The Major Acid-soluble Proteins of *Bacillus subtilis* Spores: Partial Amino Acid Sequence and Forespore Location of Their mRNAs

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In *Bacillus subtilis* the α , β , γ and δ components comprise 80–90% of the total acid-soluble spore proteins (ASSPs). Sequence analysis demonstrates that α and β share 32 of their first 36 amino acids and are closely related to the A and C ASSPs of *Bacillus megaterium* spores, confirming the results of analysis of their cloned genes. Despite the difference in apparent size of γ and δ , they have identical N-terminal sequences (37 residues). Unless γ and δ derive from very recently duplicated genes, it appears that γ is derived from δ , either *in vivo* or during isolation. Although the sequenced regions of γ and δ have no homology to α and β , outside of the previously recognized pentapeptide recognition sequence for the spore endopeptidase, they share 10 and 15 residue peptides flanking this sequence with ASSP B of *B. megaterium*, but in reverse order. At least two groups of ASSPs have, therefore, been conserved between *B. subtilis* and *B. megaterium*: the multigene AC $\alpha\beta$ family and the B $\gamma(\delta)$ group. Sequence conservation in each group implies selection for functions in addition to storage. Both the α and β components of *B. subtilis* ASSPs and their mRNAs are located in the forespore compartment of cells at $t_{5.5}$ of sporulation, the time of most rapid ASSP synthesis. The sizes of these transcripts (250–350 bp) and their ability to direct the *in vitro* synthesis of ASSPs of mature size, indicate that genes for these ASSPs are monocistronic, consistent with dispersed map location. Synthesis of ASSPs is, therefore, coordinately controlled by selective transcription in the forespore.

INTRODUCTION

Approximately 20% of the protein of dormant spores of *Bacillus megaterium* and 8% of that in spores of *Bacillus subtilis* is rapidly degraded during spore germination, providing amino acids essential for both metabolism and protein synthesis during outgrowth (Setlow, 1981; Johnson & Tipper, 1981). These proteins comprise a group of small, acid-soluble spore proteins (ASSPs), defined by solubility in acid following dry breakage (Setlow, 1975) or spontaneous rupture of mature spores in 2 M-HCl (Johnson & Tipper, 1981). They are synthesized coordinately, late in sporulation (Dignam & Setlow, 1980; Tipper *et al.*, 1981; Johnson *et al.*, 1985) under transcriptional control by early *spo* genes (Johnson *et al.*, 1985) and accumulate in the forespore (Setlow, 1981; this paper).

Three ASSPs, A, B and C in *B. megaterium* (Setlow, 1981), and α , β , and γ in *B. subtilis* (Johnson & Tipper, 1981), make up at least 80% of the total ASSPs in both species. These are the most easily extracted ASSPs, for example, in 0.5 M-acetic acid, although a number of less well-characterized minor ASSPs are found when stronger acids are used for extraction (Setlow, 1981;

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Abbreviations: ASSP, acid-soluble spore protein; DFP, diisopropyl fluorophosphate.

Johnson & Tipper, 1981). Among the predominant ASSPs, the two smallest (A and C, α and β : each 60–70 amino acid residues in length) are closely related in biochemical properties and also show inter-species similarity, as indicated by low but distinct immunological cross-reactivity (Johnson & Tipper, 1981; Yuan *et al.*, 1981). In contrast, B and γ are considerably larger and are much less closely related to the other ASSPs. δ , the major new species extracted from *B. subtilis* spores by 2 M-HCl, has an apparent size of about 105 amino acid residues, and otherwise resembles γ (about 90 residues) in biochemical properties (absence of methionine, pI, behaviour on ion-exchange chromatography; Johnson & Tipper, 1981; Johnson *et al.*, 1985).

Degradation of ASSPs is initiated by a unique endopeptidase (Setlow *et al.*, 1980), also produced during sporulation and stored in the spore, which is specific for glutamate-bounded pentapeptide sequences resembling Glu-Ile/Phe-Ala-Ser-Glu. This sequence occurs once in A and C and twice in B and γ (Yuan *et al.*, 1981) and, with minor variations, in all ASSP sequences so far identified.

Cloning of genes for C (Curiel-Quesada *et al.*, 1983) and related *B. megaterium* ASSPs (Fliss *et al.*, 1986) has identified at least seven genes, belonging to a closely related multigene family. All are expressed together, although only A and C produce easily detectable quantities of product. Cloning of related *B. subtilis* genes, initially using the *B. megaterium* C gene as probe, has recently shown that a similar family exists in *B. subtilis* (Connors *et al.*, 1986a): four closely related ASSP genes were found, including two, *sspA* and *sspB*, deletions in which result in absence of ASSPs α and β , respectively, from spores (Connors *et al.*, 1986a).

We have purified α , β , γ and δ , and obtained N-terminal sequences for their first 37–38 amino acid residues in order to compare these sequences with each other and with the related *B. megaterium* proteins. The sequences for α and β show complete agreement with the sequences predicted for the *sspA* and *sspB* gene products. The γ and δ sequences were analysed both for similarity to B and to determine whether they may represent members of a second multigene family.

mRNAs for the *B. megaterium* A and C proteins (Dignam & Setlow, 1980) and for the *B. subtilis* α and β proteins (Tipper *et al.*, 1981; Leventhal *et al.*, 1981; Leventhal & Chambliss, 1982; Johnson *et al.*, 1985), identified by *in vitro* translation, are easily recovered from cells in late stages of sporulation. Both ASSPs and their mRNAs accumulate between $t_{4.5}$ and t_6 in *B. subtilis* (from 4.5 to 6 h after the start of sporulation (Johnson *et al.*, 1985). We have now characterized the mRNAs for α and β by size fractionation and show that both α and β ASSPs and their mRNAs accumulate in the forespores of sporulating cells. Thus the ASSP genes are expressed in the forespore, the site of accumulation of their products.

METHODS

Bacterial strains. *B. subtilis* strains 168 *trpC2* (originally obtained from Dr H. O. Halvorson) and SMY (obtained from Dr R. Losick) were grown and sporulated in MSM medium at 37 °C as previously described (Johnson & Tipper, 1981). The start of sporulation, t_0 , is assumed to be the point at which the exponential increase of culture turbidity abruptly changes to a slower linear rate of increase (Johnson & Tipper, 1981). Under these conditions, spore septum formation occurs 2.5 h later (at $t_{2.5}$), and forespore engulfment is complete at $t_{3.5}$. Accumulation of ASSPs and of phase-white, UV-resistant and heat-resistant spores reaches 50% at t_6 , t_6 , t_7 and $t_{7.5}$, respectively (Johnson *et al.*, 1985).

Gel electrophoresis. ASSPs were fractionated according to size by SDS-PAGE using gradients from 7.5 to 15% (w/v) acrylamide, and according to charge by PAGE in 6 M-urea in pH 4.7 β -alanine buffer, or in aluminium lactate buffer at pH 3.6 (Lauriere & Mosse, 1982) as previously described (Johnson *et al.*, 1985).

Forespore isolation and preparation of mother cell extracts. Cultures of sporulating cells (100 ml) at t_5 to t_6 were harvested by pouring over crushed ice (100 ml) containing sufficient MgCl_2 and $\text{Na}_2\text{S}_2\text{O}_3$ to give final concentrations of 10 and 20 mM, respectively. Cells were centrifuged (5 min at 9000 g), the pellets washed in 50 mM-potassium phosphate, 10 mM- MgSO_4 , 0.6 M-sucrose, pH 7.4 (buffer S), and suspended in 10 ml buffer S containing 1 mg lysozyme ml^{-1} . After 15 min at 37 °C, cells were washed twice in buffer S at 0 °C by centrifugation. Lysozyme-treated cells in 2 ml buffer S at 0 °C were sonically disrupted, release of phase-grey forespores being followed by phase microscopy. After 5–10 treatments (10 s each) the pellet from centrifugation (4 min, 8000 g) was resuspended in buffer S, disrupted for a further two periods of 10 s, and forespores were isolated by centrifugation (4 min, 8000 g), almost free of unbroken cells and mother cell debris. Recovery, determined by cell number (phase microscopy), was about 25% at t_5 , 40–50% at $t_{5.5}$, and 50–60% at t_6 .

Mother cell extracts for ASSP isolation were prepared from the same lysozyme-treated sporulating cells as used for forespore isolation. Cells from 50 ml cultures were suspended in buffer S (1 ml) containing 5 mM-EDTA, 2.5 mM-phenylmethylsulphonyl fluoride (PMSF) and 0.1 mM-diisopropyl fluorophosphate (DFP) and sonically disrupted, as described above (two to five 10 s periods). This treatment is sufficient to disrupt about half of the lysozyme-treated mother cells, leaving at least 90% of the forespores intact. After centrifugation at 4 °C (20 min, 10000 g), the supernatant was diluted in 50 mM-potassium phosphate, 150 mM-NaCl, 2 mM-EDTA, 0.5 mM-PMSF, pH 7.4, containing 2 mg bovine serum albumin ml⁻¹ (buffer I) plus 0.1 mM-DFP for immunoprecipitation (see below).

Isolation of ASSPs. For isolation of α , β and γ , dry-broken spores were extracted with 0.52 M-acetic acid at 0 °C, as previously described (Johnson & Tipper, 1981). This extraction procedure gives optimal yields of α , β and γ , and only small quantities of more basic ASSPs. ASSPs were separated from low-molecular-mass spore metabolites, including calcium dipicolinate, unidentified oligopeptides and soluble glycan fragments, by fractionation on Sephadex G10 in 0.52 M-acetic acid. Isolation of ASSPs from sonically-isolated forespores followed essentially the same procedure. Forespores were washed in 100 mM-potassium phosphate, 5 mM-EDTA, 2.5 mM-PMSF (pH 7.4; buffer A). After centrifugation, the pellets were lyophilized, dry-broken and extracted as above.

For immunoprecipitation, the lyophilized ASSP extracts were dissolved in buffer I and precipitated with anti- $\alpha\beta$ IgG (the IgG fraction of rabbit polyclonal antiserum raised to a mixture of α and β), as previously described (Johnson *et al.*, 1985). This antibody precipitates both α and β with high efficiency. All steps were performed at 0–4 °C. Mother cell extracts, prepared in buffer S plus DFP as described above, were immunoprecipitated after dilution in buffer I in the same manner. Immunoprecipitates were assayed by SDS-PAGE and autoradiography.

For sequence analysis, ASSP preparations were fractionated on a Waters model 660 (HPLC) system using a Waters MicrobondaPak C₁₈ radial compression column and a 30 ml linear gradient from 15 to 60% (v/v) acetonitrile in 0.04% trifluoroacetic acid at 1 ml min⁻¹. Protein peaks were detected by absorbance at 210 nm. γ eluted as a pure species at 27% acetonitrile and α plus β as an unresolved mixture at 37% acetonitrile.

The mixture of α and β was fractionated by preparative slab gel electrophoresis at pH 3.6. The protein bands were detected on strips excised from the gel by staining with Coomassie brilliant blue R-250, and the appropriate unstained gel regions eluted for 6 h at 20 °C in 0.52 M acetic acid/acetonitrile (1:1, v/v). Recovery after repeated elution was 50–60%. Following lyophilization, α and β were individually repurified by HPLC as described above.

For isolation of δ , intact spores were ruptured by stirring at 20 °C in 2 M-HCl (Johnson & Tipper, 1981). Extracts were chilled to 0 °C, and after addition of acetic acid to 0.52 M, the HCl was neutralized by slow addition of 6 M-NaOH with rapid stirring. After brief dialysis (4 × 2 h) in low-porosity dialysis tubing against 0.52 M-acetic acid and lyophilization, ASSPs were desalted on a Sephadex G10 column. Fractionation at 4 °C on carboxymethylcellulose in a linear gradient of NaCl (0–0.35 M) in 50 mM Tris/maleate buffer pH 6.0 (Johnson & Tipper, 1981) produced a mixture of γ and δ essentially free of other ASSPs. They were fractionated from each other on a phosphocellulose column using a linear NaCl gradient from 0.3 to 1.2 M in the same buffer and residual impurities were removed by fractionation by HPLC, as described for α and β . Both proteins eluted at 27% acetonitrile.

Sequence analysis. Purified proteins were sequenced using a Beckman model 890C liquid-phase sequencer using the 0.1 M-Quadrol program 102474. The phenylthiohydantoin amino acid derivatives were identified and quantified by HPLC on C₁₈ columns, using both acetonitrile and methanol elution gradients. Serine was identified qualitatively as dehydroserine. Since α , β and γ are devoid of cysteine (Johnson & Tipper, 1981), and neither γ nor δ labels with [³⁵S]sulphate (unpublished observations), this identification is unique.

Isolation of RNA. Total RNA was extracted from sporulating cells by suspension of washed pellets from 1-litre cultures in *m*-cresol (5 ml), freezing at –80 °C, and passage through a French press at 10000 lbf in⁻² (69 MPa). Subsequent steps were as previously described (Tipper *et al.*, 1981; Johnson *et al.*, 1985). The quality of RNA preparations was judged from the patterns of ribosomal and other RNA species seen after agarose gel electrophoresis and staining with ethidium bromide and also by the patterns of translation products (see below). These were highly reproducible.

RNA was further fractionated on sucrose gradients and by acrylamide gel electrophoresis. The use of 40% (v/v) dimethyl sulphoxide in the sucrose gradients improved both the resolution and recovery of RNA species. Individual fractions were analysed on agarose gels, and sizes estimated from distance migrated relative to the 23S and 17S rRNAs and 4S tRNAs.

Forespore RNAs were isolated as for total-cell RNA. Selective isolation of mother cell RNA was attempted by lysozyme treatment of *t*_{5.5} cells in buffer S, as for forespore treatment, followed by suspension of the cells in *m*-cresol (5 ml) and passage through the French press at 2000 lbf in⁻² (13.8 MPa). RNA was recovered from the aqueous phases as above. Light microscopy indicated that 60–80% of the cells and apparently all of the forespores remained intact during this procedure, but the extent to which the RNA contents of mother cell and forespore compartments were extracted is unknown. RNA recoveries were also variable.

Translation, immunoprecipitation and analysis of products. RNA samples were translated in an *Escherichia coli* S30 system using L-[³⁵S]methionine as label (Arnaud *et al.*, 1980). The system was optimized, by adjustment of metal ion and RNA concentrations and reaction time, to obtain high-molecular-mass polypeptides from MS2 RNA

(Miles Research Products) and *B. subtilis* vegetative cell RNA, as previously described (Arnaud *et al.*, 1980; Johnson *et al.*, 1985). Products were analysed by SDS-PAGE and autoradiography. Patterns seen from translation of total RNA samples isolated from cells at particular stages of sporulation were highly reproducible, and incorporation into specific bands was proportional to total RNA concentration. Where indicated, products from *in vitro* translation were fractionated by precipitation with antibody to a mixture of α and β , as described above.

RESULTS

Purification of α , β , γ and δ

α and β are the least basic *B. subtilis* ASSPs (pIs 6.58 and 6.67, respectively) and are readily separated from the other ASSPs by fractionation on carboxymethylcellulose columns (Fig. 1, lane 2) (Johnson & Tipper, 1981). They co-eluted on all ion-exchange columns tested and could not be completely resolved by HPLC on C_{18} columns even under isocratic conditions. The mixture of α and β was fractionated by preparative gel electrophoresis at pH 3.6. A final HPLC fractionation removed other contaminants, including small amounts of γ , which is poorly resolved from β at pH 3.6, but well resolved on C_{18} columns. Preparations were devoid of contaminants detectable by HPLC or by electrophoresis at pH 3.6 or in SDS (Fig. 1, lanes 3 and 4).

Because δ is not extracted from dry-broken spores with 0.52 M-acetic acid, γ , extracted by this procedure, is readily purified by carboxymethylcellulose chromatography (Johnson & Tipper, 1981) or by C_{18} HPLC (see Methods). The mixture of γ and δ produced by 2 M-HCl extraction (Fig. 1, lanes 1 and 9) is just as readily fractionated by the same procedures from other ASSPs, but is poorly resolved on either type of column. Complete resolution was achieved by phosphocellulose column chromatography. After fractionation by C_{18} HPLC to remove residual impurities and salts, each gave a single band on electrophoresis in SDS (Fig. 1, lanes 5 and 6) or at pH 4.7 or 3.6 (not shown).

Amino acid analyses and sequences

Data previously reported for the approximately 2:1 natural mixture of α and β demonstrate that both label with [35 S]methionine and lack Trp, Cys, His and Tyr (Johnson & Tipper, 1981; Johnson *et al.*, 1985). Separate analyses of purified α and β confirmed this and also demonstrated the presence of Pro in each, previously thought to be absent (data not shown). Both α and β contained over 25% Glu plus Asp and their amides, consistent with their roles as storage proteins. The data were close to those predicted by the sequences of the *sspA* and *sspB* genes (Connors *et al.*, 1986a). The sequences determined for α and β are aligned and compared with those of the *B. megaterium* A and C proteins in Fig. 2. Combination of the data from two runs gave unambiguous data for more than 30 residues.

Amino acid contents determined for γ and δ were very similar except for the higher content of Glu plus Gln in δ (data not shown). Neither contained His, Met, Tyr or Pro. Previous analysis of γ indicated the absence of Cys and Trp (Johnson & Tipper, 1981). Sequence analysis of γ and δ gave identical results for 37 residues, with unambiguous data for at least 33 residues. Analysis of the first 15 residues of a preparation of δ independently isolated from spores of strain SMY also gave identical results (data not shown). A comparison of the γ and δ sequences with the sequence of *B. megaterium* ASSP B is shown in Fig. 3.

ASSPs α and β and their mRNAs are located in the forespore

Recovery of ASSPs by acid extraction of dry-broken $t_{5.5}$ or t_6 forespores was 40% of that from intact spores, and all ASSP species were represented in the same ratios (data not shown). Since the yield of these vigorously cleaned forespores was also 40–50% of intact sporulating cells, and since $t_{5.5}$ mother cell extracts were essentially devoid of ASSPs detectable by immunoprecipitation, it is concluded that, as in *B. megaterium* (Setlow, 1981), ASSPs accumulate in the forespores of *B. subtilis*.

Forespores also contained high mRNA activity for α and β ASSPs (Fig. 4, lane 4). By densitometry of the $\alpha\beta$ translation product, the actual recovery of $\alpha\beta$ mRNA activity was 55–60% of that seen in RNA prepared by the same procedure from equivalent quantities of t_6 whole

Fig. 1. Gradient (7.5–15%) SDS-PAGE of ASSP fractions. The mobilities of α , β , γ and δ are as indicated. Lanes 1 and 9, total 2 M-HCl extracted ASSPs from *B. subtilis* spores. Lane 2, fraction unabsorbed by carboxymethylcellulose (crude α plus β). Lanes 3 and 4, α and β , respectively, after preparative gel electrophoresis at pH 3.6. Lanes 5 and 6, purified γ and δ , respectively, after phosphocellulose chromatography. Lane 7, fraction eluting late from carboxymethylcellulose: predominantly ϵ (comigrating with α and β ; Johnson *et al.*, 1985). Lane 8, mixture applied to phosphocellulose column.

ASSP	2	4	6	8	10	12	14	16	18		
<i>B. subtilis</i> α	(Met) Ala Asn	Asn	Asn Ser	Gly Asn	Ser Asn	Asn	Leu	Leu Val	Pro Gly	Ala Ala Gln	
<i>B. subtilis</i> β	(Met) Ala Asn	Gln	Asn Ser		Ser Asn	Asp	Leu	Leu Val	Pro Gly	Ala Ala Gln	
<i>B. megaterium</i> A	(Met) Ala Asn				Thr Asn	Lys	Leu	Val Ala	Pro Gly	Ser Ala Ala	
<i>B. megaterium</i> C	(Met) Ala Asn	(7 residues)	Asn Ser		Ser Asn	Lys	Leu	Val Ala	Pro Gly	Ala Gln Ala	
	20	22	24	26	28	30	32	34	36	38	40
<i>B. subtilis</i> α	Ala Ile Asp Gln Met Lys	Leu	Glu Ile Ala Ser Glu	Phe Gly Val Asn Leu Gly							
<i>B. subtilis</i> β	Ala Ile Asp Gln Met Lys	Leu	Glu Ile Ala Ser Glu	Phe Gly Val Asn Leu Gly						Ala (Ser) Thr Thr	
<i>B. megaterium</i> A	Ala Ile Asp Gln Met Lys	Tyr	Glu Ile Ala Ser Glu	Phe Gly Val Asn Leu Gly						Pro Glu Ala Thr	
<i>B. megaterium</i> C	Ala Ile Asp Gln Met Lys	Phe	Glu Ile Ala Ser Glu	Phe Gly Val Asn Leu Gly						Pro Asp Ala Thr	
Conserved in ACαβ family:	Ala Ile Asp Gln Met Lys (Glu)		Glu Ile Ala Ser Glu	Phe Gly Val							
Endopeptidase recognition site:			Glu Ile Ala Ser Glu Phe(Gly)Thr Val Gln								

Fig. 2. Homology of *B. subtilis* α and β with *B. megaterium* A and C ASSPs. The data for the *B. megaterium* proteins are taken from Fliss *et al.* (1986). A dot indicates the absence of any corresponding amino acid. Residue numbers indicated are for α , assuming removal of N-terminal methionine (Connors *et al.*, 1986a) *in vivo*. Residue 37 of β is probably Asp rather than Ser (see text). The arrow shows the site of cleavage by the ASSP-specific spore endopeptidase. Its consensus pentapeptide recognition sequence (bottom line) is found in all published ASSP sequences. Parentheses indicate a residue found in one example only. The much more extensive sequence conserved in 9 of the 11 members of the AC α β family (see text) is also shown.

Fig. 3. Comparison of *B. subtilis* γ and δ with *B. megaterium* B ASSP. Most of the sequence of the *B. megaterium* B ASSP (Setlow & Ozols, 1980) is shown in the upper section, illustrating the large, near-perfect repeats (residues 19-50 and 52-85), which are centred around the pentapeptide spore endopeptidase recognition sequences (vertical box; see Fig. 2). The cleavage site is indicated by arrows. The sequence of γ and δ is aligned below to illustrate the discontinuous matching of 25 out of its 37 known residues with the repeat in B.

Fig. 4. mRNAs for ASSPs α and β are located in forespores. Total products of translation of *B. subtilis* vegetative cell RNA and of various RNA preparations from t_6 cells, corresponding to equal culture volumes, were fractionated by SDS-PAGE and detected by autoradiography. Lane 1, no RNA added to the *E. coli* S30 preparation. Lane 2, vegetative cell RNA. Lane 3, t_6 mother-cell RNA. The band running above $\alpha\beta$ but below the 12.3 kDa marker is unidentified and comigrates with a band endogenous to the *E. coli* S30 extract (lane 1). Lane 4, t_6 forespore RNA. Lane 5, t_6 whole-cell RNA. Lane 6 shows the positions of Coomassie-stained $\alpha\beta$ and of labelled marker proteins: ovalbumin (43 kDa), α chymotrypsinogen (25.7 kDa), lactalbumin (17.2 kDa), cytochrome *c* (12.3 kDa) and thrombin (5.7 kDa).

cells (Fig. 4, lane 5). Products from cells or forespores corresponding to identical volumes of culture are shown in each lane of Fig. 4. Immunoprecipitation of these *in vitro* translation products with anti- $\alpha\beta$ IgG and fractionation at pH 3.6 showed that both whole-cell and forespore RNAs contained messenger for both α and β (data not shown).

Mother-cell RNA failed to produce detectable $\alpha\beta$ mRNA activity. Since the low-pressure French press procedure produced RNA giving recognizable patterns of translation products from lysozyme-treated vegetative cells (not shown), and since $\alpha\beta$ mRNA is relatively stable, it seems possible that any $\alpha\beta$ mRNA present in the mother cell should have been detected by this technique. It is concluded that most, if not all, $\alpha\beta$ mRNA is located in the forespore of sporulating cells at t_6 .

α and β mRNAs are 250–350 bp in length

Total RNA from $t_{5.5}$ cells was fractionated on a 10–30% linear sucrose gradient. RNA was recovered from individual fractions by ethanol precipitation. The translation products from equivalent amounts of RNA are shown in Fig. 5(a) for fractions in the 12S (lane 2) to 4S (lane 9) size range. Products co-migrating with α and β were found principally in the 9–6S region, shown in lanes 5–8.

The RNA in the 9–6S region was pooled and refractionated on a second 5–20% sucrose gradient. RNA was recovered and analysed by translation, as before (Fig. 5b). Only the products

Fig. 5. Sucrose gradient fractionation of mRNAs for α and β . (a) Total RNA from a 1-litre culture of *B. subtilis* 168 at $t_{5.5}$ was fractionated in a 10–30% sucrose gradient in 40% dimethyl sulphoxide. RNA, recovered from individual fractions by ethanol precipitation, was translated and analysed as in Fig. 4. Labelled protein size markers (lane 1) are as in Fig. 4. The position of Coomassie-stained $\alpha\beta$ is also indicated. Lanes 2–9, products from successive gradient fractions in the size range from 12S (lane 2) to 4S (lane 9). (b) RNA in fractions 5–8 of (a) was pooled and re-fractionated on a 5–20% sucrose gradient. Lane 10, marker proteins, as in (a); lane 9, endogenous translation products in the S30 system; lanes 1–8, successive fractions from the 8S to 5S region of the gradient.

of RNA fractions in the 8S (lane 1) to 4S (lanes 6–9) region of the gradient are shown: the $\alpha\beta$ mRNA peaked in fractions 2 and 3, at 7S.

Total RNA from t_6 cells was denatured in 50% (v/v) formamide, 2.2 M-formaldehyde (15 min, 55 °C) and fractionated on a 7% acrylamide gel. Gel bands covering the region from 16S to 5S were excised and eluted. Translation of the recovered RNAs showed good recovery of $\alpha\beta$ mRNA activity in a narrow band corresponding to 7S RNA, about 300 bp in length (data not shown).

DISCUSSION

The sequences determined for α and β were identical to those predicted by the sequences of the *sspA* and *sspB* genes (Connors *et al.*, 1986a) except that the antepenultimate residue determined for β appeared to be Ser while the sequence indicates Asp. Since HPLC signals were becoming marginal at this point and two base changes would be required in this codon to produce Ser, Asp is almost certainly correct. The assignments of *sspA* and *sspB* to α and β , respectively, are confirmed. Like all ASSPs sequenced, mature α , and β have lost their N-terminal methionine.

The sequences of α and β are aligned and compared with those of the *B. megaterium* A and C proteins in Fig. 2. Numbering follows the α sequence assuming that the N-terminal alanine is residue 2. The best alignment of α and β is obtained by the indicated insertion of Gly Asn between residues 6 and 7 of β . Before this point, a single conservative replacement of Asn by Gln distinguishes β from α , while the only downstream change is the replacement of Asn in α (residue 11) by Asp in β . Residues 10–35 of β are identical to 12–37 of α , and the sequences of the *sspA* and *sspB* genes (Connors *et al.*, 1986a) indicate that this perfect homology extends for a total of 50 residues with only the C-terminal three residues showing a clear difference between α and β . Derivation of α and β from a recent gene duplication event seems probable.

The boxed regions in Fig. 2 show exact homology with the *B. megaterium* proteins. Comparison of the sites cleaved by the specific spore endopeptidase in γ , B, and members of the A $\text{Ca}\beta$ family (Setlow *et al.*, 1980; Yuan *et al.*, 1981) indicates requirement only for a conserved pentapeptide bounded by Glu residues (Fig. 2) within a quite varied context. The presence of this site of specific cleavage (arrow, Fig. 2) clearly indicates conservation of the role of these proteins as polymeric storage forms of amino acids and organic nitrogen to be utilized on

germination. However, members of the AC $\alpha\beta$ ASSP family share a 24-residue stretch (α residues 15–37) surrounding the cleavage site, in which only a few conservative changes occur. Marked homology within the *B. megaterium* family has previously been emphasized (Fliss *et al.*, 1986), and persistence of this homology across species lines (Connors *et al.*, 1986*a*) indicates strong functional constraints on evolution of these sequences. Secondary structure must presumably be conserved in ASSPs to optimize stability in the developing forespore and to allow access of the pentapeptide cleavage site to the specific spore endopeptidase during germination. However, sequence conservation apparently exceeds such requirements. Conservation of domains involved in protein–protein interactions seems possible. For example, cooperative binding to DNA would be consistent with the postulated role for ASSPs in UV resistance (Mason & Setlow, 1986).

γ is always the major ASSP found in acetic acid or HCl extracts of *B. subtilis* spores, whereas δ is only seen in significant quantities when stronger acids are used for extraction, and even then its recovery is somewhat variable (Johnson & Tipper, 1981). Even though δ migrates more slowly than γ on SDS-PAGE, it is possible that it is an artefactual degradation product of γ , produced during extraction in HCl. It is not a partial deamidation product since it is more basic than γ (Johnson *et al.*, 1985). Proteolytic degradation of γ to δ , or vice versa, seems unlikely in 2 M-HCl, but cannot be excluded. If neither γ nor δ is an artefact, then either γ is derived from δ by C-terminal processing *in vivo*, or they derive from independent genes of very recent divergence. The relationship of γ to δ will probably be resolved only by cloning and analysis of the gene or genes involved.

In the *B. megaterium* B protein (Setlow & Ozols, 1980), residues 21–50 are almost perfectly repeated in residues 56–85 (Fig. 3). The two spore endopeptidase recognition sites (arrow, Fig. 3) fall centrally in these repeats. There is no apparent homology between the flanking peptides and those surrounding the recognition site in the AC $\alpha\beta$ family. Although γ is known to contain two cleavage sites, as in B (Yuan *et al.*, 1981), our sequence only covers the first of these (Fig. 3). This sequence is clearly much more divergent from B than α and β are from A and C, but distinct homology can be seen for 25 of the 37 known residues. γ and δ , besides retaining exactly a decapeptide (residues 28–37) comprising the cleavage site and flanking di- and tripeptides, also retains a 15-amino-acid sequence which, with variation at two sites, follows this decapeptide in the repeated B sequences. However, this sequence precedes the decapeptide in γ and δ (residues 9–23) (Fig. 3). Whatever the function implied by this sequence conservation, it is apparently compatible with shuffling of these sequence blocks between $\gamma\delta$ and B. It appears that *B. subtilis* and *B. megaterium* have in common at least two families of ASSPs, the first represented by α , β , A and C, and the second by γ , δ and B.

Singh *et al.* (1977) demonstrated that *B. megaterium* A and B ASSPs accumulate in the forespores of sporulating cells, as we have now shown both for the α and β ASSPs of *B. subtilis* and for their mRNAs. Since ASSP synthesis is controlled at the level of transcription in both bacillus species (Dignam & Setlow, 1980; Tipper *et al.*, 1981; Johnson *et al.*, 1985), the location of ASSP accumulation is determined by exclusive expression of the ASSP genes in this cell compartment. Although this result was anticipated, ASSPs represent a considerable fraction of total forespore protein and the synthetic capacity of forespores is unknown. It was at least plausible that ASSPs could have been synthesized in the mother cell and transported across the two forespore membranes.

The roughly equivalent yields of α , β and γ originally isolated from spores of *B. subtilis* strain 168 (Johnson & Tipper, 1981) and the co-expression of ASSP components (Johnson *et al.*, 1985) made it plausible that ASSPs might derive from translation of a polycistronic transcript or that they might be derived post-translationally from a polymeric precursor (Johnson & Tipper, 1981). The *in vitro* synthesis of α , β and γ of mature size (Tipper *et al.*, 1981; Johnson *et al.*, 1985) implies the absence of significant *in vitro* processing of translation products. The estimated sizes of α and β mRNAs (about 300 bases) now indicate that each transcript is monocistronic. This is consistent with the separate map locations identified for the four *B. subtilis* ASSP genes cloned (Connors *et al.*, 1986*b*). A common forespore-specific transcriptional control system presumably exists for all coordinately controlled ASSP genes, which include, in *B. subtilis*, at least α , β , γ and δ (Johnson *et al.*, 1985). The gene products involved remain to be identified.

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