Properties of the Bacillus subtilis Spore Coat

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About 70% of the protein in isolated Bacillus subtilis spore coats was solubilized by treatment with a combination of reducing and denaturing agents at alkaline pH. The residue, consisting primarily of protein, was insoluble in a variety of reagents. The soluble proteins were resolved into at least seven bands by sodium dodecyl sulfate gel electrophoresis. About one-half of the total was four proteins of 8,000 to 12,000 daltons. These were relatively tyrosine rich, and one was a glycoprotein. There was also a cluster of proteins of about 40,000 daltons and two or three in the 20,000- to 25,000-dalton range. The insoluble fraction had an amino acid composition and N-terminal pattern of amino acids very similar to those of the soluble coat proteins. A major difference was the presence of considerable dityrosine in performic acid-oxidized preparations of insoluble coats. Coat antigen including a 60,000-dalton protein not present in extracts of mature spores was detected in extracts of sporulating cells by immunoprecipitation. This large antigen turned over in a pulse-chase experiment. Antibodies to either the array of 8,000- to 12,000-dalton coat polypeptides or to the larger coat proteins reacted with this 60,000-dalton species, suggesting a common precursor for many of the mature coat polypeptides. Spore coats seem to be assembled by processing of proteins and by secondary modifications including perhaps dityrosine formation for cross-linking.

The formation and assembly of the spore coat layers in Bacillus cereus begins early in sporulation and proceeds via synthesis and processing of a precursor (9, 32). These layers serve a protective function and perhaps have a role in germination (4-6, 10, 16, 32).

The morphology of the spore layers in B. subtilis is very different from that in B. cereus, and the B. subtilis spores respond to a different array of germinants (6, 18, 29, 34, 35). To provide a comparative assessment of spore coat structure and in the hope of exploiting the genetic system in B. subtilis, we initiated a study of the composition of the spore coats. There have been some studies of B. subtilis coat polypeptides, but these have either focused on particular polypeptides (14), or used conditions which resulted in only partial solubilization (17, 20, 21, 24, 26, 31, 36) and thus an incomplete analysis of the total profile.

In the present report, we have attempted to define conditions for the more complete solubilization and analysis of B. subtilis spore coats. Although B. subtilis shows some similarities to B. cereus, including commencement of synthesis of coat antigen early in sporulation and apparent formation of a large polypeptide precursor, there are also considerable differences. There is a more heterogeneous array of polypeptides extractable from B. subtilis coats, and there is a substantial fraction of insoluble protein that may be cross-linked.

MATERIALS AND METHODS

Media and cell growth. B. subtilis JH642 and other strains were grown in a nutrient sporulation medium (28). JH642 is a B. subtilis 168 strain obtained from J. Hoch; CU 1037 and 1038 are pigmentless strains isolated by S. Zahler. The volume of media was kept to less than 20% of the flask volume. In some experiments cells were transferred to resuspension medium (33) at indicated times. Growth was determined by measuring absorbance at 610 nm in a Zeiss spectrophotometer. Determination of spore purity was done by examining wet mounts with a Zeiss phase-contrast microscope. Various stages of sporulation are denoted as t0, t1, etc., to refer to time after the end of exponential growth.

Preparation of spores and coats. After 2 to 3 days of incubation, spores were harvested in an SS34 rotor by centrifuging in a Sorvall refrigerated centrifuge at 10,000 × g for 10 min. Pellets were washed once with deionized water and then incubated for 30 to 60 min at 37°C in 0.05 M Tris, pH 7.8, containing 100 μg of lysozyme/ml. Lysis of vegetative cells with lysozyme was followed by one or two washings each with 1 M NaCl, 0.14 M NaCl, 0.1% (wt/vol) sodium dodecyl sulfate (SDS), 1 mM NaOH, and antibody buffer (9), followed by five washings with deionized water. Antibody buffer consisted of 0.05 M sodium
phosphate containing 0.05 M NaCl, 0.1% (wt/vol) sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1% Triton X-100, pH 7.8. Occasionally, preparations were also washed with Sepharose-hemoglobin and 1 mM HgCl2 to help inactivate proteases (27) before being washed with deionized water. Final preparations contained greater than 99% refractile spores. Clean spores were broken by shaking at 4°C with 200- to 270-μm glass beads (equal weight of glass beads and spore pellet) and a drop of Dow antifoam spray in a Brannon disintegrator, type 2876. Microscopic examination showed that 6 to 7 min of shaking (2 min of cooling after every 1 to 2 min of shaking) was sufficient for total disruption of spores. The suspensions were decanted and washed from beads with 0.03 M Tris, pH 7.8. Crude spore coats were pelleted by centrifugation at 12,000 × g for 15 min. Pellets were suspended in 0.05 M Tris, pH 7.8, plus 100 to 200 μg of lysozyme/ml for 1 to 2 h at 37°C and then subjected to the sequence of washings described above for the preparation of spores. As previously discussed (6), readily solubilized spore coat proteins would be lost in this procedure. The isolated coats do appear in thin-section electron micrographs, however, to have retained all of the major morphological features of the coat layers as seen in sections of intact spores (4, 6, 14).

Solubilization of spore coat protein. For solubilization of coat protein, clean spores (5 × 10^6 to 8 × 10^6 spores/ml) or the isolated coats were suspended in freshly prepared buffer containing 0.05 M dithioerythritol-1% SDS-8 M urea in 0.005 M cyclohexyl amino-methane sulfonic acid (CHES), pH 9.8 (UDS), and were incubated for 2 to 3 h at 37°C with occasional blending in a Vortex mixer. Pellets were reextracted as above where mentioned. Extractions were also done with UDS at higher pH and various concentrations of dithioerythritol or SDS at 27 or 37°C. Occasionally, Sepharose-hemoglobin and PMSF were included in the extraction buffer. The SDS was precipitated by adding a drop of saturated KCl solution to the extract at 0°C. A fraction of the solubilized material or residue was suspended in 15% trichlooroacetic acid and incubated at 0°C for 40 min. After centrifuging in a refrigerated Sorvall at 10,000 rpm for 10 min, the pellets were dissolved in 0.2 M NaOH and assayed for protein content by the procedure of Lowry et al. (23). The polysaccharide content of the extract was determined by use of the anthrone reagent (13), with a glucose standard.

UDS-urea gel electrophoresis. Immediately after extraction, the spore coat proteins were fractionated by electrophoresis in either a slab apparatus or tubes in 15% acrylamide–UDS-urea gels by the procedure of Laemmli and Favre (22), with the inclusion of 6 M urea in separating and spacer gels (6). Samples containing 10 to 50 μl of coat extract in UDS (made up to 50 μl with UDS) plus 5 μl of 0.001% bromophenol blue (made in 50% glycerol) were heated for 1 to 3 min at 100°C before loading on the gels. Electrophoresis was performed at 25 mA for slabs (or at 5 mA per tube) in 0.025 M Tris–0.19 M glycine–0.1% SDS–0.009 M mercaptoethanol at pH 8.5 until the dye marker had moved three-quarters of the way down the tube. Gels were stained with 0.025% Coomassie brilliant blue in 25% isopropanol–10% acetic acid for 10 to 12 h. They were destained in 0.0025% Coomassie brilliant blue in 10% isopropanol plus 10% acetic acid for 6 to 8 h, followed by a similar soak in 10% acetic acid.

Glycoprotein was stained on gels with periodic acid–Schiff reagent (3). Gels were immersed for 12 to 16 h in a solution of 25% isopropanol and 10% acetic acid to fix proteins and remove SDS before staining. They were then preconditioned by soaking for several hours in 0.5% sodium arsenite and 5% acetic acid. Destaining was done by soaking gels for 16 h in a solution of 0.1% sodium metabisulfite and 0.1 N HCl. For radioactive determinations, gels were frozen on dry ice immediately after electrophoresis, cut into 1-mm slices with a Yeda microtome GTS-1, swollen by incubation in NCS scintillation fluid, and counted.

Labeling of sporulating cells. Cultures (10 to 20 ml) of sporulating cells at the desired stage were labeled by adding one or more of [3H]isoleucine (2 μCi/ml), [3H]-labeled reconstituted protein hydrolysate (2 μCi/ml), or [3H]tyrosine (0.5 μCi/ml) for time periods as described with specific experiments. Pulse-labeled sporulating cells were harvested, washed with antibody buffer, and broken with a Branson Sonifier model 200 employing a microtip at maximum output for a total of 2 min. After centrifugation at 12,000 × g for 15 min, the pellet was suspended in UDS and incubated at 37°C for 1 h. After centrifugation as above, the solubilized fraction was pooled with the original supernatant fluid and dialyzed for 16 h at 27°C against 1,000 volumes of antibody buffer. An excess (100 μg/ml) of unlabeled amino acid or Casamino Acids (in case of labeling with [3H]-labeled reconstituted protein hydrolysate) was added simultaneously for steady-state labeling or after the labeling period for pulse-chase experiments. [3H]- and [14C]-labeled spores were mixed (to give a ratio of [3H] to [14C] of 3:1 or 4:1) before extraction or were extracted separately (7, 22).

125I-labeled bovine serum albumin was prepared as for antibodies (19).

Immunological experiments. Antiserum was prepared by the method of Horn et al. (19) by immunizing rabbits against either total coat or different classes of proteins (40,000-dalton plus 20,000- to 25,000-dalton class and 8,000 to 12,000 class; see Fig. 6 and 7) fractionated on a Sepharose 6B-CL column. The activity of antiserum was tested on Ouchterlony plates (11). For immunoprecipitation of cell extracts, various amounts of anti- or preimmune sera (20 to 100 μl) were added to a fixed amount of antigen (50 to 100 μl), incubated at 37°C for 1 h, and then incubated at 4°C for 16 h. An amount of 20 to 50 μl of goat antirabbit antibody was added to each tube, and the tubes were incubated at 37°C for 1 h and then at 4°C for 4 h. Pellets were collected at 12,000 × g for 15 min in a Sorvall refrigerated centrifuge, washed twice with antibody buffer, dissolved in UDS, heated for 1 min at 100°C, and subjected to electrophoresis.

Amino acid analysis. After removal of SDS as the potassium salt from UDS extracts, the proteins were precipitated with excess 15% (wt/vol) trichloroacetic acid followed by two washings with ether to remove trichloroacetic acid, and the pellets were dried. Solubilized or residue fractions were oxidized by dissolving in 0.5 ml (per 1 to 2 mg of protein) of performic
acid and incubating at 0°C for 1 h (25). The samples were diluted with water and dried in vacuo. Protein was hydrolyzed in constant boiling HCl at 110°C for 20 h in sealed and evacuated tubes. The hydrolysates were dried, washed several times with distilled water, and analyzed on a Durrum column by the procedure of Spackman et al. (30).

Synthesis and detection of dityrosine. Dityrosine was prepared by oxidizing N-acetyl tyrosine (dissolved in 0.2 M sodium borate buffer, pH 9.5) with a solution of hydrogen peroxide and horseradish peroxidase (1). The preparation was concentrated by evaporation, passed over a Sephadex G10 column (25 by 85 cm) in deionized water, and eluted with water for the separation of N,N'-diacetyl dityrosine from peroxidase and oxidation products. For further purification, ascending chromatography was done on silica plates (20 by 20 cm; layer thickness, 0.25 mm), with butanol-acetic acid-water (4:1.1:1) as the solvent system. N,N'-diacetyl dityrosine was deacetylated by acid hydrolysis as for amino acid analysis. Fluorescence determinations of different concentration of the sample were done by suspending 0.4 to 0.8 μg/ml of protein/ml in 0.2 M sodium borate buffer, pH 9.5, and measuring excitation and emission spectra on a Perkin-Elmer model MPF-4 fluorescence spectrophotometer.

Chemicals. ["C]tyrosine (460 mCi/mmoll), ["H]isoleucine (40 Ci/mmol), ["H]-labeled reconstituted protein hydrolysate, and urea were purchased from Schwarz/Mann. Lysozyme was from Worthington Biochemicals Corp.; SDS, from British Drug House, Ltd.; NCS, from Amersham-Searle; diethioerythritol, from Pierce Chemical Co.; PMSF, deoxycholate, and horseradish peroxidase, from Sigma Chemical Co.; N-acetyl tyrosine, from Eastman Organic Chemical Div., Eastman Kodak Co.; Sepharose 6B-CL, from Pharmacia Fine Chemicals, Inc.; Sepharose-hemoglobin, from P-L Biochemicals, Inc.; Freund adjuvant (complete), from Grand Island Biological Co.; and CHES and goat antirabbit serum, from Calbiochem. Other chemicals were reagent grade.

RESULTS

Solubilization of spore coat protein. Spore coat protein was solubilized either directly from washed spores or from isolated coats. Approximately 30% and 63% of the total protein could be extracted from spores and coats, respectively (Table 1). Increasing the pH to 10.5 resulted in solubilization of 75% of the protein from coats. On the basis of labeling studies, it is estimated that spore coat protein is at least 50% of the total spore protein (6, 21, 31). The less extensive solubilization from spores is believed to be due to the presence of a tightly fitting exosporial layer (6, 18). A variant of the wild type that formed spore colonies with a rougher appearance was picked, and in this case 46% of the intact spore protein could be solubilized (Table 1). In addition to proteins, some polysaccharide, equivalent to about 6% of the dry weight of the total soluble fraction, was solubilized. Subsequent extractions of the residue with UDS did not result in any further solubilization. A variety of attempts were made to solubilize the spore coat completely or to solubilize the residue further by suspension in solutions of 1 M lithium bromide-20% (vol/vol) glacial acetic acid-concentrated trifluoroacetic acid, by extraction for longer periods at 27 or 37°C in UDS, or by using higher concentrations of dithioerythritol and/or SDS. None was successful since 25 to 30% of the coat protein remained insoluble.

Gel electrophoresis of solubilized coat protein. Protein extracted from spores or isolated coats with UDS was fractionated by electrophoresis on a 15% acrylamide-SDS-urea slab gel (Fig. 1). Six to seven major polypeptides were reproducibly found, with a less complete extraction if urea were omitted. A polypeptide of about 40,000 daltons moved as a broad or diffuse band on most of the gels. Four polypeptides migrated faster than cytochrome c, in the 8,000- to 12,000-dalton range, and there was a group of polypeptides of intermediate size (20,000 to 25,000 daltons). The fastest-moving band in the low-molecular-weight region reacted as a glycoprotein with the periodate-Schiff reagent (3). Some minor bands seen on the gel may have been coat polypeptides present in only a few hundred copies per spore. Alternatively they may have been contaminants from cell fragments or lysed spores or tyrosine cross-linked dimers or trimers (see below). Noncovalently linked aggregates are unlikely because the identical gel profile resulted from extracts in UDS buffer containing 1 to 3% SDS. Similar gel patterns were obtained by extracting clean spores or the coats under a variety of conditions and from the spores of three other strains of B. subtilis (B. subtilis 168, CU1037, and CU1038). In all cases, a vigorous washing protocol (see Materials and Methods) with salts and buffer containing protease inhibitors, i.e., EDTA, PMSF, and Sepharose-hemoglobin (27),

<table>
<thead>
<tr>
<th>Material extracted</th>
<th>Percentage of total protein solubilized by UDS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spores of wild type</td>
<td>30</td>
</tr>
<tr>
<td>Isolated coats from wild-type spores</td>
<td>63</td>
</tr>
<tr>
<td>Spores of rough variant</td>
<td>46</td>
</tr>
<tr>
<td>Isolated coats of rough variant</td>
<td>60</td>
</tr>
<tr>
<td>Spores of wild type</td>
<td>33</td>
</tr>
<tr>
<td>Isolated coats from wild-type spores</td>
<td>75</td>
</tr>
</tbody>
</table>

* Treatment at 37°C for 3 h. Eighty-three percent of soluble fraction dry weight is protein and 6% is polysaccharide.

**Picked from wild type on basis of colony morphology. See text for further details.

* UDS at pH 10.5.

**UDS a
VOL. 137, plus cytochrome employed. cereus B. serine proteases spores preparations during proteins of and Komberg strated by distribution of rich (11.4 mol%, (14). To an of about 12,500 daltons). (E) Bovine serum albumin (65,000 daltons) plus RNase (13,700 daltons). (G) Trypsin (23,800 daltons) plus cytochrome c (12,700 daltons).

was employed. Absence of protease activity from preparations during extraction was demonstrated by the lack of hydrolysis of 125I-labeled bovine serum albumin that was added to washed spores or coats prior to extraction (Fig. 2). The serine proteases present in spore extracts of B. cereus and B. subtilis are inactive in UDS (O.P. Srivastava, personal communication).

Tyrosine-rich coat polypeptides. Spudich and Kornberg (31) reported that soluble coat proteins of B. subtilis strain SB113 were tyrosine rich (11.4 mol%, see Table 3). A coat component of about 12,500 daltons has been found to have an unusually high tyrosine (32 mol%) content (14). To study further the location of tyrosine-rich coat polypeptides, we analyzed the relative distribution of tyrosine in different coat fractions. Spores steady-state labeled with 3H-labeled protein hydrolysate and [14C]tyrosine were washed and then extracted with UDS; the extracts were fractionated by electrophoresis on 15% gels, sliced, and counted (Table 2). The lower-molecular-weight proteins incorporated relatively greater amounts of tyrosine. A qualitatively similar distribution was obtained when spores were labeled with [3H]tyrosine and [14C]isoleucine. The results are consistent with a relatively higher tyrosine content of the lower-molecular-weight spore coat proteins.

Amino acid analysis. As previously mentioned, about 30% of the total protein of isolated coats remained insoluble after exposure to a variety of extraction conditions. The amino acid analyses of performic acid-oxidized soluble and insoluble fractions were similar (Table 3) except for substantially lower proline and tyrosine contents of the latter. Table 3 also includes the results of amino acid analyses from other laboratories. There are significant differences in mo-

**TABLE 2. Relative distribution of tyrosine in spore coat polypeptides of B. subtilis**

<table>
<thead>
<tr>
<th>Gel band</th>
<th>Percentage of total recovered cpm</th>
<th>3H/14C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3H</td>
</tr>
<tr>
<td>40,000</td>
<td>21.0</td>
<td>21.8</td>
</tr>
<tr>
<td>20,000-25,000</td>
<td>20.7</td>
<td>150</td>
</tr>
<tr>
<td>8,000-12,000</td>
<td>33.7</td>
<td>46.8</td>
</tr>
</tbody>
</table>

See Fig. 1.

Data are the averages of two separate experiments.

3H-labeled protein hydrolysate (2 μCi/ml) and [14C]tyrosine (0.5 μCi/ml) were added to cells in nutrient sporulation medium at 37°C at the end of exponential growth plus 100 μg each of Casamino Acids and tyrosine/ml. After 12 h of incubation, spores were harvested, washed, and extracted as described in Materials and Methods. Extracts were subjected to electrophoresis in 15% acrylamide-urea-SDS gels. Gels were sliced and counted in NCS-Omnifluor cocktail, and the percentage of radioactivity recovered is shown.
**Table 3. Amino acid composition of purified spore coat fractions**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>This study</th>
<th>Mitani and Kadota</th>
<th>Spudich and Kornberg</th>
<th>Goldman and Tipper</th>
<th>Munoz and Doi</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>8.5</td>
<td>7.8</td>
<td>12.2</td>
<td>11.9</td>
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<tr>
<td>Threonine</td>
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<td>3.2</td>
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<td>5.0</td>
<td>4.6</td>
</tr>
<tr>
<td>Serine</td>
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<td>3.0</td>
<td>7.3</td>
<td>9.2</td>
<td>7.6</td>
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<tr>
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<td>10.0</td>
<td>6.8</td>
<td>8.8</td>
<td>6.0</td>
</tr>
<tr>
<td>Proline</td>
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<td>2.0</td>
<td>3.6</td>
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<td>2.8</td>
</tr>
<tr>
<td>Glycine</td>
<td>12.4</td>
<td>11.2</td>
<td>14.3</td>
<td>10.3</td>
<td>21.1</td>
</tr>
<tr>
<td>Alanine</td>
<td>7.8</td>
<td>11.5</td>
<td>8.7</td>
<td>7.7</td>
<td>7.0</td>
</tr>
<tr>
<td>Cysteic acid</td>
<td>2.8</td>
<td>4.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Valine</td>
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<td>7.9</td>
<td>5.2</td>
<td>5.7</td>
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<tr>
<td>Methionine</td>
<td>0.6</td>
<td>0.3</td>
<td>1.7</td>
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<tr>
<td>Isoleucine</td>
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<td>3.8</td>
<td>4.3</td>
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<tr>
<td>Leucine</td>
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<tr>
<td>Tyrosine</td>
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<td>7.1</td>
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<tr>
<td>Phenylalanine</td>
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<tr>
<td>Lysine</td>
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<td>6.1</td>
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<tr>
<td>Histidine</td>
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<td>3.0</td>
<td>2.0</td>
<td>3.4</td>
<td>3.0</td>
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<td>+</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.7</td>
<td>2.4</td>
<td>4.9</td>
<td>3.5</td>
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</table>

*Values of amino acids are expressed as mole percent. S, Soluble fraction; I, insoluble fraction. Different conditions were used for solubilization of proteins as noted below.

1. Coat protein solubilized in UDS for 3 h at 37°C (Materials and Methods). Analyses on a Durrum column after performic acid oxidation. Values are averages of three determinations on independently isolated preparations.

2. Coat protein solubilized in 0.01 M sodium borate buffer, pH 10.0, containing 1% SDS and 0.1 M dithiothreitol (30 min at 50°C), dialyzed against deionized water, and analyzed on a Hitachi autoanalyzer (24).

3. Coat protein solubilized with a mixture of 0.01 M dithioerythritol, 1% SDS, and 0.06 M NaOH for 50 min and analyzed on a Spinco model B amino acid analyzer (31).

4. Coat protein initially solubilized with 0.05 M sodium carbonate-bicarbonate buffer, pH 10.0, containing 1% SDS plus 50 mM dithiothreitol for 20 min at 37°C (pellet reextracted and supernatant fractions pooled). A second solubilization was achieved by heating for 3 min at 100°C in 0.06 M Tris-hydrochloride buffer, pH 6.8, with 3% SDS and 5% 2-mercaptoethanol (14).

5. Data are for pure spore coat fraction. Spores extracted in a solution containing 0.1 M dithiothreitol, 0.1 M NaCl, 1% SDS, 2 mM EDTA, 2 mM PMSF, and 5 g of Sepharose-hemoglobin, pH 9.8, for 2 h at room temperature. Supernatant neutralized to pH 7.8 with HCl and treated for 20 min at 4°C with 5 mM diisopropylfluorophosphate followed by dialysis (against 0.03 M Tris-hydrochloride, 4.5 M urea, 0.01 M mercaptoethanol, 1% SDS, pH 7.8). Extracts were then passed over a DEAE-cellulose column, dialyzed against deionized water, purified on 8% SDS-urea preparative gels, and analyzed on a Durrum column (L. E. Munoz and R. H. Doi, J. Biol. Chem., in press).

6. Peak eluting as dityrosine (Fig. 5).

Lar amounts of several amino acids such as proline, glycine, and isoleucine, and in fact no one analysis completely agrees with any other. In general, aspartate, glutamate, glycine, and alanine are high. The discrepancies may arise from differences in methods employed for solubilization of proteins and their subsequent treatment. Coat proteins are least soluble in the pH range of 6 to 8 and most soluble at pH 11.0 (24). A pH higher than 9.8 is not recommended for solubilization because at least in *B. cereus*, it has been found that some lysinoalanine is formed (6). In our efforts to define the optimal conditions for solubilization of coat proteins, we determined the percent solubilization after extracting spores or isolated coats in UDS buffer or UDS lacking urea. A 30-min extraction of spore coat with UDS resulted in 27% solubilization versus greater than 60% in 3 h. Only 15% of the protein was solubilized from coats in a 30-min extraction with the same buffer lacking urea. Electrophoresis of these extracts on 15% gels showed that relatively lower-molecular-weight proteins of the 8,000- to 12,000-dalton class were more readily extracted. There is evidence that at least one of these small proteins has an unusual amino acid composition, being very rich in tyrosine, glycine, and proline (14). The different methods of extraction (see footnotes to Table 3) and further treatments of proteins may result in
selective enrichment for certain polypeptides, thus accounting for the differences in amino acid composition.

A new broad ninhydrin-positive peak was observed in the region of the chromatogram between histidine and arginine on a Durrum column where dityrosine is eluted (Fig. 3). This new peak was detected in performic acid-oxidized hydrolysates of both soluble and insoluble coat fractions but was more prominent in the latter. When the proteins were not oxidized with performic acid prior to hydrolysis, the tyrosine content of the insoluble fraction was almost the same as that of the soluble fraction, but the dityrosine was still detected in some analyses of the former.

To demonstrate the synthesis of dityrosine in vivo, sporulating cells (at t3, t5, or t7) were incubated with [14C]tyrosine for 20 min. After centrifugation and washing once with 1 M NaCl and three times with 100 µg of unlabeled tyrosine/ml in distilled water, the pellets were hydrolyzed, and the hydrolysates were chromatographed on silica plates as described in Materials and Methods. Two radioactive areas with R’s of 0.26 and 0.6 comigrating with dityrosine and tyrosine, respectively, were found at these three times (Fig. 4). No radioactive dityrosine was detected when cells were labeled at t2 or earlier. Approximately 10% of the total radioactivity recovered from the plates is in the region of dityrosine. The fraction of total protein synthesized during any of these times that is insoluble spore coat is not known, but it is unlikely to be greater than 10%. Since only a small fraction of the tyrosine residues in the insoluble coat are cross-linked as dityrosines (Fig. 3 and 5), only a few percent of the total tyrosines incorporated into protein should be present as dityrosine. The resolution achieved (Fig. 4) is not adequate for quantitative assessments; only the synthesis of some dityrosine in sporulating cells is demonstrated.

Fluorescence spectra also provided suggestive evidence for the presence of dityrosine in spore coat fractions (Fig. 5). Based on the excitation spectrum of dityrosine (Fig. 5f), 320 nm was used in all emission studies. The emission spectrum for performic acid-oxidized total coat (or insoluble coat, not shown) was similar to the standard (Fig. 5a and b) and very different from unoxidized total coat (Fig. 5d). This difference is further evidence that oxidation increases the amount of dityrosine in coat preparations, as was found in amino acid analyses (Fig. 3).

The spectra for unoxidized insoluble and soluble coat fractions are different (Fig. 5c and e). The asymmetrical curve in Fig. 5c is interpreted as resulting from the differential contribution of two components, with maxima at about 375 and 385 to 395 nm. The spectra for total and soluble coat (Fig. 5d and e) are very similar, with maxima at about 365 and 390 nm. The spectra of the unoxidized samples were obtained at the maximum sensitivity of the spectrofluorometer, thus increasing the possibility of a nonspecific contribution from the buffer. At higher concentrations of total or insoluble coat, scattering may be a major problem, but the emission spectrum of unoxidized insoluble coat at 50 µg/ml was very similar to that in Fig. 5b. Assuming all emission between 390 and 400 nm to be due to dityrosine, there would be about 3 dityrosines per 60,000 daltons of insoluble coat protein. The relative concentration in the soluble fraction (Fig. 5e) is at least 10-fold less, with no detectable increase after performic acid oxidation.

The approximate amounts of dityrosine in oxidized samples were determined by calculating areas under the peaks and comparing them with
A when extracts of plates after the 40,000-dalton (Fig. 4) of radioactivity in the two size classes of spore coat proteins (see also Fig. 9).

**DISCUSSION**

Although most investigators have reported that the majority of the extractable spore coat protein in *B. subtilis* is of low molecular weight, there is some discrepancy as to the extent of heterogeneity (8, 14, 24, 26). Urea was found to be essential for complete extraction (Fig. 1), and it was necessary to examine extracts immediately (no dialysis, etc.) because of the ready aggregation of these proteins. About half the areas for known amounts of dityrosine. Performic acid-oxidized total coat had about 3 dityrosines per 12,000 daltons.

**Synthesis of spore coat antigen.** To examine the synthesis of spore coat antigen, antisera were prepared (see Materials and Methods) by immunizing rabbits with total coat or with two different coat fractions separated on Sepharose 6B-CL (Fig. 6), i.e., 40,000-dalton plus 20,000- to 25,000-dalton class and the 8,000 to 12,000 molecular weight class (Fig. 7). Synthesis of coat antigen was detected from *t*2 to *t*6 when labeled extracts of sporulating cells were treated with antiserum to total coat. A prominent component of about 60,000 daltons was found in immunoprecipitates (Fig. 8A) that was not seen on the gel profile of mature spore coat protein. Radioactivity in this peak disappeared after incubation of cells with excess unlabeled isoleucine (Fig. 8B). A relative decrease in the radioactivity of the 40,000-dalton region and an increase in the low-molecular-weight region was also noted. A 60,000-dalton antigen was also precipitated when extracts from sporulating cells were incubated with antisera prepared against different coat fractions (Fig. 9).

One major precipitation band and one minor precipitation band were present on Ouchterlony plates after immunodiffusion of the 20,000- to 40,000-dalton spore coat proteins or 8,000- to 12,000-dalton proteins and antibodies to the 20,000- to 40,000-dalton species or to total spore coat. These bands were confluent, suggesting common antigenic components in the two size classes of spore coat proteins.

Fig. 4. Detection of dityrosine in hydrolysates of sporulating cells. At various times cells in nutrient sporulation medium were incubated for 20 min with [14C]tyrosine, harvested, washed, hydrolyzed, and chromatographed as described in the text and Materials and Methods. The silica gel plate was cut into 1-cm squares, and each was counted in a toluene-Omnifluor cocktail. Symbols ○, △, and □ represent counts per minute in hydrolysates of cells labeled 3, 5, and 7 h after the end of growth, respectively; ● counts per minute in [14C]tyrosine. Arrows A and B represent the positions of dityrosine and tyrosine, respectively.

Fig. 5. Fluorescence emission spectra (excited at 320 nm; 5-nm band pass) of (a) dityrosine at 0.5 μmol/ml; (b) total spore coat performic acid oxidized at 0.8 μg of protein/ml; (c) unoxidized insoluble coat; 0.4 μg of protein/ml suspended in buffer; (d) unoxidized total coat at 0.8 μg of protein/ml; (e) unoxidized soluble coat at 0.8 μg of protein/ml; (f) inset excitation spectrum of dityrosine at 390 nm; 5-nm band pass. All samples in 0.2 M sodium borate, pH 9.5. Sensitivity for (a) was 1/10 that for (b) through (e). All values for (a) through (e) initiate at zero. Arrows denote wavelength of presumed maxima.
extractable protein is present as four low-molecular-weight polypeptides when isolated spore coats are extracted with UDS. One of these is a glycoprotein (perhaps present in the tight fitting exosporium). One is probably the collagen-like protein purified by Goldman and Tipper (14), and one may be the protein characterized by Munoz et al. (26). This array of specific classes suggests that the group of low-molecular-weight proteins is not generated by nonspecific proteolysis of a larger coat protein. In our studies, we have attempted to minimize such artifactual proteolysis by washing spores thoroughly and employing inhibitors. We appear to have been successful since $[^{185}]$T-albumin was not degraded when added to spore coat prior to solubilization (Fig. 2).

In contrast to nonspecific proteolysis, there is the possibility that these smaller polypeptides are processed from a larger precursor. Such a potential precursor was found in extracts of sporulating cells, and it turned over in pulse-chase experiments. We have not identified the breakdown products, but a 60,000-dalton coat antigen is precipitable by antibody to either major size class of coat polypeptides, i.e., 25,000 to 40,000 daltons or less than 12,000 daltons, implying either that antigenic sites for both size classes (or sites shared) are present in the large species or that there are different species of precursors of about the same size. If cleavage of a precursor provides most or all of the small coat polypeptides, then the sites of hydrolysis may be very specific since there are only two major N-terminal amino acids (arginine and histidine) in total soluble coat.

The insoluble fraction is similar to the soluble fraction in its overall amino acid composition and N-terminal amino acids (major arginine and histidine; unpublished results). The most significant difference between the fractions was noted when performic acid-oxidized preparations were compared. In this case, most of the tyrosine was absent from hydrolysates of the insoluble fraction, and there was a corresponding increase in dityrosine (Fig. 3). The more sensitive fluorescence analysis revealed a definitive dityrosine emission spectrum in oxidized total or insoluble coat. No such emission was detected in oxidized

![Figure 6](http://jb.asm.org/)

**Fig. 6.** Sepharose 6B-CL column chromatography of UDS extract of spore coat. The column (2 by 90 cm) was equilibrated with 0.005 M CHES buffer containing 0.1 M mercaptoethanol, 0.4% SDS, and 8 M urea at pH 9.6. About 5 mg of protein was loaded immediately after extraction and eluted with the same buffer. The first 50 ml (void volume) not containing any protein was discarded, and thereafter 3-ml fractions were collected. The column fractions were assayed for protein by a turbidimetric procedure: 0.3-ml samples were added to 1.2 ml of 4% trichloroacetic acid and incubated for 30 min at 27°C; absorbance was read at 450 nm.

![Figure 7](http://jb.asm.org/)

**Fig. 7.** SDS-polyacrylamide gel electrophoresis pattern of the spore coat proteins eluted from a Sepharose 6B-CL column as in Fig. 6. Slots A through G represent fractions 34, 28, 24, 22, 9, 7, and 5, respectively, from the column run of Fig. 6. Arrows (from top) indicate the position of bovine serum albumin and RNase. B and C were pooled for 8,000- to 12,000-dalton antigen; fractions F and G were pooled to provide 40,000-dalton plus 20,000- to 25,000-dalton antigen.
preparations of B. cereus spore coats nor in a crude extract of B. subtilis vegetative cells. The spectrum of unoxidized insoluble coat certainly differs from that of soluble coat and is suggestive of the presence of dityrosine. About 2 to 3 dityrosines could be present per 60,000 daltons of insoluble protein in unoxidized preparations (limit of sensitivity of the analysis), with a four- to fivefold increase following oxidation. The concentration of N-terminal arginine (determined as the dansyl derivative) in the polypeptides of the insoluble fraction is consistent with an average molecular weight of 35,000 to 40,000 daltons.

The capacity of sporulating cells to synthesize dityrosine was shown by chromatography of acid hydrolysates of extracts incubated with [14C]tyrosine. These cells apparently have the hydrogen peroxide and peroxidase activity necessary to form dityrosine. A puzzling result is the marked increase in dityrosine following oxidation of the total coat or insoluble fraction. Either the denatured proteins have tyrosine runs within some of the polypeptides or possibly the chains align with tyrosine residues in close proximity, thus enhancing the probability of cross-linking during oxidation. As mentioned above, performic acid oxidation of proteins does not generally lead to the formation of dityrosine.

Dityrosine has been shown to cross-link structural proteins in several cases (1, 2, 15), including a recent demonstration for the sea urchin fertilization membrane (12). Such cross-links could account for the insolubility of about 30% of the coat and may contribute to the overall structural stability of the spore. Attempts to block the formation of dityrosine selectively by adding metafluorotyrosine during sporulation were only partially successful. At concentrations that permitted spores to form (10 to 20 μg/ml), the analogue had only a marginal effect on the amount of extractable spore coat (18 to 22% of the total spore protein in the control versus 24 to 31% for the treated spores). The stability and rate of germination of these treated spores were unaltered.

The overall pattern of spore coat proteins in B. subtilis is certainly very different from that in B. cereus and related species (6). There is little if any insoluble coat protein in B. cereus and a much less heterogeneous array of soluble proteins. Disulfide bonds are a principal mech-
anism for organizing the keratin-like coat proteins of *B. cereus*. Although *B. subtilis* spore coats may be stabilized by disulfide bonds, the presence of a substantial insoluble fraction and a collagen-like protein (14) implies that other means for conferring stability have been developed.

These differences may not be surprising, considering the differences in the appearance of the coats as seen in thin sections and by freeze etching of spores (6, 14, 18, 20, 29). In comparison with *B. cereus* spores, *B. subtilis* spores contain a smaller proteoplasm with a much thicker multi-layered coat and a different surface packaging. Although all free-living soil sporeformers need both protective layers and the capacity to respond rapidly to germinants, these two species have clearly employed very different mechanisms to achieve these functions. Perhaps a more detailed understanding of the natural environments where these species are found would help to account for the differences.

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