

Coat Protein Synthesis During Sporulation of *Bacillus subtilis*: Immunological Detection of Soluble Precursors to the 12,200-Dalton Spore Coat Protein

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Antibody specific to the 12,200-dalton spore coat protein of *Bacillus subtilis* was used to detect the synthesis of cross-reacting material during sporulation. Cross-reacting protein was first detected by immunoprecipitation after 4 h of development and represented at least 1 to 2% of the total soluble protein synthesis at 5.5 h. A polypeptide of 21,000 daltons was detected in immunoprecipitates by gel electrophoresis. This polypeptide did not accumulate in sporulating cells and was rapidly turned over at the time of coat deposition. In contrast, a 32,000-dalton polypeptide reacted with antibody when unlabeled cell protein was denatured with sodium dodecyl sulfate, separated by gel electrophoresis, and transferred to nitrocellulose paper. This polypeptide was not detected during cell growth or the first 3.5 h of development but was found to accumulate in sporulating cells at 5.5 h. The lack of detection of this polypeptide by immunoprecipitation of undenatured protein indicates that the antigenic sites which cross-reacted with antibody to the 12,200-dalton protein sequence were not exposed unless the molecular conformation was altered. The 32,000-dalton protein may be a primary translation product which is proteolytically processed into mature spore coat protein via a 21,000-dalton intermediate.

Bacterial sporulation is a process of cell differentiation initiated in response to appropriate environmental stimuli. Nutrient deprivation leads to cessation of normal cell division and initiation of a developmental program resulting in sequential biochemical and morphological alterations, the end product of which is the spore. The spore is a specialized cell, characterized by dormancy and maintenance of viability under harsh environmental conditions.

Evidence has accumulated which indicates that transcriptional (3, 18) and translational (4, 12, 19) controls operate during sporulation. New RNA sequences are synthesized during development, and some RNA sequences found in dividing cells are no longer made (3, 18). As a result, sporulation-specific polypeptides are synthesized and some polypeptides, characteristic of growing cells, are not (14). In addition, RNA polymerase is structurally altered during development with a subsequent change in artificial template specificity (5, 13), suggesting that promoter specificity *in vivo* may also be altered. In this regard, a 0.4-kilobase RNA was recently found in sporulating cells, and a 5-kilobase pair segment of DNA, containing the complementary

sequence, has been cloned (7). RNA polymerase from growing cells does not transcribe 0.4-kilobase RNA from the 5-kilobase cloned segment; however, it does transcribe a different sequence within the 5-kilobase segment which is expressed in growing cells. In contrast, a modified form of RNA polymerase lacking the 55,000-dalton sigma subunit, but containing a 37,000-dalton sigma subunit isolated from sporulating cells, transcribes 0.4-kilobase RNA in addition to the RNA specific to growing cells. These data suggest that modification of RNA polymerase promoter specificity is involved in the regulation of development; however, the functional significance of these observations cannot really be assessed until specific functions have been assigned to these RNA species.

Control of development at the level of mRNA translation is suggested by the isolation of mutations which affect ribosomal proteins and which simultaneously affect sporulation but not cell growth (4, 12, 19). Strains with mutant ribosomal protein L-17 are concurrently resistant to erythromycin and are temperature sensitive during the latter half of the sporulation period (19). Although one may conclude that require-

ments for translation during sporulation are different from those during growth, it is difficult at present to assess the regulatory significance of these observations.

Definitive experiments designed to test the significance of the observations mentioned above, with respect to transcriptional and translational control of gene expression during development, cannot be performed until assays for sporulation-specific gene products (protein and mRNA) are available. The easily accessible and well-characterized spore coat proteins are reasonable candidates for such studies. These proteins represent approximately 50% of the total spore protein (2, 6, 16, 17) and are probably synthesized as precursors which are proteolytically processed into mature coat proteins (1, 2, 16, 17). These precursors may be synthesized several hours before assembly of spore coats (1, 2, 16, 17).

We purified a 12,200-dalton spore coat protein from *Bacillus subtilis* (6) with the intent of examining regulation of its synthesis during development. We now report the identification of material cross-reacting with antibody to the 12,200-dalton coat protein and describe the time of synthesis and accumulation in sporulating cells.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. subtilis* 168 was grown and induced to sporulate in a nutrient sporulation medium at 37°C as previously described (6). The number of hours after cessation of exponential turbidity increase (determined with a Klett colorimeter, no. 66 filter) is hereafter represented by a T and a numerical subscript. Phase-bright spores appear at T_{5.5}, reaching half-maximal yield at T_{6.5}, and these kinetics were used as an internal marker for the timing of developmental events. *Staphylococcus aureus* Cowan I was grown as described by Kessler (11).

Preparation of cell extracts. Cells (10 ml) were harvested by centrifugation (10,000 × g for 5 min), washed in 0.05 M Tris-hydrochloride buffer (pH 7.5) containing 0.01 M EDTA, 10% (vol/vol) glycerol, 1 M NaCl, and 5% (vol/vol) freshly prepared solution of phenylmethylsulfonyl fluoride (6 mg/ml in ethyl alcohol) (buffer A). Cells were suspended in 5 ml of 0.02 M maleic acid-NaOH buffer (pH 6.5) containing 0.02 M MgSO₄, 0.5 M sucrose, and 0.001 M NaN₃ (buffer B). Cells were converted to spheroplasts by the addition of lysozyme to 50 μg/ml for vegetative cells and 100 μg/ml for sporulating cells and by incubation at 37°C for 7 min for vegetative cells and 15 min for sporulating cells. Conversion of cells to spheroplasts was 80 to 90% complete under these conditions. After centrifugation (10,000 × g for 5 min) and three washes (5 ml each) with buffer B, the spheroplast pellet was lysed by addition of 0.05 M Tris-hydrochloride buffer (pH 7.4) containing 0.15 M NaCl, 0.005 M EDTA, 0.02% NaN₃, and 0.05% (vol/vol) Nonidet P-40. (This

buffer was designated NET by Kessler [11].) After centrifugation at 15,000 × g for 20 min, the supernatants were assayed by immunoprecipitation (see below). Cell extracts were prepared and assayed on the same day.

Cell extracts were also prepared by passing cells four times through a French press at 16,000 lb/in² in buffer A, followed by centrifugation at 100,000 × g for 1 h at 4°C. Supernatants were saturated with (NH₄)₂SO₄ at 4°C, and precipitated protein was collected by centrifugation (10,000 × g for 10 min). Precipitates were suspended in either NET buffer containing 0.05% Nonidet P-40 for immunoprecipitation or in 0.06 M Tris-hydrochloride buffer, containing 3% (wt/vol) sodium dodecyl sulfate (SDS) and 5% (vol/vol) 2-mercaptoethanol for gel electrophoresis. The latter were heated at 100°C for 3 min before electrophoresis.

Protein content of cell extracts was determined as follows. Protein from 1 volume of cell extract was precipitated with 9 volumes of acetone and collected by centrifugation. The pellet was dried with a stream of nitrogen gas and assayed by the method of Lowry et al. (15), with bovine serum albumin as the standard.

Antibody preparation. The 12,200-dalton spore coat protein was purified as previously described (6), and rabbits were immunized with 100 μg in Freund complete adjuvant by intradermal injection. After 30 days, the rabbits were injected intradermally with 100 μg of purified protein in Freund incomplete adjuvant and bled 7 days later by cardiac puncture. Boosting and bleeding were repeated every 5 weeks. Sera (stored at -20°C) from four rabbits were pooled and processed as described by Harboe and Ingild (8) up to the final acetate dialysis. The precipitate of lipoprotein was removed by centrifugation. The supernatant was dialyzed against 10 mM phosphate buffer (pH 7.2) containing 15 mM NaCl and applied to a column of DEAE-cellulose (25 ml) overlaid with carboxymethylcellulose (25 ml) previously equilibrated with the same phosphate buffer. Antibody was recovered from the material flowing through the column and precipitated with 25 g of (NH₄)₂SO₄ per 100 ml of solution. The precipitate was collected, dissolved in 0.1 M NaCl containing 15 mM NaN₃, and dialyzed against the same solution. The final preparation (2.07 g of protein) from 200 ml of serum was stored at a concentration of 200 mg/ml at 4°C. The antibody was found to react specifically with the 12,200-dalton spore coat protein by immunodiffusion in agarose (data not shown). Preimmune serum was processed in the same manner. These antibody preparations were 35 to 40% immunoglobulin G (IgG), as determined by binding to excess *S. aureus* cells.

Preparation and characterization of the *S. aureus* immunoadsorbent. *S. aureus* Cowan I was grown and prepared as a specific IgG adsorbent as described by Kessler (11) and stored in 2-ml portions in liquid nitrogen. These cells bound a maximum of 70 μg of rabbit IgG per 100 μl of cell suspension (10%, wt/vol). No deterioration of IgG binding capacity was observed after 4 months of storage.

Immunoprecipitations. Cell extracts were assayed by immunoprecipitations as described by Kessler (11). Radioactivity in immunoprecipitates was

determined after solubilization with 1% SDS in 0.06 M Tris-hydrochloride (pH 6.8) at 37°C for 15 min and removal of *S. aureus* cells by centrifugation. Immunoprecipitated protein was prepared for gel electrophoresis by solubilization with 0.06 M Tris-hydrochloride (pH 6.8) containing 3% (wt/vol) SDS and 5% (vol/vol) 2-mercaptoethanol at 100°C for 3 min. *S. aureus* cells were removed by centrifugation.

Gel electrophoresis. Polyacrylamide gel electrophoresis with SDS was performed as previously described (6), using 1-mm-thick slab gels. After they were stained and destained, the gels were dried and exposed to Kodak X-Omat RP film.

Electrophoretic transfer of protein to nitrocellulose paper and subsequent antibody assay. Protein was transferred electrophoretically from polyacrylamide gels to nitrocellulose paper as described previously (20). Transfer sheets were processed as described previously (20), with 100 µg of IgG from either preimmune or immune serum used for the primary antibody reaction. Proteins to which IgG had bound were identified by a second reaction with peroxidase-conjugated goat anti-rabbit IgG (20).

Radiochemical methods. Cells (10 ml of culture) were labeled with 20 µCi of [³⁵S]methionine (790 Ci/mmol) per ml or 50 µCi of [³H]tyrosine (70 to 100 Ci/mmol) per ml for 15 min at 37°C. Less than 1% of the radioactivity was incorporated under these conditions. Pulse-chase experiments were performed by labeling with 20 µCi of [³⁵S]methionine per ml for 15 min followed by the addition of 2 mg of unlabeled methionine per ml to stop incorporation and effect a chase. The efficiency of this procedure in preventing further incorporation of label was determined by measuring incorporation into trichloroacetic acid-insoluble material after the simultaneous addition of labeled (20 µCi/ml) and unlabeled (2 mg/ml) methionine. This procedure reduced incorporation by more than 95%.

Radioactivity in polyacrylamide gel slices was determined after digestion with 0.5 ml of Protosol for 20 h at 42°C. Radioactive samples were counted in Aquasol. Radionucleotides, Protosol, and Aquasol were obtained from New England Nuclear Corp.

RESULTS

Assay for material cross-reacting with antibody to the 12,200-dalton spore coat protein. The mature 12,200-dalton spore coat protein is insoluble except at high pH (>10) and contains no methionine or cysteine (6). Thus, labeling sporulating cells with [³⁵S]methionine and immunoprecipitating protein soluble at pH 7.4 with antibody to the 12,200-dalton spore coat protein would not be expected to detect the mature form of this molecule. However, if the 12,200-dalton spore coat protein is initially synthesized as a higher-molecular-weight precursor which is soluble at pH 7.4 and does contain methionine, it might be detectable by labeling and immunoprecipitation.

The time of synthesis of cross-reacting protein was determined by immunoprecipitation of sol-

uble protein extracts from growing and sporulating cells which had been labeled for 15 min with [³⁵S]methionine just before harvesting. Increasing amounts of these extracts were incubated with constant amounts of antibody to the 12,200-dalton coat protein or with antibody from preimmune serum, followed by isolation of immune complexes with *S. aureus* cells (Table 1). *S. aureus* cells alone bound amounts of input cell protein only slightly less than those bound in the presence of antibody from preimmune serum (data not shown), and this was interpreted as nonspecific binding. The difference between counts bound in the presence of antibody from immune and preimmune sera [(SA + I) - (SA + PI); Table 1] was assumed to be a measure of specific labeled antigen. This value was relatively independent of the amount of input protein, suggesting that antibody was in excess over the range of concentrations employed. Only extracts from cells labeled at T_{5.5} of sporulation showed a significant increase in the fraction of labeled protein bound in the presence of antibody to the 12,200-dalton spore coat protein. This increase corresponded to 1 to 2% of the

TABLE 1. Immunoprecipitation of cell extracts from growing and sporulating cells labeled with [³⁵S]-methionine

Sample ^a	Protein input to assay (µg)	% Input (cpm) bound by: ^b		% Specific labeled antigen (cpm) [(SA + I) - (SA + PI)]
		SA + PI	SA + I	
Vegetative	5	3.2	3.7	0.5
	49	1.3	1.7	0.4
	53	1.3	1.8	0.5
	105	1.8	2.0	0.2
T ₂	3	2.3	2.9	0.6
	31	1.2	1.6	0.4
	31	1.5	2.0	0.5
	62	1.2	1.6	0.4
T _{3.5}	32	0.7	1.1	0.4
	307	0.5	0.9	0.4
T ₄	5	1.6	2.1	0.5
	48	0.5	0.9	0.4
	42	0.6	0.7	0.1
	84	0.8	1.0	0.2
T _{5.5}	5	1.2	3.5	2.3
	46	0.5	2.3	1.8
	46	0.6	2.3	1.7
	91	0.6	2.2	1.6

^a Vegetative, Cells harvested during exponential growth; T₂, T_{3.5}, T₄, and T_{5.5}, cells harvested at 2, 3.5, 4, and 5.5 h, respectively, after growth had ceased. Extracts were prepared from spheroplasts.

^b SA, *S. aureus*-immune adsorbent; PI, antibody from preimmune serum; I, antibody from immune serum.

[³⁵S]methionine incorporated into soluble protein at T_{5.5} (Table 1).

We attempted two additional immunoprecipitation procedures: (i) immunocompetition with the 12,200-dalton spore coat protein and (ii) immunoprecipitation of the mature 12,200-dalton spore coat protein after solubilization from extracts of sporulating cells by raising the pH and adding SDS. As yet, we have been unable to overcome a major technical difficulty in performing these experiments: the 12,200-dalton spore coat protein is only soluble under conditions which denature antibody.

Characterization of cross-reacting material by gel electrophoresis. Although the *S. aureus* immunoabsorbent allows rapid processing of samples, some nonspecific binding of protein from crude extracts occurred (Table 1). The significance of the immunoprecipitation reactions was therefore assessed by gel electrophoresis.

S. aureus cells alone release a heterogeneous mixture of protein in the range of 20 to 30 μg/100 μl of a 10% (wt/vol) suspension, and in these amounts, they do not interfere with electrophoretic separation of bound IgG or antigen (9). However, we found that a maximum of 15 to 20 μg of IgG could be applied to each slot of a

standard slab gel (1-mm thickness) without serious distortion of protein bands during electrophoresis. Exceeding this amount led to distortion of protein bands and nonspecific trapping of labeled protein in the immunoglobulin heavy- and light-chain regions of the gel (data not shown).

Immunoprecipitates from cell extracts labeled with [³⁵S]methionine were characterized by gel electrophoresis (Fig. 1). Extracts from T_{5.5} sporulating cells contained a single polypeptide of 21,000 daltons which cross-reacted specifically with antibody to the 12,200-dalton spore coat protein (Fig. 1, lane 1). This cross-reactive protein did not react with antibody from preimmune serum (Fig. 1, lane 2) nor with antibody from immune or preimmune serum and extracts of vegetative cells or T₂ sporulating cells (Fig. 1, lanes 5 through 8). A very small amount of protein with the same mobility as the cross-reacting component in T_{5.5} samples was detected at T₄ (Fig. 1, lane 3).

We observed slight variations in the results of immunoprecipitations performed on different days with different extracts (data not shown); however, the 21,000-dalton protein (Fig. 1, lane 1) was the only protein reproducibly immunoprecipitated. This method of immunoprecipita-

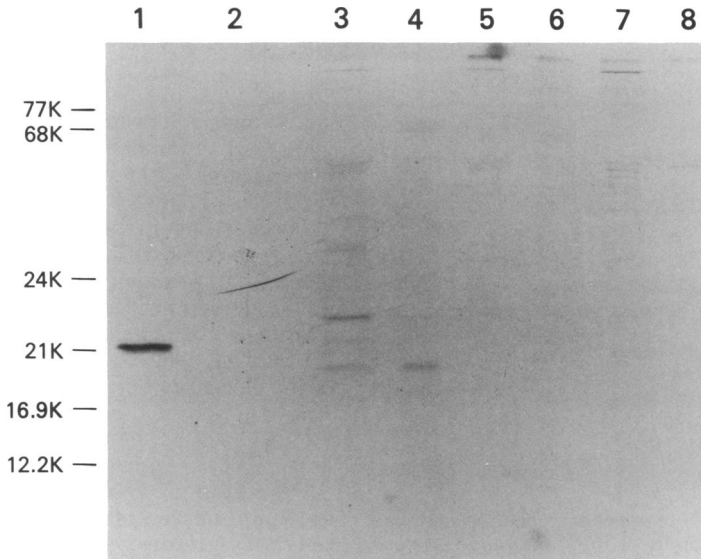


FIG. 1. Gel electrophoresis of immunoprecipitates from cell extracts prepared from spheroplasts. Immunoprecipitates from extracts labeled with [³⁵S]methionine were solubilized and separated by electrophoresis on a 15% acrylamide gel. Labeled proteins were detected by autoradiography. Lanes 1, 3, 5, and 7 were precipitated with immune serum; lanes 2, 4, 6, and 8 with preimmune serum. Lanes 1 and 2, T_{5.5} sporulating cells; lanes 3 and 4, T₄ sporulating cells; lanes 5 and 6, T₂ sporulating cells; lanes 7 and 8, vegetative cells. Molecular weight markers are conalbumin, bovine serum albumin, trypsinogen, and myoglobin (77,000, 68,000, 24,000, and 16,900 daltons, respectively). The relative position of the purified 12,200-dalton spore coat protein is indicated.

tion (IgG in excess, but complete recovery of IgG completely precipitates antigen (11); no additional 21,000-dalton protein was detected by immunoprecipitation of $T_{5.5}$ soluble cell protein, from which the 21,000-dalton protein was previously immunoprecipitated (data not shown).

The cross-reacting protein from $T_{5.5}$ sporulating cells was 21,000 daltons and had the same electrophoretic mobility as a pulse-labeled soluble protein present in extracts from $T_{5.5}$ sporulating cells (Fig. 2, lanes 4 and 5, respectively). Some label was observed in this region of the gel after electrophoresis of extracts from T_2 sporulating cells (Fig. 2, lane 2); however, this probably represented an unrelated polypeptide of similar molecular weight, as it did not react with antibody from immune serum. Note that the gel shown in lane 5 of Fig. 2 was more heavily exposed to film than that shown in lane 1 of Fig. 1, yet the immunoprecipitate still consisted almost exclusively of the 21,000-dalton species.

Tyrosine and methionine labeling of immunoprecipitated protein. The antigen to which antibody was produced is 12,200 daltons

and contains 32 mol% of tyrosine, but lacks methionine (6). The cross-reacting protein of 21,000 daltons was 1.7 times larger and did contain methionine. If it also contains the 12,200-dalton spore coat protein sequence and an average amount of methionine in its additional 8,800-dalton peptide, it should have a relatively high tyrosine-to-methionine ratio.

Cells were labeled simultaneously with [^3H]tyrosine and [^{35}S]methionine for 15 min at $T_{5.5}$ of sporulation, and soluble protein was prepared from spheroplasts. The 21,000-dalton protein was immunoprecipitated, identified by gel electrophoresis as before, and cut from the gel for quantitation of ^3H and ^{35}S isotopes. The tyrosine-to-methionine ratio of the 21,000-dalton protein (Fig. 3, lane 3) was 250% greater than the average of three other protein bands from the cell extract separated on the same gel (Fig. 3, lane 1), supporting the inference derived from the results of immunoprecipitation that the 21,000-dalton protein contains the tyrosine-rich 12,200-dalton spore coat protein sequence. Furthermore, the use of tyrosine to label cell protein did not reveal any additional cross-reacting material from $T_{5.5}$ cells (Fig. 3, lane 3). In particular, no band was seen corresponding to the mature 12,200-dalton protein.

Stability of the 21,000-dalton cross-reacting protein. The stability of the 21,000-dalton polypeptide *in vivo* was determined by labeling cells at $T_{5.5}$ with [^{35}S]methionine and chasing for 10 min with unlabeled methionine. Since the simultaneous addition of unlabeled methionine with [^{35}S]methionine reduced incorporation by more than 95%, the chase was considered effective.

Radioactivity in the 21,000-dalton polypeptide rapidly decreased during the chase when soluble cell protein was analyzed by gel electrophoresis and immunoprecipitation (Fig. 4). We observed a definite 21,000-dalton protein band when the gel shown in lane 3 of Fig. 4 (immunoprecipitate of the chased sample) was exposed to film for a longer period of time; however, as determined by quantitative densitometry, it represented only 5% of the control value (data not shown).

Detection of cross-reacting material by electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose paper. We anticipated that a precursor to the 12,200-dalton spore coat protein might elude immunochemical detection because of the masking of antigenic sites present in the 12,200-dalton spore coat protein sequence. Thus, the 21,000-dalton polypeptide, detected by standard immunoprecipitation, may not represent the primary translation product of a spore coat protein precursor but rather a processing intermediate

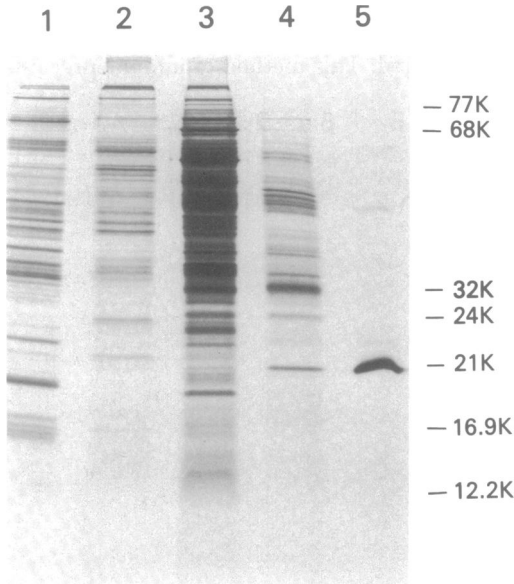


FIG. 2. Gel electrophoresis of pulse-labeled protein prepared from protoplasts and immunoprecipitated protein from $T_{5.5}$ sporulating cells. Samples were separated on a 15% acrylamide gel, and proteins were detected by autoradiography. Lane 1, pulse-labeled protein from vegetative cells; lane 2, sporulating cells at T_2 ; lane 3, sporulating cells at T_4 ; lane 4, sporulating cells at $T_{5.5}$; lane 5, the 21,000-dalton protein immunoprecipitated from $T_{5.5}$ cell extracts. Molecular weight markers are the same as in Fig. 1. The relative position of the purified 12,200-dalton spore coat protein is indicated.

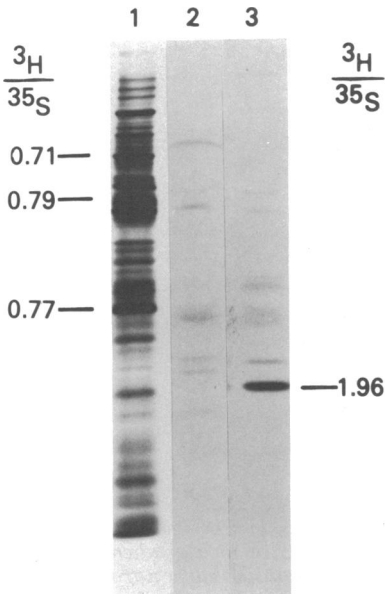


FIG. 3. Tyrosine and methionine labeling of immunoprecipitated protein. Cells were labeled with [^3H]tyrosine and [^{35}S]methionine for 15 min at $T_{5.5}$ of sporulation. Soluble protein was prepared from spheroplasts and a portion of protein immunoprecipitated with antibody to the 12,200-dalton spore coat protein. Duplicate samples of the extract (before immunoprecipitation) and the immunoprecipitates were subjected to electrophoresis on a 12.5% acrylamide gel. After the gel was stained and destained, one of each duplicate set was dried and exposed to X-ray film by fluorography. Gel slices (2 mm) were cut from the remaining duplicate samples at the regions indicated and counted for radioactivity after digestion with Protosol. Lane 1, Soluble protein from the cell extract; lane 2, protein immunoprecipitated with preimmune serum; lane 3, protein immunoprecipitated with immune serum. The band with the $^3\text{H}/^{35}\text{S}$ ratio of 1.96 is the 21,000-dalton cross-reacting component. Bands with $^3\text{H}/^{35}\text{S}$ ratios of 0.71, 0.79, and 0.77 are components of approximate molecular weights 64,000, 50,000, and 25,000, respectively.

in which the 12,200-dalton spore coat protein sequence is accessible to antibody. We therefore assayed for cross-reacting protein in cell extracts after denaturation with SDS, electrophoresis in polyacrylamide gels containing SDS, and transfer to nitrocellulose paper.

A polypeptide of about 32,000 daltons cross-reacted with spore coat protein antibody when samples of $T_{5.5}$ sporulating cells were assayed by this method (Fig. 5, lane 10), whereas no such reaction was observed with antibody from preimmune sera (data not shown). In contrast, no significant cross-reacting material was detected in growing cells or cells harvested at $T_{3.5}$ of sporulation (Fig. 5, lanes 8 and 9, respec-

tively). The faint reactions seen for proteins greater than 32,000 daltons (Fig. 5, lanes 8 through 10) were identical to nonspecific reactions observed with antibody from preimmune sera (data not shown). Rabbit IgG retained its reactivity with goat anti-rabbit IgG (Fig. 5, lane 7). The 12,200-dalton spore coat protein reacted specifically with antibody produced against it (Fig. 5, lane 11).

The 21,000-dalton protein, which is immunoprecipitated by antibody raised against the 12,200-dalton spore coat protein, was not detected by the electrophoretic transfer method. Since the 32,000-dalton protein was detectable as a Coomassie blue-stained band at $T_{5.5}$ and the 21,000-dalton component was not (Fig. 5, lane 5), we believe that the lack of detection of the 21,000-dalton protein by electrophoretic transfer reflected the much lower amount of this component in $T_{5.5}$ sporulating cells. Lack of immunochemical detection of the 21,000-dalton protein could not have been due to inefficient transfer, because the expected amount was detected by autoradiography after transfer of labeled protein (data not shown). Extracts prepared with

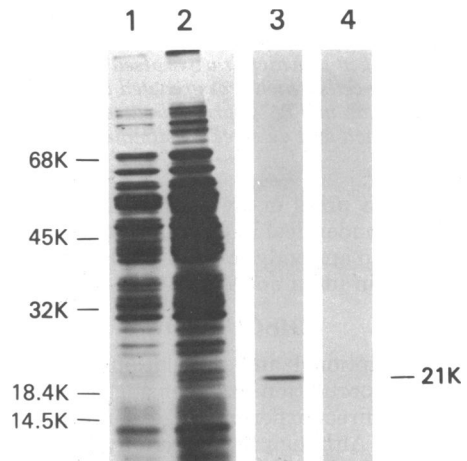


FIG. 4. Stability of the 21,000-dalton cross-reacting protein. Cells were labeled with [^{35}S]methionine, and soluble protein was prepared after passage of cells through a French press. Samples were electrophoretically separated on a gradient gel of 7.5 to 20% acrylamide. Lane 1, Soluble protein from cells labeled with [^{35}S]methionine for 15 min and then chased with unlabeled methionine for 10 min; lane 2, same as lane 1 but without a chase; lane 3, protein immunoprecipitated from the sample shown in lane 2 with antibody to the 12,200-dalton spore coat protein; lane 4, protein immunoprecipitated from the sample shown in lane 1. Molecular weight markers are bovine serum albumin, egg albumin, β -lactoglobulin, and egg lysozyme (68,000, 45,000, 18,900, and 14,500 daltons, respectively).

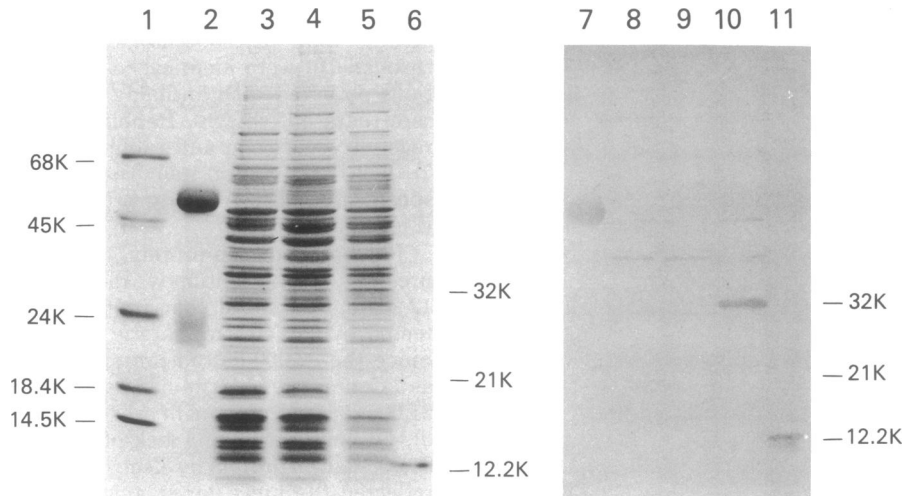


FIG. 5. Identification of cross-reacting protein after electrophoresis in SDS gels. Soluble cell protein, prepared by lysis with a French press, was electrophoretically separated in a gradient gel of 7.5 to 20% acrylamide containing SDS. Samples were then electrophoretically transferred to nitrocellulose paper for reaction with antibody. Lanes 1 through 6, Standard SDS gel stained for protein. Duplicate samples of lanes 2 through 6 were electrophoretically transferred to nitrocellulose and reacted first with antibody to the 12,200-dalton spore coat protein, followed by peroxidase-conjugated goat anti-rabbit IgG. Immunoglobulin complexes were visualized by staining for peroxidase. Lane 1, Molecular weight standards; lanes 2 and 7, rabbit IgG; lanes 3 and 8, soluble protein from vegetative cells; lanes 4 and 9, soluble protein from $T_{3.5}$ sporulating cells; lanes 5 and 10, soluble protein from $T_{5.5}$ sporulating cells; lanes 6 and 11, the purified 12,200-dalton spore coat protein. Goat anti-rabbit IgG reacted with rabbit IgG (lane 7) and with rabbit IgG bound to the 12,200-dalton antigen to which it was raised (lane 11). Lane 10 shows a component of 32,000 daltons present in $T_{5.5}$ sporulating cells which cross-reacted with antibody to the 12,200-dalton spore coat protein. Molecular weight standards, as in Fig. 4, are indicated, along with the relative position of the 21,000-dalton cross-reacting protein, which was detected by standard immunoprecipitation (see Fig. 1 through 4).

the French press or by conversion to spheroplasts were identical in protein composition and reactions to antibody to the 12,200-dalton spore coat protein (data not shown).

DISCUSSION

Transcriptional and translational control of bacterial sporulation has been suggested on the basis of indirect experimental observations (3, 4, 12, 18, 19). Although this cumulative evidence is rather compelling, no direct evidence has yet been obtained for transcriptional or translational control of a sporulation-specific gene of known function. As a first step to performing such definitive experiments, we previously characterized the structural proteins of the spore coat and purified one such component (6). This polypeptide of 12,200 daltons represents 5% of the total spore protein (12% of the total spore coat protein), contains 32 mol% of tyrosine, but lacks methionine (6). We have obtained antibody from rabbits injected with this coat protein and now report the identification of putative precursors which specifically cross-react with such immune sera.

Antibody against the 12,200-dalton spore coat protein specifically immunoprecipitated a polypeptide of 21,000 daltons from the soluble protein from $T_{5.5}$ sporulating cells. We believe this molecule represents a precursor to the 12,200-dalton, tyrosine-rich spore coat protein because: (i) it was first detected about 15 min before the morphological appearance of spore coats; (ii) it was relatively rich in tyrosine; (iii) it was not detected during cell growth nor at T_1 or $T_{3.5}$ of sporulation; (iv) it cross-reacted specifically with antibody to the 12,200-dalton spore coat protein but was not detected with preimmune serum; and (v) it was actively synthesized and rapidly turned over *in vivo* at the time of coat deposition. Since this molecule contained methionine and was only 21,000 daltons, it did not appear to represent merely a dimer of the 12,200-dalton, tyrosine-rich but methionine-free spore coat protein. The additional 8,800 daltons may represent one of the other low-molecular-weight coat proteins (2, 6, 16, 17) or assist in maintaining solubility of the precursor before coat assembly *in vivo* or both.

In addition, a polypeptide of approximately

32,000 daltons cross-reacted with antibody to the 12,200-dalton coat protein after SDS denaturation and gel electrophoresis of soluble protein from cells at $T_{5.5}$ of sporulation. Since this is the largest component we detected which specifically cross-reacted with antibody to the 12,200-dalton spore coat protein, it may represent the primary translation product of mRNA encoding the 12,200-dalton spore coat protein sequence. The lack of detection of this molecule by immunoprecipitation indicates that the antigenic sites of the 12,200-dalton protein sequence were buried within the 32,000-dalton molecule in the native state and thus were inaccessible to antibody unless the molecular conformation was altered.

In addition to the 12,200-dalton protein, spore coats contain four additional proteins in the approximate range of 8,000 to 16,000 daltons (2, 6, 16, 17). One of these, a protein of 13,500 daltons, was purified and shown to be different from the 12,200-dalton protein by amino acid analysis (16). A 25,000-dalton protein, immunoprecipitated with antibody to this 13,500-protein, was detected in vivo as early as T_1 of sporulation (16). Proteins of 60,000, 40,000, and 25,000 daltons, immunoprecipitated with antibody against a mixture of low-molecular-weight spore coat proteins, were also detected as early as T_1 of sporulation (2, 17). These studies used standard, indirect immunoprecipitation methods. When compared with the method which uses *S. aureus* cells, these indirect methods have the disadvantages of higher background of non-specifically bound protein, potential proteolysis during the extended incubation times, and the presence of large amounts of IgG in immunoprecipitates (9, 11). The latter can adversely affect gel electrophoresis of precipitates, owing to an excess of heavy and light chains of IgG. The 21,000-dalton precursor, which we detected, was immunoprecipitated and ready for electrophoresis within 3 h after the cells were harvested. No more than 15 to 20 μg of IgG was applied to the gel, and the precursor was identified as a sharp band by autoradiography.

Our results indicate that the pathway for synthesis and assembly of the 12,200-dalton spore coat protein is separate and substantially different from other spore coat proteins (2, 16, 17). The polypeptides which specifically cross-reacted with antibody to the 12,200-dalton spore coat protein were not detected during growth or the first 3.5 h of development; however, they were detected by $T_{5.5}$ of sporulation, just before the appearance of assembled spore coat structures within the cell. These molecules are likely candidates for soluble precursors to the very insoluble 12,200-dalton spore coat protein. The

32,000-dalton component accumulated in sporulating cells and may represent the primary translation product of a spore coat protein gene which is proteolytically cleaved to yield a 21,000-dalton component. The 21,000-dalton fragment, containing the 12,200-dalton coat protein sequence, would now be recognized in its native state by antibody to the 12,200-dalton coat protein, owing to exposure of the relevant antigenic sites. The 21,000-dalton polypeptide rapidly turned over in vivo, did not accumulate in sporulating cells, and may represent a processing intermediate which is proteolytically cleaved to yield the mature 12,200-dalton spore coat protein.

These data all indicate that spore coat proteins are synthesized as higher-molecular-weight precursors which are used in the assembly of spore coats. The purification of the remaining spore coat proteins and use of peptide mapping will allow the pathway of spore coat synthesis and assembly to be worked out in detail. Furthermore, in light of the current desire to clone developmental genes, antibody to sporulation-specific gene products of known function will be useful as probes. In this way, transcriptional and translational control of a developmental gene with known functions could be investigated.

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