino acid analysis were performed as described in the text. Each value is the mean of four separate analyses of oxidized and unoxidized samples. Val was used as the conserved amino acid to convert oxidized to unoxidized values. Mol% is the number of moles of each amino acid per 100 moles of amino acids. Residues/molecule is based on an average mass of 110 daltons per amino acid residue and the data in Table 2. Neither α + β nor γ contained significant amounts of Pro, His, Cys, or Tyr. They also lacked Trp (Fig. 5).

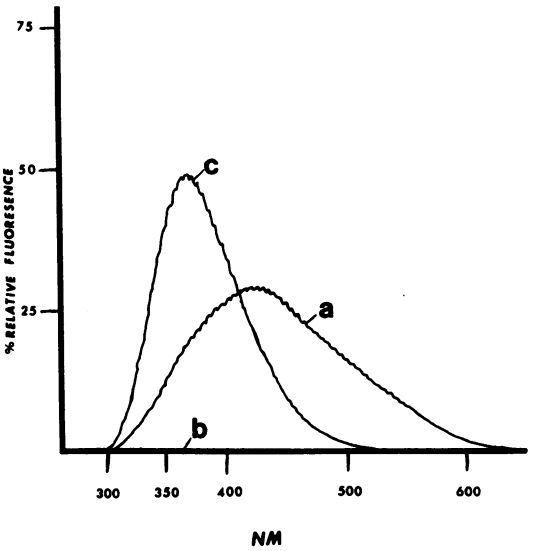

FIG. 5. *Fluorescence emission spectra. Fluorescence emission was determined with a Aminco-Bowman spectrofluorimeter, model 233. All samples were in 0.1 N NaOH and were excited at 290 nm. (a) α + β, 1 mg/ml; (b) γ, 1 mg/ml; and (c) N-acetyltryptophanamide, 0.1 mg/ml.*

TABLE 3. *Protein contents*[a]

Sample	Protein contents by:			
	Amino acid analysis	Lowry procedure	Fluorescamine binding	Dye binding
α + β	100	75	97	<5
γ	100	92	46	<5

Assays were performed as described in the text and are presented as milligrams per milliliter, relative to the data from amino acid analyses of the same samples, assuming an average molecular weight per amino acid residue of 110. The standard for each assay procedure (see text) was bovine serum albumin.

low efficiency, and it visualized α + β very poorly, compared with protein standards. The Lowry procedure offered the best compromise for assaying yields of the major ASSPs (Table 4).

Complexity of total *B. subtilis* ASSPs released by acid rupture. Because the *B. subtilis* ASSPs have no known enzymatic or other function assayable in vitro, the only criterion by which the authenticity of repeated preparations can be judged is the constancy of their physical and chemical properties. Two primary characteristics have been employed in assessing the natural complexity of ASSPs. These are the total yield of acid-soluble proteins and their gel electrophoretic patterns.

It was anticipated that, as for *B. megaterium* (17), extraction with stronger acids would result in more complex ASSP patterns. Testing of many techniques showed that the most reproducible recoveries and patterns were obtained by acid rupture (24) in 2 N HCl. Up to twelve bands could be distinguished by pH 4.7 PAGE, although some were relatively minor (Fig. 6). The diminishing quantities of ASSPs derived from repeated extraction (Fig. 6, lanes 2 and 3) contained no additional components, but did contain considerable α + β and γ, which were the components most slowly solubilized by this technique. By densitometry, these components comprised about 18, 18, and 36%, respectively, of the total acid-rupture ASSPs. Total yield of ASSPs (3 to 3.2% of spore dry weight) was constant, and very little of this was dialyzable (Table 4). SDS-PAGE patterns were clear and highly reproducible (Fig. 4, lanes 1 to 4), and

TABLE 4. *Yields of ASSPs*[a]

	Extraction procedure	Yield with strain			
		168	168 (*trp*$^+$)	BR151	SMY
A.	HOAc after dry break	6.5	6.8	7.0	5.8
	HCl after HOAc	0.4	1.0	0.9	1.1
	Total	6.9	7.8	7.9	6.9
	Total after dialysis	3.2	3.4	3.3	2.6
B.	HCl after dry break	5.0	5.9	6.0	4.5
	HOAc after HCl	0.4	0.5	0.3	0.5
	Total	5.4	6.4	6.3	5.0
	Total after dialysis	3.1	3.0	3.2	2.4
C.	HNO_3 after dry break	0.4	0.5	6	
	HOAc after HNO_3	0.2	0.2	0.1	
	Total	0.6	0.7	6.1	
D.	HCl rupture	3.0	2.9	3.1	2.5
	HOAc after HCL	0.3	0.6	0.2	0.7
	Total	3.3	3.5	3.3	3.2
	Total after dialysis	3.1	3.2	3.0	2.6
E.	HNO_3 rupture	2.0	1.1	2.5	
	HOAc after HNO_3	0	0	0.1	
	Total	2.0	1.1	2.6	

[a] Data for protein are the percent dry weight of spores. These spores contain 65 to 70% of their dry weight as protein. Mature spores (100 mg) of strains 168, 168 (*trp*$^+$), BR151, and SMY were extracted with HOAc, HCl, or HNO_3 after dry breakage and subsequently extracted with HCl, HOAc, or HOAc, respectively. Intact spores were also ruptured in HCl at 20°C or in HCO_3 at 0°C, followed by extraction with HOAc (see text). Total protein (7) was determined after lyophilization of extracts and, except for HNO_3 extracts, after subsequent dialysis against 1% acetic acid. Samples (40 μg) of the undialyzed extracts were fractionated by SDS-PAGE (Fig. 7A and B).

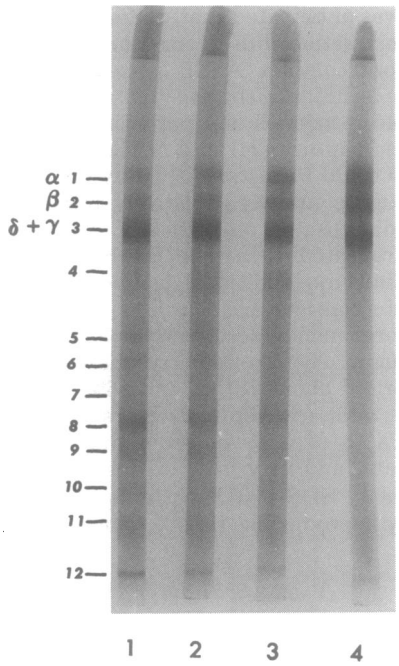

FIG. 6. *Fractionation at pH 4 of ASSPs produced by acid rupture of strain 168 spores. Cleaned spores (20 mg) were ruptured in 2 N HCl (2 ml). The insoluble residue was extracted twice more with 1 ml of 2 N HCl. Samples (40 μg) of these sequential extracts were fractionated at pH 4.7 (lanes 1, 2, and 3). The samples correspond to 0.7, 3, and 10 mg of spores, respectively. The final lane contains a mixture of purified α + β and γ.*

artifactual proteolysis appeared to be minimized by this technique.

A prominent band of low mobility (estimated size, 33 kilodaltons) was released by acid rupture of T_{10} to T_{15} spores (Fig. 4, lanes 3 and 4), but was only a minor component in extracts of older spores (Fig. 4, lanes 1 and 2). It probably corresponded to pH 4.7 PAGE band 4 (Fig. 6), and its relationship to other ASSPs is unknown. Its disappearance may reflect a very late stage in spore maturation.

Yield of component δ (Fig. 4) was somewhat variable (see below). This component migrated with γ on pH 4.7 gels (Fig. 6) and is possibly related, since it also lacked Met. The most basic ASSP (Fig. 6, band 12) migrated with α + β on SDS-PAGE, but was absent in acetic acid extracts. This and the other relatively basic ASSPs (Fig. 6, bands 5 to 11) labeled with [^{35}S]methionine and could be separated from α + β and γ + δ by fractionation of CM cellulose (data not shown). Bands 5 to 11 (Fig. 6) comprised the components of mobility intermediate between α + β and γ on SDS-PAGE (Fig. 4), of which three are prominent and three are minor.

Variation of ASSP pattern with extraction procedure. Spores of four different *B. subtilis* strains were extracted by acid rupture in HCl or HNO_3, or by acetic acid, HCl, or HNO_3 after dry breakage. Combinations of acids were used in an attempt at optimizing yields of total ASSPs (Table 4) or SDS-PAGE patterns (Fig. 7). These undialyzed preparations gave less clear patterns, but the following conclusions can be drawn from these and several similar experiments. After dry breakage, acetic acid extraction (Fig. 7A, lanes 2, 5, and 8) gave the best recovery of ASSPs, consisting mostly of α, β, and γ. Extraction with 2 N HCl (Fig. 7A, lanes 3, 6, and 9) gave some additional components, but not the amount of δ or the total complexity seen after rupture in 2 N HCl (Fig. 7A, lanes 11, 13, and 15), nor was the extraction of dialyzable, presumably degraded protein prevented (Table 4). Extraction of broken spores with HNO_3 (Fig. 7A, lanes 4, 7, and 10) or rupture in HNO_3 (Fig. 7A, lanes 12, 14, and 16) gave irreproducible recoveries (Table 4). In contrast to rupture in 2 N HCl, rupture in even 0.5 N HNO_3 was virtually instantaneous at 0°C. However, such brief exposure did not improve recoveries. Spores did not rupture efficiently in 2 N H_2SO_4, $HClO_4$, or formic acid.

Secondary extraction with an alternate acid (Table 4) improved yields. For example, acetic acid solubilized mostly α, β, and γ from HCl-ruptured spores (Fig. 7B, lane 12) and α + β from HCl-extracted dry-broken spores (Fig. 7B, lane 2). However, recovery of other ASSPs by HCl extraction of acetic acid-extracted dry broken spores (Table 4; Fig. 7B, lane 1) was considerably less than after rupture in HCl.

In conclusion, acid rupture in 2 N HCl gave the most consistent recovery of total ASSPs from all strains tested. No significant variation in yield or pattern of ASSPs was seen in several preparations from spores of strain 168 grown in Difco sporulation medium, MSM, or potato extract medium.

Strain variation. Differences between ASSPs recovered from spores of strains 168, 168 (*trp*$^+$), and BR-151 (Fig. 7A) were minor, and no greater than between different preparations of strain 168 spores. Yields of total ASSPs from strain SMY, however, were consistently about 25% lower (Table 4). Although the patterns seen by SDS-PAGE were similar (Fig. 8), a relative reduction in α + β content was seen in all extracts. Among several other *B. subtilis* strains investigated, this was the major variation seen.

Accumulation and degradation of ASSPs. Sporulating and germinating spores failed to rupture in 2 N HCl. Cleaned, lyophilized

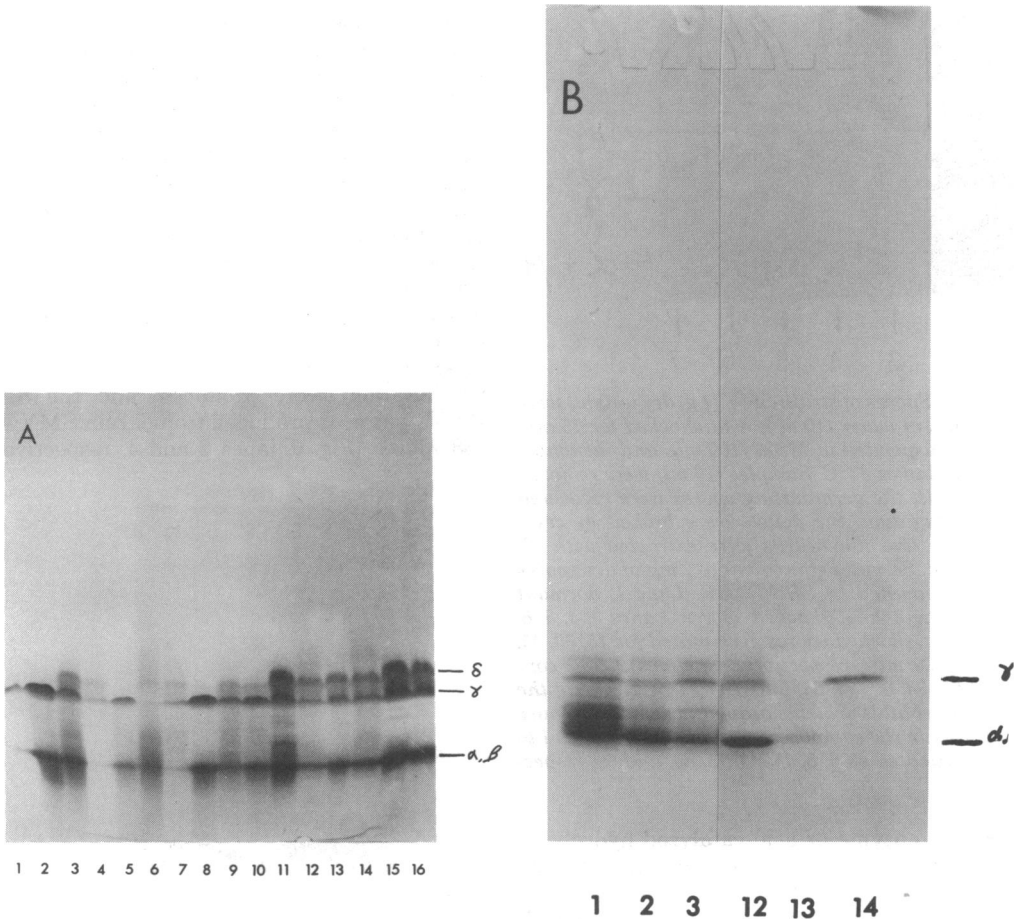

FIG. 7. *ASSP extraction procedures. (A) Primary extracts. Acid-soluble extracts were prepared from mature spores as described in Table 4, footnote a, and fractionated on a 7.5 to 15% gradient SDS-polyacrylamide gel. Each lane contained 30 μg of protein except lane 4, which contained 15 μg. Lanes 2 to 10 contained acid extracts from dry broken spores; lane 1 contained a mixture of purified α, β, and γ. Lanes 2, 3, and 4 contained acetic acid, HCl, and HNO_3 extracts, respectively, of dry broken strain 168 spores; lanes 5, 6, and 7 were similar extracts of strain 168 (trp^+) spores; lanes 8, 9, and 10 were extracts of strain BR151 spores. Samples in lanes 11 to 16 were obtained by acid rupture of intact spores. Lanes 11 and 12, HCl and HNO_3 extract of strain 168 spores; lanes 13 and 14, HCl and HNO_3 extracts of ruptured strain 168 (trp^+) spores; lanes 15 and 16, HCl- and HNO_3-ruptured strain BR151 spores. (B) Secondary extracts. A sample (40 μg) of each secondary extract (Table 4) was lyophilized and suspended in 40 μl of SDS sample buffer just before electrophoresis on a 7.5 to 15% gradient gel. Only data for strain 168 are shown. Data for the other strains were similar. Lane 1, HCl after acetic acid; lane 2, acetic acid after HCl; lane 3, acetic acid after HNO_3; lane 12, acetic acid extract of HCl-ruptured spores. Lane 13 contained 10 μg of purified α and β. Lane 14 contained 10 μg of purified γ.*

cells at these stages were also much more difficult to break in the dry state than were spores, and since some degradation of ASSPs frequently occurred during dry breakage, especially when prolonged (presumably because of transient proteolysis), a more efficient method for ASSP extraction applicable to cells was sought. Breakage of cells in the Bronwill mill in dry ethanol or isopropanol was more efficient, but breakage of spores under these conditions produced lower yields of ASSPs. Breakage of spores in 2 N HCl gave good yields and patterns of ASSPs, but since it is impossible to achieve accurate temperature control with this mill, hydrolytic damage might occur. It was found that shattering of wet strain 168 spore pellets, frozen in liquid nitrogen, by shaking in a steel vessel (21), produced normal ASSP yields and patterns (Fig. 8). Melting of these deep-frozen fragments in acid at 0°C appeared to guarantee minimal oppor-

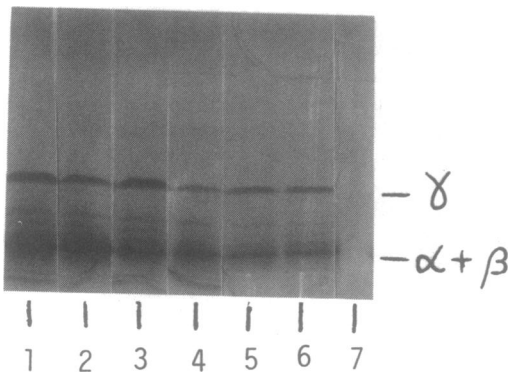

FIG. 8. *Spores of strain SMY (1 g, dry weight) were suspended in water (10 ml), heat shocked for 15 min at 80°C, suspended in MSM (100 ml), and incubated with shaking at 37°C. Samples (10 ml) were removed at intervals, the germinating spores were recovered by centrifugation, the pellets were broken by cryo-impaction, and the ASSPs were extracted with 3% acetic acid. Samples equivalent to 2 mg of dry spores were fractionated by SDS-PAGE. Lane 1, dormant spores; lane 2, heat-shocked spores. Lanes 3, 4, 5, 6, and 7, heat-shocked spores germinated for 15, 30, 45, 60, and 120 min, respectively. Samples 2 to 7 contained 95, 84, 62, 33, 25, and 12% respectively, of the total acid-soluble proteins present in sample 1. Phase darkening in the samples corresponding to lanes 1 to 7 was estimated as 5, 5, 15, 60, 75, 80, and 98, respectively.*

tunity for enzyme or acid-catalyzed hydrolysis. Under these conditions, extraction with 3% HOAc produced mostly $\alpha + \beta$ and γ (Fig. 8), whereas extraction with HCl (at 0°C, which precludes acid-catalyzed rupture) gave a pattern closely resembling HCl rupture extracts (data not shown). Use of these acids in sequence produced the same yield of non-dialyzable ASSPs (3.2%) as did acid rupture (Table 4), and there was no evidence of degradation.

With this procedure, it was demonstrated that accumulation of ASSPs was detectable in cells of strain 168 sporulating in MSM at 37°C as early at T_4, with pronounced accumulation by T_5 (1 h before fore-spores became phase bright). Similarly, ASSPs disappeared during germination in MSM with approximately the same kinetics as phase darkening (Fig. 8).

Location of ASSPs. Spore coats comprise about 50% of the total protein of *B. subtilis* spores, and 70% of this coat protein can be solubilized from spore coat preparations by a combination of high pH, reducing agent, and denaturant (SDS) (4). Of the solubilized coat components, 75% consist of three major and several minor low-molecular-weight species (4). The same fraction of spore coats can be solubilized from intact spores at 37°C by urea-dithiothreitol-SDS (UDS) buffer, pH 9.8 (8). Such extracts of cleaned strain SMY and 168 spores gave the patterns shown in Fig. 9, lanes 1 and 3, respectively. The extracted spores, having lost 35% of their protein (assayed after acetone precipitation of the UDS extract) remained refractile, viable, still temporarily dormant and heat-resistant, and responsive to normal germinants. They thus retained many of the properties of unextracted spores. However, the extracted spores were now germinated rapidly by lysozyme, indicating that this 14,500-dalton protein has access to their cortices. These extracted spores still ruptured in 2 N HCl. Normal yields of ASSPs (4% of total spore protein after acetone precipitation) were produced, and the SDS-PAGE patterns produced from strain SMY and 168 spores (Fig. 9, lanes 2 and 4, respectively)

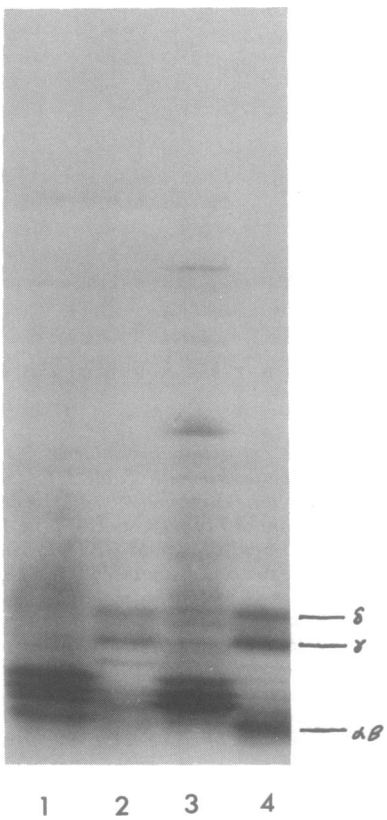

FIG. 9. *Sequential extraction of coat proteins and ASSPs. Mature, cleaned spores were extracted with UDS butter (8), washed, and then ruptured in 2 N HCl. Samples (30 μg) of the proteins solubilized by UDS from spores of strains SMY and 168, fractionated by SDS-PAGE, and detected by Coomassie brilliant blue are shown in lanes 1 and 3, respectively. ASSPs solubilized by acid rupture of UDS-extracted strain SMY spores (30 μg) are shown in lane 2, and ASSPs from UDS-extracted strain 168 spores (60 μg) are shown in lane 4.*

were essentially normal, although the normally low content of $\alpha + \beta$ in ASSPs from SMY spores was further reduced (note the low protein load in this lane). $\alpha + \beta$ and γ comigrate with minor coat species, but all of these coat proteins are insoluble in acid, and solubilized coat proteins fail to compete with labeled $\alpha + \beta$ and γ for precipitation by antisera to these ASSPs (unpublished data). Moreover, antisera to coat protein (R. C. Goldman and D. J. Tipper, unpublished data) fail to precipitate ASSPs. The coat protein migrating with $\alpha + \beta$ (reported to be a glycoprotein [8]) stained a characteristic purple color with Coomassie brilliant blue, quite different from the typical dark blue of the $\alpha + \beta$ band. It can be concluded that the major ASSPs are not part of the coat. They would presumably be lost from UDS-extracted spores, if they were outside of the cortex. Their probable location is in the spore cytoplasm, as previously surmised (14).

DISCUSSION

The specific acid-soluble proteins described in this paper make up 3 to 3.5% of the dry weight, or about 5% of the total protein, of *B. subtilis* spores. This is considerably less than the 15 to 20% reported for *B. megaterium* spores (3), but still represents a large fraction of the spore cytoplasmic protein, since 50% of the spore protein is found in coats and another 20% is estimated to reside in membranes (4). Additional low-molecular-weight protein extracted after dry breakage which was dialyzable and did not give discrete bands on PAGE was presumably an artifact of proteolysis since it was not found after acid rupture or cryo-impact breakage. This fraction was increased (with reduction of $\alpha + \beta$ and especially of γ) unless great care was taken to exclude moisture during breakage. Yields of ASSPs obtained by acid rupture from spores of strain 168 and related strains have been very consistent. Yields from strain SMY were somewhat smaller, the most consistent reduction being in the $\alpha + \beta$ component. We believe that patterns derived by SDS-PAGE after rupture in 2 N HCl represent the true complexity and content of spore ASSPs.

The α, β, and γ components make up about 18, 18, and 36% of the ASSPs, respectively, in acid-rupture extracts of strain 168. In relative charge and size, these resemble the A, C, and B components of *B. megaterium* ASSPs; however, the relationship is not close (26). Indeed, whereas antisera raised separately against $\alpha + \beta$ and γ cross-react with $\alpha + \beta$ and γ (Johnson and Tipper, manuscript in preparation) and antisera raised against the *B. megaterium* proteins also cross-react, neither set of antisera gives significant interspecific cross-reaction (26). Neither the amino acid compositions of $\alpha + \beta$ and γ nor the N-terminal sequence of purified γ bears profound similarity to the *B. megaterium* ASSP sequences (18, 19, 26; Setlow and Ozols, in press). However, function as storage proteins seems to be conserved since the ASSPs were degraded during germination (Fig. 8). Also, γ is cleaved twice by the exquisitely specific *B. megaterium* spore endopeptidase whose only other known natural substrates are the *B. megaterium* ASSPs (26). $\alpha + \beta$ is also cleaved (C. W. Johnson et al., unpublished data). Conservation of this degradation mechanism, which is presumably activated during germination, indicates its functional importance and the primacy of the storage protein role of ASSPs. In this light, the complexity of ASSPs in both *B. subtilis* and *B. megaterium* is surprising, if they represent the products of distinct genes. In vitro translation of RNA from sporulating cells (D. J. Tipper, C. W. Johnson, G. Chambliss, I. Mahler, M. Arnaud, and H. O. Halvorson, *in* H. S. Levinson, A. L. Sonenshein, and D. J. Tipper, ed., *Sporulation and Germination*, in press) indicates that the major ASSPs are derived from discrete messengers, and in vivo labeling (Johnson and Tipper, in preparation) indicates that synthesis of *B. subtilis* ASSPs during sporulation is not completely coordinated, suggesting independent expression of multiple genes.

The less abundant ASSPs, δ and components 6, 7, and 8 (Fig. 1), presumably provide amino acids missing in $\alpha + \beta$ and γ. Their fractionation and further analysis are in progress.

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