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Synthesis of Acid-Soluble Spore Proteins by *Bacillus subtilis*

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The major acid-soluble spore proteins (ASSPs) of *Bacillus subtilis* were detected by immunoprecipitation of radioactively labeled in vitro- and in vivo-synthesized proteins. ASSP synthesis in vivo began 2 h after the initiation of sporulation (t_2) and reached its maximum rate at t_7. This corresponded to the time of synthesis of mRNA that stimulated the maximum rate of ASSP synthesis in vitro. Under the set of conditions used in these experiments, protease synthesis began near t_0, alkaline phosphatase synthesis began at about t_2, and refractile spores were first observed between t_7 and t_8. In vivo- and in vitro-synthesized ASSPs comigrated in sodium dodecyl sulfate-polyacrylamide gels. Their molecular weights were 4,600 (α and β) and 11,000 (γ). The average half-life of the ASSP messages was 11 min when either rifampin (10 μg/ml) or actinomycin D (1 μg/ml) was used to inhibit RNA synthesis.

The acid-soluble spore proteins (ASSPs) of *Bacillus subtilis* represent about 5% of the total protein of mature spores (13). They are degraded at germination and are sensitive to the highly specific *Bacillus megaterium* endoprotease that degrades *B. megaterium* ASSPs at germination (13). As in the case of *B. megaterium*, the ASSPs appear to function as storage proteins. Comparison of the major ASSPs of *B. subtilis* (α, β, and γ) with those of *B. megaterium* (A, C, and B) has shown that the endoprotease-sensitive amino acid sequences are conserved. Based on amino acid composition, immunological cross-reactivity, and primary sequence comparison, the major ASSPs can be divided into two groups. Group I contains *B. subtilis* α and β and *B. megaterium* A and C proteins. Although the *B. subtilis* group I proteins α and β cannot be distinguished from one another on the basis of molecular weight, their isoelectric points have been reported to differ (3). Group II contains *B. subtilis* γ and *B. megaterium* B proteins. All of these proteins have low molecular weights (no greater than 11,000). They all lack cystine, cysteine, and tryptophan. The ASSPs from these two *Bacillus* species are not identical, however, as shown by the variation in their amino-terminal sequences and the low level of their immunological cross-reactivity (13).

In spite of the functional and structural similarities between *B. megaterium* and *B. subtilis* ASSPs, the factors regulating the time of their synthesis seem to differ when the two species are grown in complex sporulation medium and sporulate due to nutrient exhaustion. It was reported that the synthesis of mRNA coding for *B. megaterium* ASSPs and the in vivo synthesis of A, C, and B antigens peak at about 3 h after the initiation of sporulation (t_3) (2). Previous work on *B. subtilis* demonstrates that the synthesis of mRNA capable of directing the in vitro synthesis of its ASSPs peaks late in sporulation (t_6 to t_8) (7). This paper presents further documentation of the synthesis of *B. subtilis* ASSPs and their mRNAs.

Because neither *B. megaterium* nor *B. subtilis* ASSPs or their mRNAs were present in vegetative cells, ASSPs appear to be the products of sporulation-specific genes. As such, they should be of use in the study of the molecular biology of the sporulation process.

MATERIALS AND METHODS

Growth and sporulation of bacteria. *B. subtilis* 168 (Trp) was grown and sporulated due to nutrient exhaustion in complex sporulation medium (10). Exponentially growing cells were harvested when the culture reached an optical density at 570 nm (OD_{570}) of 0.5. The end of exponential growth occurred approximately 1 h later; therefore, the time of an optical density of 0.5 plus 1 h was referred to as t_0 in all of the following experiments.

RNA extraction. At appropriate times during exponential growth and sporulation, *B. subtilis* 168 (Trp) was harvested, lyophilized, broken, and added to water-saturated phenol as indicated by Dignam and Setlow (2). After the addition of an equal volume of buffer VI (0.02 M Tris-hydrochloride [pH 7.5], 0.01 M KCl, 5 mM $MgCl_2$, 0.01 M NaN_3), RNA was extracted by the procedure of Legault-Demare and Chambliss (5) with the exception that all extractions were done with water-saturated phenol rather than with *meta*-cresol. The dried RNA pellets were dissolved in water at concentrations between 10 and 25 μg/ml.

In vitro protein synthesis. All conditions for in vitro protein synthesis were carried out as previously published (6) with the following exceptions. When the in vitro protein-synthesizing reaction mixture contained L-[^{35}S]methionine (Amersham Corp.; 1,490 Ci/mmol), the 19 unlabeled amino acids were present at 812 μM each. When L-*U*-^{14}C-amino acid mixture (Amersham; >50 mCi per milligram-atom of carbon) was used to label in vitro-synthesized proteins, the 19 unlabeled amino acids were present at 12 μM. Unlabeled methionine was present at 51 μM.

Preparation of in vivo-labeled cell extracts. *B. subtilis* 168 (Trp) was grown as indicated above. At appropriate times during exponential growth and sporulation, 1 ml of cell culture was transferred to a 25-ml flask containing 5 or 10 μCi of L-*U*-^{14}C-amino acid mixture. This flask was shaken at 37°C for 5 or 10 min before 10-μl samples were added to 2 ml of cold 5% trichloroacetic acid (TCA) to determine the extent of protein synthesis. These TCA-precipitated samples were later filtered and washed with more 5% TCA and then with 80% ethanol. The dried filters were counted in toluene-based scintillation fluid. The rest of the 1 ml of labeled cell culture was added to 2 ml of cold 0.15 M NaCl, centrifuged (5 min, 6,000 × *g*), frozen, and lyophilized as described by Dignam and Setlow (2). The cells were broken in the presence of 100 mg of glass beads (75 to 150 μm) with a dental amalgamator (LP-60 Wig-L-Bug). Eight bursts of 1 min each with 30 s of cooling between bursts resulted in breakage of 10 to 20% of the cells. The mixture of broken cells and glass beads was extracted one time for 30 min on ice with 1 ml of buffer A (0.1 M Tris-hydrochloride [pH 8.0], 20 mM EDTA, 0.2 mM diisopropyl fluorophosphate). The samples were then microfuged (Beckman microfuge B) for 2 min, and supernatants were stored at −80°C.

For determination of ASSP mRNA stability, in vivo-labeled samples were prepared in the above manner with the following modifications. Rifampin (10 μg/ml, final concentration) or actinomycin D (1 μg/ml, final concentration) was added to the sporulating *B. subtilis* culture at t_7. One-milliliter samples were removed at intervals up to 20 min after the addition of the inhibitor. The samples were labeled with 7.5 μCi of L-*U*-^{14}C-amino acid mixture or with 0.1 μCi of [2-^{14}C]uracil for 5 min. After labeling, the [2-^{14}C]uracil-labeled samples were precipitated with 1 ml of cold 10% TCA. Filters were washed, dried, and counted as described above.

Raising antiserum. ASSPs for injection into a New Zealand white female rabbit were prepared as follows. *B. subtilis* BR151 was grown and allowed to sporulate at 30°C in complex sporulation medium. Three days after the medium was inoculated, the spores were harvested by centrifugation (5,000 × *g*, 5 min). The pellets were washed two times with buffer I and one time with buffer II (5) before treatment with a lysozyme solution (0.1 mg of lysozyme per ml, 10 mM EDTA, 3.45 mM phenylmethylsulfonyl fluoride, 0.2 mM diisopropyl fluorophosphate) for 15 min at 37°C. The spores were next washed successively with (i) 1.0 M NaCl–10 mM EDTA, (ii) 0.14 M NaCl–10 mM EDTA, (iii) 0.1% sodium dodecyl sulfate (SDS) (wt/vol)–10 mM EDTA, and three times with (iv) 10 mM EDTA. All of these washing solutions contained 3.45 mM phenylmethylsulfonyl fluoride and 0.2 mM diisopropyl fluorophosphate. After lyophilization, the spores were stored at −80°C until the time of breakage. The spores were broken in a dental amalgamator in batches of 200 mg with 200 mg of glass beads and a small metal pestle. Microscopic examination of the spores after eight bursts of 1 min each indicated that most of the spores broke when treated this way. The broken spores were extracted two times for 30 min each at 4°C with 3% acetic acid. This extract was centrifuged (12,000 × *g*, 10 min), and the supernatants were pooled before being lyophilized.

To increase the immunogenicity of the ASSPs, their molecular weight was increased by cross-linking the ASSP molecules to each other. The lyophilized ASSPs were dissolved in 4 ml of phosphate buffer (0.1 M, pH 7.0) at a final concentration of 0.85 mg/ml. Glutaraldehyde was added to this solution to give a final concentration of 3.6 mM. Incubation took place at room temperature for 90 min. The reaction was stopped by adding 0.5 ml of 1 M L-lysine. The reaction mixture was dialyzed overnight against two changes of 500 volumes of phosphate buffer.

The rabbit was injected subcutaneously in the back with 0.75 mg of cross-linked protein and an equal volume of complete Freund adjuvant. Thirty days later another set of subcutaneous injections with 0.3 mg of protein and an equal volume of incomplete Freund adjuvant was given. The rabbit was bled 6, 9, and 13 days later. The clot was removed after the blood was incubated overnight at 4°C. The immunoglobulins were precipitated by the addition of 1/2 volume of saturated $(NH_4)_2SO_4$ and incubation at room temperature for 30 min. The suspension was centrifuged (12,000 × *g*, 10 min), and the pellet was redissolved in 0.85% NaCl. This precipitation was repeated two times. After the final precipitation, the pellet was dissolved in PBS-N_3 (0.1 M sodium phosphate buffer [pH 7.4], 0.85% NaCl, 0.05% NaN_3) to its original volume. The antiserum was stored in 1-ml aliquots at −80°C.

Immunoprecipitation reactions. Radioactively labeled in vitro- or in vivo-synthesized protein was diluted with immunoprecipitation buffer (50 mM KPO_4 [pH 7.4], 0.15 M NaCl, 2 mM EDTA, 2 mg of bovine serum albumin per ml) before the addition of a saturating amount of antiserum. This mixture was incubated on ice for at least 1 h. Except where otherwise indicated, washed *Staphylococcus aureus* Cowan I cells (4) were added, and incubation on ice was continued for 15 min. In one case, goat anti-rabbit immunoglobulin G (GARGG; Calbiochem) was substituted for *S. aureus*. These samples were incubated at 37°C for 30 min and then at 4°C for 30 min. After this point, identical procedures were followed for the *S. aureus* and GARGG samples. Pellets formed by 1 min of microfugation were washed four times to remove weakly bound radioactivity. Washing buffers contained 50 mM Tris-hydrochloride (pH 7.4), 5 mM EDTA, 0.02% NaN_3, 0.5% Triton X-100, and 1 mg of ovalbumin per ml. In addition, the first wash contained 0.5 M NaCl, the second and third contained 0.25 M NaCl, and the last wash contained 0.15 M NaCl. When the sample to be immunoprecipitated was labeled with L-[^{35}S]methionine, these buffers also included 5 mM L-methionine. Immunoprecipitated samples labeled with L-*U*-^{14}C-amino acids were washed with the same buffers containing each amino acid in the labeled mixture at 0.5 mM. Antigen bound to the *S. aureus* was eluted by boiling in the presence of 4% (wt/vol) SDS–6 M

urea for 4 min. The samples were then either counted in aqueous counting scintillant (Amersham) or precipitated by acetone. Acetone precipitates were washed one time in fresh acetone, dried, and then either (i) redissolved directly in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer or (ii) extracted with 3% acetic acid, lyophilized, and redissolved in SDS-PAGE sample buffer.

SDS-PAGE. SDS-polyacrylamide gels (12 to 20% linear acrylamide gradient) with a Tris buffer system were run as described by Adoutte-Panvier et al. (1). Molecular weight standards from high- and low-molecular-weight calibration kits (Pharmacia Fine Chemicals, Inc., Bethesda Research Laboratories) were run at one side of the gel. After electrophoresis at 150 V for 36 to 48 h, the gel was cut vertically to remove the molecular weight standards. The rest of the gel was treated with En^{3}Hance (New England Nuclear Corp.), dried, and exposed to Kodak X-OMAT AR X-ray film. The portion of the gel containing the molecular weight standards was stained (0.1% Coomassie blue, 25% isopropanol, 10% acetic acid, and 0.1% cupric acetate). Destaining solution consisted of 10% acetic acid and 30% methanol.

Miscellaneous procedures. Protease activity was determined by incubation of the supernatant of harvested *B. subtilis* 168 (Trp) in the presence of Azocoll (Calbiochem) and Tris-hydrochloride–$CaCl_2$ buffer (0.1 M Tris-hydrochloride [pH 7.3], 2 mM $CaCl_2$) at 37°C for 60 min. The reaction was stopped with 7% perchloric acid. Absorbance was read at 520 nm. Specific activity = OD_{520} of protease assay/OD_{570} of culture.

Alkaline phosphatase activity was measured in a reaction mixture containing 1 ml of *B. subtilis* 168 (Trp) resuspended in 1.0 M Tris-hydrochloride (pH 8.0), 1 drop of toluene, and 1 ml of phosphatase substrate (Sigma Chemical Co.) dissolved in 1.0 M diethanolamine. After incubation at 37°C for 15 min, the reaction was stopped by the addition of 1.5 ml of 2 M NaOH. Absorbance was read at 410 nm. Specific activity = OD_{410} of alkaline phosphatase assay/OD_{570} of culture.

Protein concentrations were determined by the method of Lowry et al. (8) with bovine serum albumin as the standard.

RESULTS

Comparison of in vivo and in vitro protein synthesis. In vitro protein synthesis reaction mixtures containing 3,600 cpm of L-[^{35}S]methionine per pmol of total methionine were incubated at 37°C in the presence of *B. subtilis* RNA. This RNA had been extracted from cells harvested during vegetative growth (OD_{570} = 0.50) at the initiation of sporulation (t_0) and at 2-h intervals thereafter (t_2 to t_{12}). After incubation, the addition of anti-ASSP antiserum to the reaction mixtures and the subsequent addition of *S. aureus* Cowan I resulted in the immunoprecipitation of up to 10% of the total protein synthesized. Although the anti-ASSP antiserum could immunoprecipitate all three major ASSPs (see below), the 11,000-molecular-weight protein (γ) contains no methionine (3) and therefore was not labeled. As confirmed by the molecular weight of the single radioactively labeled band in SDS-polyacrylamide gels of these immunoprecipitated proteins, only the presence of ASSPs α and β was detected in this experiment. Figure 1 shows that the synthesis of α and β in vitro depended on the presence of RNA extracted from cells late in sporulation. A small amount of mRNA capable of directing the in vitro synthesis of α and β began to appear at t_2. The concentration of this mRNA peaked between t_6 and t_8, and a small amount was still present at t_{12}. Repetition of this in vitro synthesis with RNA from two independently grown and extracted batches of *B. subtilis* gave similar results. The optimal magnesium concentration for in vitro α and β synthesis was 13 mM and coincided with the optimum for total in vitro protein synthesis.

The time of ASSP synthesis in vivo was determined with extracts prepared from *B. subtilis* pulsed with L-*U*-^{14}C-amino acid mixture for 10 min before harvesting. This procedure labeled γ as well as α and β. Immunoprecipitation of in vivo-synthesized protein was carried out in the same manner as that of in vitro-synthesized protein. Initial experiments indicated that no ASSP antigen was synthesized during vegetative growth or at t_0. When sporulating cells were labeled for 10 min at 1-h intervals between t_2 and t_{10}, immunoprecipitation of ASSPs showed that their rate of synthesis peaked between t_6 and t_8 (Fig. 1). This confirmed the results obtained in vitro.

Relationship between the time of ASSP synthesis and other events. Because the choice of a time to call t_0 is somewhat subjective, the time of ASSP synthesis was related to events other than the cessation of logarithmic growth. Supernatant and cell pellet samples were taken from the *B. subtilis* cultures used as the source of RNA in the in vitro protein synthesis reactions described above. The supernatant was assayed for protease activity, and the pellet was assayed for alkaline phosphatase activity (Fig. 2). We found that protease synthesis began near t_0. Alkaline phosphatase synthesis began shortly after t_2, the same time as the appearance of the first detectable quantity of ASSP-encoding mRNA. Microscopic examination of sporulating cultures revealed that refractile spores first appeared between t_7 and t_8, the time at which ASSP synthesis was at its maximum.

Evidence for the specificity of the anti-ASSP antiserum-antigen reaction. ASSPs extracted with 3% acetic acid from broken mature spores were electrophoresed along with solutions of protein molecular weight standards on SDS-polyacrylamide gels (12 to 20% linear gradient) containing Tris buffers (1). Figure 3 shows that the semilogarithmic plot of molecular weight versus distance traveled by a protein was bipha-

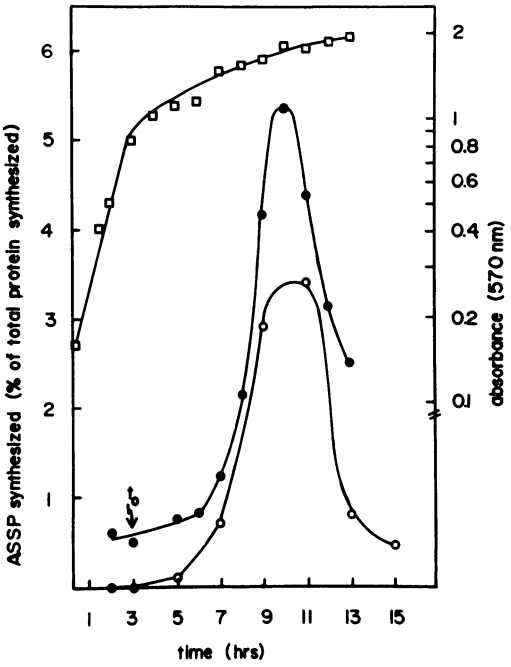

FIG. 1. In vitro and in vivo synthesis of ASSPs. In vitro protein synthesis reaction mixtures contained *B. subtilis* RNA extracted from cultures harvested during vegetative growth and at 2-h intervals between t_0 and t_{12} during sporulation. The concentration of RNA that gave the maximum amount of total in vitro protein synthesis was used in each reaction mixture (50 to 260 μg of RNA). After incubation of in vitro protein synthesis reaction mixtures at 37°C for 30 min, 50 μl of each L-[^{35}S]methionine-labeled sample was immunoprecipitated with anti-ASSP antiserum as described in the text. A 5-μl amount of the immunoprecipitate was spotted on glass fiber filters and counted. The remainder was run on SDS-polyacrylamide gels as seen in Fig. 5. About 0.5% of the protein synthesized in the presence of 150 μg of SPO1 RNA (i.e., RNA from *B. subtilis* harvested 5 min after infection with phage SPO1) or in the absence of exogenous RNA was found to be precipitated by the above procedure. The amount of ASSP synthesized as a percentage of total protein synthesized was therefore calculated to be (counts per minute immunoprecipitated by anti-ASSP/total counts per minute incorporated into protein in vitro) × 100% − 0.5%. Samples containing 5,000 cpm of in vivo-synthesized proteins labeled with L-*U*-^{14}C-amino acid mixture were immunoprecipitated under the same conditions used for precipitation of in vitro-synthesized proteins. A control containing preimmune serum in place of anti-ASSP was run for each sample. ASSP synthesized in vivo as a percentage of total protein synthesized = {[(counts per minute precipitated by anti-ASSP) − (counts per minute precipitated by preimmune serum)]/5,000 cpm} × 100%. Symbols: ○, protein synthesized in vitro; ●, protein synthesized in vivo; □, OD_{570} of *B. subtilis* culture used as the source of cells for in vivo labeling.

sic. The slope of the line changed at approximately molecular weight 14,500, but both portions of the plot were linear. It was therefore possible to determine the molecular weights of even very small proteins.

In addition, in vivo- and in vitro-synthesized proteins labeled with L-*U*-^{14}C-amino acids were electrophoresed on SDS-polyacrylamide gels (Fig. 4). Each sample gave two bands with molecular weights of 4,600 (α and β) and 11,000 (γ), although the γ band in the in vitro-synthesized t_6 sample appeared to be relatively faint. The bands seen in the gel comigrated with the two major bands seen in gels of ASSPs that were acetic acid extracted from unlabeled spores and used to raise anti-ASSP antiserum as described above. Protein that was synthesized in vitro in the presence of t_6 and t_8 RNA and L-[^{35}S]methionine, immunoprecipitated, and run on an SDS-polyacrylamide gel gave one band with a molecular weight corresponding to that of α and β (Fig. 5). The band was also faintly present in the immunoprecipitate of samples synthesized in vitro in the presence of t_4, t_{10}, and t_{12} RNA. This single band was seen whether the entire immunoprecipitate of the in vitro-synthesized sample or just the acetic acid extract of the immunoprecipitate was electrophoresed. This result indicated that the protein precipitated from in vitro-synthesized products had the same characteristic solubility in 3% acetic acid as its in vivo-synthesized counterpart. The α and β band was not immunoprecipitated from in vitro protein-synthesizing reaction mixtures that contained phage SPO1 RNA or RNA from *B. subtilis* harvested earlier than t_4. This result supported that described above in Fig. 1; i.e., ASSP synthesis was directed only by RNA found in sporulating cells.

Further proof that the in vivo-synthesized L-*U*-^{14}C-amino acid-labeled proteins were immunoprecipitated in specific antigen-antibody reactions is shown in Fig. 6. Increasing amounts of anti-ASSP antiserum or normal serum were added to 8,000 cpm of t_8 sporulating-cell extract. The amount of labeled material precipitated by the addition of anti-ASSP antiserum increased linearly with the increase in antiserum concentration up to a point of saturation. A uniform low level of labeled material was precipitated regardless of the concentration of normal serum in the immunoprecipitation reaction mixture. This indicated that nonspecific binding of labeled protein to components of normal serum or directly to *S. aureus* itself was not responsible for the immunoprecipitation results presented above.

The necessity for protein synthesis to occur in order for L-*U*-^{14}C-amino acids to be incorporated into immunoprecipitable material was demonstrated by TCA precipitation and immunopre-

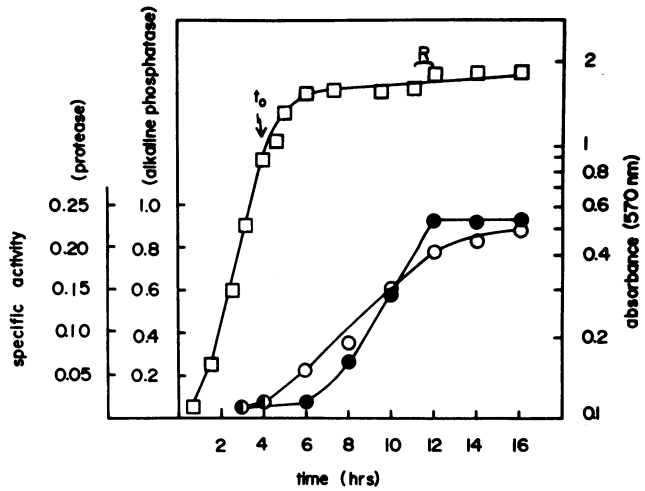

FIG. 2. Alkaline phosphatase and protease synthesis. Symbols: □, OD_{570} of growing and sporulating *B. subtilis* culture; ○, specific activity of protease; ●, specific activity of alkaline phosphatase. R is the time of first appearance of refractile spores.

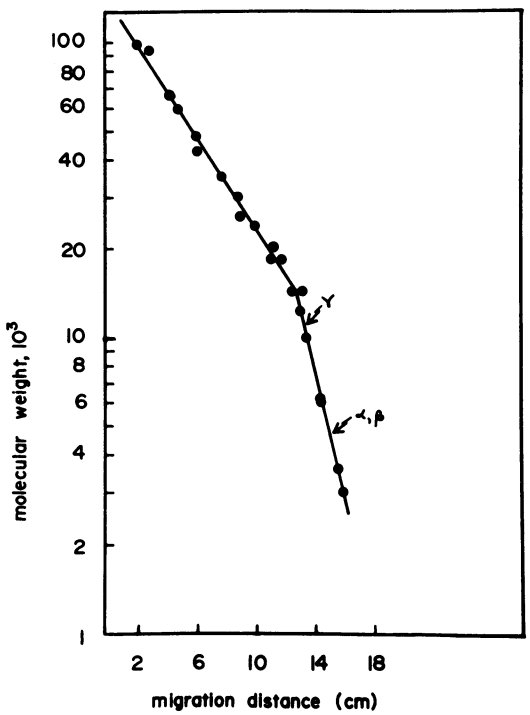

FIG. 3. Molecular weight calibration curve. Proteins of known molecular weights were run on a Tris-buffered SDS-polyacrylamide gel (12 to 20% linear gradient). The gel was stained, and the distance traveled by each protein from the top of the gel was measured. Arrows indicate the positions at which proteins extracted from spores by 3% acetic acid were found.

cipitation of the proteins synthesized in a chloramphenicol-treated *B. subtilis* culture. At t_7, chloramphenicol (100 μg/ml) was added to a sporulating culture. After 20 min, 1 ml of the culture was labeled for the next 5 min. A 10-μl sample was TCA precipitated for protein synthesis determination. The rest of the 1-ml culture

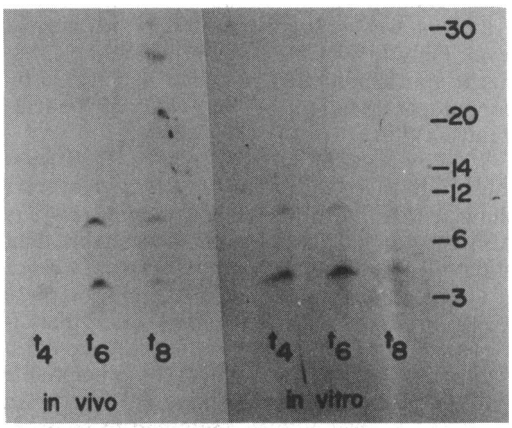

FIG. 4. Fluorogram of SDS-polyacrylamide gel of immunoprecipitated in vivo- and in vitro-synthesized protein. In vitro-synthesized proteins made in the presence of t_4, t_6, or t_8 RNA and extracts of in vivo-synthesized proteins labeled at t_4, t_6, and t_8 were immunoprecipitated with anti-ASSP. Before immunoprecipitation, in vitro- and in vivo-synthesized samples contained 35,000 and 75,000 cpm of L-*U*-^{14}C-amino acids, respectively. The immunoprecipitates were run on an 18-cm SDS-polyacrylamide gel. Molecular weights of standards ($\times 10^3$) are given at the side of the figure.

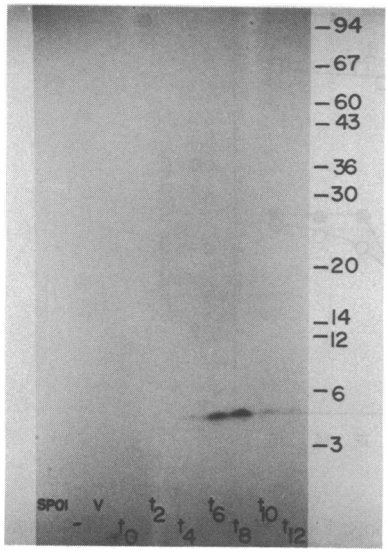

FIG. 5. Fluorogram of SDS-polyacrylamide gel of immunoprecipitated, in vitro-synthesized, L-[^{35}S]methionine-labeled protein. The in vitro-synthesized samples run on this gel were those described in the legend to Fig. 1. The RNA template used in each reaction mixture is indicated at the bottom of the figure. Molecular weights of standards ($\times 10^3$) are given at the side.

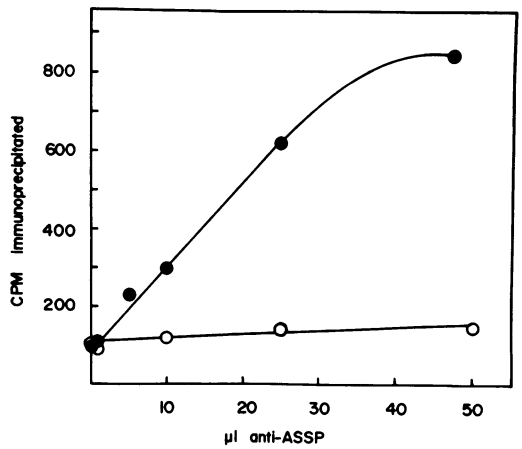

FIG. 6. Antibody saturation curve. Between 1 and 50 µl of anti-ASSP antiserum or preimmune serum was added to immunoprecipitation reaction mixtures containing 8,000 cpm of in vivo-synthesized protein labeled at t_8 with L-*U*-^{14}C-amino acids. Immunoprecipitates eluted from *S. aureus* were counted in aqueous counting scintillant (Amersham). Symbols: ●, anti-ASSP; ○, preimmune serum.

was prepared for immunoprecipitation. Under these conditions, the amount of total protein synthesis was reduced to 30% of its normal level. Only 11% of the amount of ASSP synthesized in an uninhibited culture was detected by immunoprecipitation of the chloramphenicol-treated culture.

Stability of ASSP mRNA. In vivo ASSP synthesis during inhibition of RNA synthesis showed that the mRNAs encoding *B. subtilis* ASSPs were, on the average, more stable than other mRNAs. Sporulating cultures were treated at t_7 with rifampin at a final concentration of 10 µg/ml. Preliminary experiments showed that after 5 min of this treatment in the presence of [2-^{14}C]uracil, the amount of cold TCA-precipitable material was reduced to background levels. An untreated control culture continued to incorporate [2-^{14}C]uracil into cold TCA-precipitable material at a linear rate for at least 60 min. Sporulating (t_7) cells were next pulse-labeled (for 5-min periods) with L-*U*-^{14}C-amino acids. Labeled cells were harvested, and their extracts were immunoprecipitated with anti-ASSP antiserum. The amount of ASSP synthesized (as a percentage of total protein synthesized) rose during the first 8 min after rifampin addition to 1.5 times its initial level (Fig. 7). Repetition of this experiment with actinomycin D (at 1 µg/ml, final concentration) instead of rifampin also reduced the amount of [2-^{14}C]uracil-labeled, cold TCA-precipitable material to background level. The amount of L-*U*-^{14}C-amino acid-labeled ASSP detected (as a percentage of total protein synthesized) doubled in the first 8 min after actinomycin D addition.

The half-life of ASSP mRNA was calculated by graphing the data from the amino acid label-

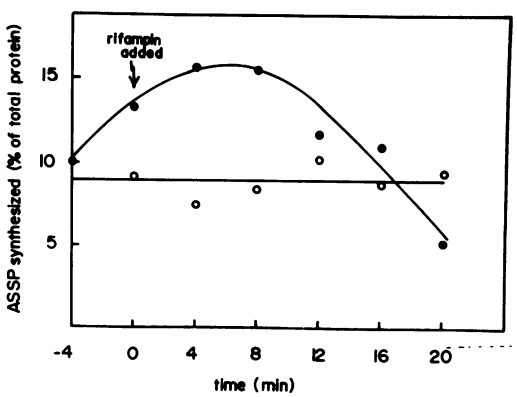

FIG. 7. ASSP synthesis in the presence of rifampin. *B. subtilis* was labeled with L-*U*-^{14}C-amino acids at the peak of ASSP synthesis (t_7) in the presence and absence of rifampin (10 µg/ml). Rifampin was added at time zero, and 1-ml samples were labeled at 4-min intervals. Cell extracts containing 2,000 cpm were immunoprecipitated. Symbols: ●, 10 µg of rifampin present per ml; ○, no rifampin added.

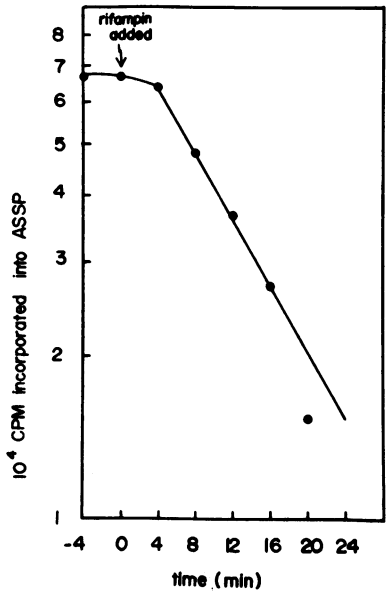

FIG. 8. Half-life of ASSP mRNA. In vivo-synthesized proteins were labeled in the presence of rifampin as described in the legend to Fig. 7. Rifampin was added at time zero. Samples containing 1,500 cpm were immunoprecipitated. Counts per minute of L-*U*-^{14}C-amino acids incorporated into ASSP = (percentage of sample containing 1,500 cpm that was immunoprecipitated) × (total counts per minute incorporated into 1-ml culture).

ing experiments described directly above, as shown in Fig. 8. The half-life equalled the time it took for the amount of ASSP synthesized to drop to half the amount synthesized during the 5-min labeling period that began at 4 min after the addition of the RNA synthesis inhibitor. Table 1 gives the results of five half-life determinations. These data indicate that the average half-life of α, β, and γ mRNA was approximately 11 min. This was true whether RNA synthesis was inhibited by rifampin or actinomycin D and whether *S. aureus* or GARGG was used to precipitate the antigen-antibody complexes. When the average half-life of total cellular mRNA was calculated in the same way from the amino acid incorporation data gathered with either rifampin or actinomycin D, it was found to be only 6 min.

DISCUSSION

The results presented in this paper show that the ASSPs of *B. subtilis* are synthesized during the intermediate and late stages of sporulation. Under the set of conditions used in this study, protease synthesis began near t_0, alkaline phosphatase synthesis began at about t_2, and refractile spores began to appear between t_7 and t_8. In terminology commonly used to refer to the stages of sporulation, protease synthesis begins at stage I and alkaline phosphatase synthesis begins at stage II (9). Pulse-labeling of sporulating cells at hourly intervals showed that the synthesis of anti-ASSP-immunoprecipitable material began at about t_2. This corresponds to the time at which alkaline phosphatase was first synthesized, indicating that the start of ASSP synthesis in *B. subtilis* is a stage II event.

SDS-PAGE of anti-ASSP immunoprecipitates of in vivo- and in vitro-synthesized, L-*U*-^{14}C-amino acid-labeled proteins gave two bands that comigrated with the two major bands appearing in gels of 3% acetic acid extracts of mature *B. subtilis* spores. When RNA from cells harvested late during sporulation (specifically t_6 or t_8) was present in the in vitro protein-synthesizing reaction mixture, the maximum amount of ASSP synthesis occurred. The in vitro synthesis of proteins that were identical to in vivo-synthesized proteins in terms of both antigenicity and molecular weight attests to the fidelity of the in vitro protein-synthesizing system. The time of appearance of the maximum quantity of ASSP-encoding mRNA coincided with the time of maximum ASSP synthesis in vivo, suggesting that control of ASSP synthesis is mainly at the transcriptional level. The alternative explanation that ASSP mRNA is synthesized in an inactive form at an earlier time and is not

TABLE 1. Half-life of ASSP mRNA

Sample prepn no.[a]	Antigen-antibody complex precipitant	RNA synthesis inhibitor[b]	Half-life[c] (min)
1	*S. aureus*	Rifampin	10
2	*S. aureus*	Rifampin	10
2	GARGG	Rifampin	11
3	*S. aureus*	Rifampin	11
4	*S. aureus*	Actinomycin D	12

[a] *B. subtilis* cultures were treated with an RNA synthesis inhibitor, and extracts were prepared as in the legend to Fig. 7. In experiments with sample preparations 1, 2, and 4, cell extracts containing 1,500 to 2,000 cpm were immunoprecipitated. In the experiment with sample preparation number 3, immunoprecipitation reactions contained 200 μl of sample (7,000 to 50,000 cpm).

[b] The concentrations of rifampin and actinomycin D used were 10 and 1 μg/ml, respectively.

[c] Half-lives were determined from the data obtained from each experiment by plotting as in Fig. 8. The half-life was calculated as the time it took for the amount of ASSP synthesized to drop to half the amount synthesized at 4 min after the addition of the inhibitor.

activated until stage II cannot be ruled out, but inhibition of ASSP mRNA synthesis by rifampin at t_7 showed that at least a portion of the ASSP mRNA was synthesized at the time of its translation.

The molecular weights of the immunoprecipitated proteins were determined using a Tris buffer-based SDS-polyacrylamide gel system. Although it has been reported that the molecular weights of small proteins can be more accurately determined using phosphate buffer gels (11), we continued to use Tris gels because of their superior resolution of individual protein bands. Our molecular weight standards showed that the molecular weights of small proteins could be determined accurately provided that enough standards in the low-molecular-weight range were run. The two major ASSP bands in gels of extracts from unlabeled mature *B. subtilis* spores as well as the two bands immunoprecipitated from in vivo- or in vitro-synthesized protein labeled with L-*U*-^{14}C-amino acids had molecular weights of 4,600 (α and β) and 11,000 (γ). As expected due to the lack of methionine in γ (3), only the 4,600-molecular-weight protein band appeared when immunoprecipitates of L-[^{35}S]methionine-labeled in vitro-synthesized protein were run on gels. One of our molecular weight determinations agreed with that of Johnson and Tipper (3) who also assigned a molecular weight of 11,000 to γ, but disagreed with their α and β molecular weight of 5,900. This discrepancy may be due to the differences in the gel systems used.

Immunoprecipitation of in vivo-synthesized protein labeled with L-*U*-^{14}C-amino acids at the peak of ASSP synthesis showed that 5 to 10% of protein synthesized at that time was ASSP (α, β, and γ). It is possible that this is an underestimation of the actual amount of ASSP synthesized in vivo because ASSPs are highly subject to proteolytic degradation (3). Their yield from in vivo-labeled cell extracts might therefore be artificially lowered. The immunoprecipitation of protein synthesized in vitro in the presence of t_8 RNA and L-[^{35}S]methionine demonstrated that up to 10% of this protein was ASSP (α and β). This, however, might be an overestimation of the ASSP mRNA concentration present in vivo. The above-average stability of ASSP mRNA might artificially raise the in vitro concentration of ASSP mRNA. If proportionally more ASSP mRNA survives the RNA extraction procedure, the percentage of mRNA coding for ASSP that is presented to the in vitro protein-synthesizing system would be artificially elevated.

Many similarities have been found between *B. subtilis* ASSPs and those of *B. megaterium*. These include their low molecular weight, amino acid composition, sites of amino acid sequence homology, sensitivity to a specific *B. megaterium* spore protease, and a low level of immunological cross-reactivity (13). Important differences between the two groups of ASSPs are apparent from the data presented in this paper. *B. megaterium* ASSP synthesis has been shown to peak early in sporulation at about t_3 (2), whereas ASSP synthesis in *B. subtilis* did not peak until t_7. Sporulation in both cases took place due to nutrient exhaustion after growth in supplemented nutrient broth. The only readily apparent difference between *B. megaterium* and *B. subtilis* growth and sporulation conditions was that *B. megaterium* was cultured at 30°C as opposed to 37°C for *B. subtilis*. Lowering the temperature of *B. subtilis* cultures to 30°C did not hasten the synthesis of ASSP mRNA, but rather delayed the maximum synthesis until t_{12} (data not shown).

Another apparent difference between *B. subtilis* and *B. megaterium* concerns the stability of ASSP mRNA. Half-life determinations for *B. subtilis* ASSP mRNA were initially carried out with rifampin. This resulted in a calculated average half-life of 11 min for ASSP mRNA versus 6 min for total cellular mRNA. To eliminate the possibility that the initiation of ASSP mRNA synthesis was less sensitive to rifampin than the initiation of the average mRNA and to eliminate any residual RNA synthesis occurring due to elongation of RNA molecules initiated before the addition of rifampin, the half-life determination was repeated with actinomycin D as the inhibitor of RNA synthesis. The results of this experiment corroborated the evidence previously obtained from the rifampin experiments; i.e., the average half-life of ASSP mRNA was longer than that of the average mRNA molecule. The greater than average stability of ASSP was also demonstrated by the one- to twofold increase in the amount of ASSP synthesized (as a percentage of total protein synthesized) during the first 8 min after the addition of either RNA synthesis inhibitor. It has been reported that the half-life of the mRNA coding for the *B. megaterium* ASSP A is no longer than that of the reported average mRNA half-life of 4 min (2). The experiment performed to determine the *B. megaterium* ASSP half-life used a concentration of actinomycin D 100 times greater than was used in our *B. subtilis* experiment. We used 1 μg/ml as the final concentration of actinomycin D because this concentration was reported to be the optimum for inhibiting sporulation without causing *B. subtilis* to lyse (12). No more than 1 μg/ml was necessary to prevent RNA synthesis at t_7 under our sporulation conditions. It should be emphasized that we did not determine the half-life of the mRNA of individual ASSPs, but the average half-life of the mRNAs for α, β, and γ. Because

α and β each make up 18% of the ASSPs of *B. subtilis* and γ makes up 36% (3), it can be assumed that α, β, and γ mRNA each constitute a significant portion of the total mRNA examined in our half-life experiments. The *B. megaterium* mRNA half-life experiment specifically determined the half-life of the mRNA coding for the A protein (2). It is possible that the difference between *B. subtilis* and *B. megaterium* ASSP mRNA in terms of resistance to degradation reflects the differences in the mRNA nucleotide sequences coding for the two species' ASSPs. Such differences might be expected from the variations in their primary structures as exemplified by their amino-terminal sequences (13).

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