Synthesis and Order of Assembly of Spore Coat Proteins in *Bacillus subtilis*

By H. F. JENKINSON, W. D. SAWYER† AND J. MANDELSTAM*

Microbiology Unit, Department of Biochemistry, University of Oxford, Oxford OX1 3QU

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Analysis of mature spores or their integuments by extraction with sodium dodecyl sulphate/dithioerythritol followed by electrophoresis shows that the coat contains four major proteins and about ten others. Nine of the 14 proteins begin to be synthesized in stage II or stage III and their synthesis must be controlled by stage II or stage III operons. Some of these proteins are incorporated into the spore structure from about t_4 onwards (i.e. 4 h after induction of sporulation). Their deposition in the coat between t_4 and t_7 is not stopped by chloramphenicol, with the exception of one protein (mol. wt 36 000) which begins to be synthesized only at t_6 . Spores were isolated at various stages from about t_5 onwards, and the surface proteins were labelled with ^{125}I . The labelling patterns show that proteins which are exposed on the surface at $t_{5,3}$ are successively overlaid as the spores mature. It appears that the coat of the mature spore contains at least three layers. The outermost layer is mainly composed of an alkali-soluble protein (mol. wt 12 000), which is synthesized early (t_2), and the 36 000 mol. wt protein, which is synthesized very late. Deposition of the former seems to require processing by proteolytic action, and both proteins are apparently necessary for the acquisition of resistance to lysozyme, though not for resistance to heat or organic solvents. The results are discussed in relation to the classification of sporulation events and the nomenclature of the genetic loci controlling sporulation.

INTRODUCTION

Late events in sporulation of *Bacillus subtilis* preceding the release of the mature spore specifically include the deposition of spore protein coats and the development by the spores both of their germination properties and of their resistances to organic solvents, heat and to lysozyme. The order in which these properties appear in resuspension cultures has been described by Jenkinson *et al.* (1980) and Dion & Mandelstam (1980). The evidence presented by these authors suggests that these late events, occurring during stages V and VI of sporulation, involve some form of processing and self-assembly of proteins transcribed and translated before or during stage IV. Only one of these late events, the development of lysozyme resistance, appears to require *de novo* protein synthesis during stage V.

The coat proteins make up 50 to 80% of the total protein in mature spores (Spudich & Kornberg, 1968; Goldman & Tipper, 1978), and their appearance as seen under the electron microscope occurs concomitantly with the development of the resistance properties of the spores. Earlier observations suggest that some of the spore coat proteins in *B. subtilis* may be synthesized from as early as stage II of sporulation either as soluble precursors (Munoz *et al.*, 1978; Pandey & Aronson, 1979) or as mature coat protein (Wood, 1972). However, an understanding of the structure of the spore coat and the regulation of its assembly requires (i) characterization of the component polypeptides and (ii) a method for determining their order of deposition. In this paper the sequence of synthesis and deposition of the spore coat proteins

† Present address: Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, Indiana 46223, U.S.A.

is described. The results suggest that the times of synthesis of the coat proteins are determined by an order of gene expression beginning at stage II but that their incorporation into the structure of the spore does not necessarily follow the same order.

METHODS

Chemicals. Acrylamide (purified) and *N,N'*-methylenebisacrylamide were obtained from BDH. Radioactively labelled compounds were purchased from The Radiochemical Centre, Amersham. All other chemicals were from Sigma unless otherwise specified.

Bacteria. *Bacillus subtilis* 168 *trpC2*, which requires indole or tryptophan and sporulates normally, was used. Stocks were kept at 4 °C as dilute spore suspensions in distilled water.

Induction of sporulation. Cells in the exponential phase of growth in a hydrolysed casein medium at 37 °C were harvested by centrifugation when the culture contained about 0.25 mg dry wt bacteria ml⁻¹. The pellet was resuspended to the same density in warm resuspension medium containing glutamate, inorganic salts and 20 µg tryptophan ml⁻¹ (Sterlini & Mandelstam, 1969) and incubated with shaking. About 80% of the cells contained refractile spores at 8 h after resuspension. The time of resuspension is denoted *t*₀ and subsequent times (h) as *t*₁, *t*₂, etc.

Determination of spore incidence. Spores were counted by phase-contrast microscopy and expressed as a percentage of the total number of cells. The term 'phase-bright' describes all gradations from dull white to bright spores; earlier visible sporal inclusions are termed dark forespores.

Measurement of resistance to toluene, heat and lysozyme. For the determination of toluene resistance (Toluene^R) or heat resistance (Heat^R), samples from cultures were diluted 100-fold in sporulation medium and treated with toluene (0.02 vol.) or heated (80 °C, 15 min) as previously described (Jenkinson *et al.*, 1980). The numbers of spores surviving after incubation with lysozyme (250 µg ml⁻¹) (EC 3.2.1.17) for 10 min at 37 °C were determined by plating suitable dilutions on nutrient agar (Oxoid).

Preparation of spores. Spores from cultures at *t*₂₀ were harvested by centrifugation (8000 g, 20 min, 4 °C). The pellets were washed with KCl (1 M) and NaCl (0.5 M), and incubated for 60 min at 37 °C in Tris/HCl buffer (50 mM, pH 7.2) containing lysozyme (50 µg ml⁻¹). The spores were then cleaned by alternate centrifugation (5000 g, 10 min) and washing with: NaCl (1 M); deionized water; sodium dodecyl sulphate (SDS; 0.05%, w/v); TEP buffer [Tris/HCl (50 mM, pH 7.2) containing EDTA (10 mM) and phenylmethylsulphonyl fluoride (PMSF; 2 mM)]; and deionized water (three times). The final preparations contained >98% phase-bright spores.

Spores from cultures at *t*₀ or earlier were prepared as follows. The sporangia were harvested by centrifugation (5000 g, 10 min), washed once with KCl (1 M), and suspended to a density of about 5 mg dry wt ml⁻¹ in TEP buffer. The suspension was (i) passed twice through an ice-cold French pressure cell at 83 MPa, which released >90% of the spores from their mother cells, (ii) diluted with 10 vol. TEP buffer, and then (iii) centrifuged at low speed (800 g, 3 min) to pellet spores. The spores were washed twice with TEP buffer and in some experiments purified further by centrifugation (20000 g, 30 min) through Urographin (40%, w/v) (Keynan *et al.*, 1976). Coat fragments do not pellet under these conditions (see also Stelma *et al.*, 1980).

Preparation of spore coat fractions. Spores or sporangia were suspended to a density of 2 to 5 mg dry wt ml⁻¹ in TEP buffer containing Dow antifoam (0.02%, w/v), mixed with glass beads (0.10 to 0.11 mm diam.; 10 ml suspension plus 7 ml beads) and broken in a Braun model MSK cell homogenizer. Microscopic examination showed that 4 min of shaking at full speed (4000 rev. min⁻¹) with 10 s of cooling in CO₂ every 30 s of shaking was sufficient for total disruption.

The glass beads were allowed to settle out of suspension and the supernatant was removed by pipetting. The beads were washed with a further 2 to 3 ml TEP buffer. The washings and original supernatant were pooled, and the insoluble fraction was pelleted by centrifugation (15000 g, 20 min, 4 °C). The pellet was suspended in TEP buffer containing lysozyme (50 µg ml⁻¹) and incubated for 1 h at 42 °C to hydrolyse cortical and mother-cell peptidoglycan. The suspension was again centrifuged (15000 g, 10 min) and the insoluble fraction was cleaned by alternate centrifugation and washing with: TEP buffer containing KCl (0.5 M) and glycerol (1%, w/v); NaCl (1 M; twice); SDS (0.1%, w/v) in TEP buffer; TEP buffer; NaCl (1 M); and twice with deionized water containing Tween 80 (0.01%, w/v), PMSF (2 mM), EDTA (5 mM) and iodoacetamide (0.5 mM). The residue was termed the 'spore coat fraction'.

Extraction of spore coat proteins. Spores (about 5 × 10⁹ spores ml⁻¹) or spore coat fraction were suspended in freshly prepared DS buffer [cyclohexylaminoethanesulphonic acid (CHES)/NaOH buffer (5 mM, pH 9.8) containing SDS (1%, w/v), dithioerythritol (50 mM) and PMSF (2 mM)] and incubated for 30 min at 70 °C. After centrifugation (5000 g, 5 min) the supernatant was retained. Other extraction methods are described with specific experiments.

Alkali extraction of spores. Spores were suspended to a density of about 5 × 10⁹ spores ml⁻¹ in NaOH (0.1 M) at 4 °C and left for 15 min with occasional vortex mixing (Wood, 1972). After centrifugation (5000 g, 10 min), the

supernatant was dialysed against 100 vol. sodium acetate/acetic acid buffer (0.5 M, pH 5) for 16 h at 4 °C, and then against 100 vol. deionized water for 12 h at 4 °C. The contents of the dialysis sac were then centrifuged (18 000 g, 30 min) and the pellet was redissolved in half the original volume of DS buffer (70 °C, 10 min). This was termed the 'alkali-soluble fraction'.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were fractionated by electrophoresis in a slab apparatus on 1.5 mm thick SDS (0.1%, w/v)-acrylamide gels, prepared as described by Laemmli & Favre (1973). The separating gel contained acrylamide (15%, w/v) and *N,N'*-methylenebisacrylamide (0.25%, w/v). In the stacking gel the corresponding concentrations were 5% and 0.38%. The samples to be electrophoresed were mixed with 'sample buffer' (4 vol. sample plus 1 vol. sample buffer), which contained Tris/HCl (0.2 M, pH 6.8), glycerol (30%, w/v) and bromophenol blue (0.01%, w/v), and were heated for 5 min at 100 °C. Samples were then applied to wells formed in the stacking gel, under the electrode buffer which contained Tris (0.025 M), glycine (0.192 M) and SDS (0.1%, w/v). Electrophoresis was performed at 8 mA per slab until the bromophenol blue tracking-dye had penetrated the separating gel, and then at 25 mA per slab until the dye marker had moved to about 0.5 cm from the end of the gel. The gels were stained with Coomassie Brilliant Blue R250 (0.2%, w/v) in propan-2-ol/acetic acid/water (25:10:65, by vol.) for 14 h and destained first in propan-2-ol/methanol/acetic acid/water (10:5:10:75, by vol.) until the background was colourless, and then finally destained and stored in methanol/acetic acid/water (5:10:85, by vol.). Stained gels were scanned with a double-beam recording microdensitometer (Joyce, Loebel & Co, Gateshead) in which the pen deflection was proportional to the absorbance. Molecular weights of proteins were estimated from their distances of migration by reference to a graph relating migration distances for six marker proteins to log molecular weight. The markers were bovine serum albumin (mol. wt 66 000), ovalbumin (45 000), pepsin (34 700), trypsinogen (24 000), β -lactoglobulin (18 400) and lysozyme (14 300).

For visualization of radioactively labelled proteins separated by SDS-PAGE, the stained gels were impregnated with scintillant (Bonner & Laskey, 1974), dried on to Whatman 3MM paper, and then exposed to pre-flashed Fuji Medical X-ray film (Laskey & Mills, 1975) at -70 °C for an appropriate time (2 to 5 d).

Determination of protein. The sample to be assayed (10 to 200 μ l) was added to 5 ml of ice-cold trichloroacetic acid (TCA; 5%, w/v) and kept at 4 °C for 15 min. After centrifugation (12 000 g, 30 min) the protein was suspended in 0.5 ml NaOH (1 M) and heated for 5 min at 100 °C. Protein was then determined by the Lowry method using bovine serum albumin as standard. For electrophoresis of samples in the same series, the volumes applied were adjusted so that all samples contained the same amount of protein.

Radioactive labelling. Spores were radioactively labelled by adding one of the following to sporulation medium at t_0 : *N*-acetyl-D-[1- 14 C]glucosamine (0.05 μ Ci ml $^{-1}$, 100 μ g *N*-acetyl-D-glucosamine ml $^{-1}$), L-[4,5- 3 H]leucine (0.2 μ Ci ml $^{-1}$, 100 μ g L-leucine ml $^{-1}$), L-[35 S]methionine (0.1 μ Ci ml $^{-1}$, 30 μ g L-methionine ml $^{-1}$), L-[U- 14 C]tyrosine (0.1 μ Ci ml $^{-1}$, 40 μ g L-tyrosine ml $^{-1}$) or [35 S]sulphate (0.5 μ Ci ml $^{-1}$, 74 μ g sulphate ml $^{-1}$) (1 μ Ci = 37 kBq).

Other radioactive labelling methods are described with specific experiments. Whenever $^{35}\text{SO}_4^{2-}$ was used, the MgSO_4 in sporulation medium was replaced by MgCl_2 ; this had no effect on the rate or degree of sporulation. Radioactivity in aqueous samples (0.1 to 0.5 ml) was measured by adding ethanol (6 ml) followed by 2-(4'-*tert*-butylphenyl)-5-(4''-biphenyl)-1,3,4-oxadiazole (butyl-PBD; 0.5%, w/v) in toluene (8 ml) and counting in a LKB Wallac 81 000 liquid scintillation counter. After separation of radioactively labelled proteins by SDS-PAGE, the channels were excised, frozen and sliced into 1 mm sections. The slices were incubated for 14 h at 42 °C in Protosol (New England Nuclear; 0.45 M, 0.5 ml) after which Omnifluor (New England Nuclear; 0.5%, w/v) in toluene (6 ml) was added, and the radioactivity in each slice was determined with a liquid scintillation counter.

Carbohydrate staining. After electrophoresis, gels were stained for carbohydrate using the periodic acid-Schiff (PAS) procedure of Fairbanks *et al.* (1971) and also using Stainsall (BDH) (King & Morrison, 1976).

^{125}I surface-labelling. Cleaned spores (about 3 mg dry wt) or spore coat fraction from an equivalent amount of spores were labelled with ^{125}I (0.15 mCi) using lactoperoxidase (EC 1.11.1.7; Sigma) by the method of Marchalonis (1969) and the procedure described by Swanson *et al.* (1975). After labelling, the samples were washed three times in $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer (0.05 M, pH 7.3) containing NaCl (0.15 M) and NaI (5 mM). The proteins of the spore coat fractions were then extracted with DS buffer, which solubilized >80% of the ^{125}I , and separated by SDS-PAGE. The radioactivity associated with each protein was determined by counting 1 mm slices in a LKB Wallac 1270 Rackgamma counter. In one experiment, spores were surface-labelled with ^{125}I using the sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a,6a-di-phenylglycoluril (Fraker & Speck, 1978) deposited as a film on the bottom inner surface of a glass vial.

Cyanogen bromide (CNBr)-activated dextran surface-labelling. Spores labelled with [^{35}S]methionine (3 mg dry wt) or spore coat fractions were suspended in $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ buffer (0.1 M, pH 8.5; 1 ml) and incubated with freshly prepared CNBr-activated dextran (Pharmacia) for 60 min at 23 °C (Kamio & Nikaido, 1977). For each sample treated with CNBr-activated dextran, a parallel sample was treated similarly but omitting the labelling agent. After coupling, the samples were washed and the proteins of the spore coat fraction were separated by

SDS-PAGE. The migration profile was determined by scanning the stained gel and by the distribution of radioactivity.

RESULTS

Fractionation and solubilization of spore coat proteins

Sporulation in *B. subtilis* is essentially complete 8 to 10 h after transfer of exponentially growing cells to resuspension medium at 37 °C (see Methods). The spores at this time are morphologically similar to free spores at t_{20} and about 90% are resistant to heating (80 °C, 15 min) and to treatment with lysozyme (250 µg ml⁻¹, 37 °C, 10 min) (Jenkinson *et al.*, 1980). Initial experiments were done (i) to compare the proteins extracted from the insoluble integument fraction (Murrell, 1967), obtained after breakage of the spores, with those extracted from the outside of intact spores, and (ii) to compare different methods for removing or solubilizing the coat proteins.

To identify the proteins present in the insoluble spore layers, spores from a culture at t_9 were purified and broken with glass beads. After removal of the beads, the insoluble integuments – containing cortical peptidoglycan, membrane components and spore coat proteins – were treated with lysozyme and extensively washed. The resultant spore coat fraction contained <20% of the initial peptidoglycan, as estimated by the content of incorporated *N*-acetyl-D-[¹⁴C]glucosamine, and about 75% of the total spore protein (Table 1).

Treatment of this spore coat fraction with DS buffer (see Methods) solubilized about 50% of the [³⁵S]sulphate and 65% of the [³H]leucine which had been incorporated into the protein during sporulation (Table 1; see also Goldman & Tipper, 1978). The residue could not be solubilized further by re-extracting with DS or by re-extracting and alkylating, but was partly (20 to 40%) degraded to acid-soluble material by treatment with Pronase (100 µg ml⁻¹, 37 °C, 20 min) (Table 1).

The SDS-PAGE pattern of proteins extracted with DS buffer from the spore coat fraction is shown in Fig. 1, lanes 1 and 2. The pattern was reproducible but changes in the washing procedures sometimes affected the position and intensity of some minor bands. The pattern of major bands was not affected either by alkylating the DS extracts with 100 mM-iodoacetamide (30 °C, 15 min) before application, or by varying the heating time of the samples from 1 to 5 min at 100 °C before application. Each protein band is referred to by its apparent molecular weight (mol. wt × 10⁻³ = K) primarily as an aid to identification and not necessarily as an accurate molecular weight determination.

Four major proteins – 36K, 20K, 12K and 11K – dominated the coat fraction. An additional ten proteins – 65K, 59K, 33K, 30K, 26K, 24K, 15K, 10.5K, 9K and 8K – were

Table 1. *Distribution of N-acetyl-D-[¹⁴C]glucosamine, [³⁵S]sulphate, [³H]leucine and protein during the preparation of spore coat fractions from t_9 spores*

Sample	<i>N</i> -Acetyl-D-[¹⁴ C]glucosamine (c.p.m. ml ⁻¹)	[³⁵ S]Sulphate (c.p.m. ml ⁻¹)	[³ H]Leucine (c.p.m. ml ⁻¹)	Protein (µg ml ⁻¹)
Total before fractionation	15 320 (100)*	13 140 (100)	5 900 (100)	4 550 (100)
Integuments	11 470 (74.8)	6 165 (46.9)	5 240 (88.8)	3 640 (80.0)
Spore coat fraction, cleaned and washed	2 910 (19.0)	4 465 (34.0)	4 732 (80.2)	3 504 (77.0)
Residue after incubation with DS buffer	1 024 (6.7)	2 215 (16.8)	1 670 (28.3)	1 437 (31.6)
Residue after treatment with Pronase	NM	1 405 (10.7)	1 290 (21.9)	NM

NM, Not measured.

* Figures in parentheses are percentages of the total radioactivity before fractionation.

Fig. 1. SDS-PAGE patterns (stained with Coomassie blue) of proteins extracted from spore coat fractions and whole spores. The positions of molecular weight markers are indicated for reference and the coat protein bands are referred to by their apparent molecular weights (see text).

Lane 1: proteins extracted with DS buffer from the coat fraction obtained after breakage of t_9 spores (20 μ g protein applied). Lane 2: as for lane 1 (41 μ g protein). Lane 3: proteins extracted with DS buffer from whole spores at t_9 (34 μ g protein). Lane 4: proteins extracted with 0.1 M-NaOH from whole spores at t_{30} (8 μ g protein). Lane 5: proteins extracted with DS buffer from whole spores at t_{30} (38 μ g protein). Lane 6: proteins extracted with DS buffer containing 8 M-urea from whole spores at t_{30} (36 μ g protein). Lane 7: proteins extracted with DS buffer from the coat fraction obtained after breakage of t_{30} spores (30 μ g protein). The various extraction procedures are described in Methods and in the text.

always visible (Fig. 1, lane 2). The 12K band often ran as a doublet and the 20K component contained varying amounts of three polypeptide bands at 20K, 19K and 17.5K. Quantitative densitometry of gels stained with Coomassie blue showed that the four major protein bands accounted for about 50% of the total integrated density in the stained profile. When stained for carbohydrate using the PAS reagent (see Methods), only a region just behind the tracking-dye was stained. Therefore either the 8 to 9K components are glycoproteins or the PAS-positive activity may be due to non-specific association of carbohydrate and protein. The same region stained positively for carbohydrate using the Stainsall method.

As an independent measure of whether any of the proteins of the spore coat fraction were covalently linked to peptidoglycan, sporulating cells were labelled with *N*-acetyl-D- $[^{14}\text{C}]$ glucosamine and spore coat fractions were prepared with or without lysozyme treatment. After extraction with DS buffer and SDS-PAGE, none of the proteins specifically had radioactivity associated with it. In addition, neither the distribution of radioactivity nor the migration distance of the polypeptides was affected by lysozyme treatment, and so none of the proteins seemed to be covalently linked to peptidoglycan. Any proteins non-covalently linked to peptidoglycan were likely to be released during the extraction of the coat fraction. About 25% of the total spore protein was removed when intact t_9 spores were incubated with DS buffer, and the protein pattern after SDS-PAGE of this extract was similar to that described above (Fig. 1, lane 3). The spores remained phase-bright and heat-resistant after this treatment, but became sensitive to lysozyme (see also Gould *et al.*, 1970; Wood, 1972).

Spores from cultures at t_{30} were also treated with 0.1 M-NaOH or incubated with DS

Fig. 2. SDS-PAGE patterns (stained with Coomassie blue) of proteins extracted in DS buffer from the insoluble fractions obtained after breakage of complete sporangia at various times during sporulation. Each lane was loaded with $40 \pm 5 \mu\text{g}$ protein. The arrows in lane 1 denote probable membrane proteins of mother-cell origin (see text).

Lanes 1 to 5: sporangial extracts at t_4 , t_5 , t_6 , t_7 and t_9 as indicated. Lane 6: proteins extracted from the coat fraction of isolated spores at t_9 (see Fig. 1, lanes 1 and 2). Lanes 7 to 9: sporangial extracts at t_9 from cultures to which chloramphenicol (CAM) had been added at t_5 (lane 7), t_6 (lane 8) or t_7 (lane 9).

buffer or with DS buffer containing 8 M-urea at 37°C for 3 h (Pandey & Aronson, 1979). The proteins in these later spores were more resistant to extraction. Alkali treatment (see Methods) removed $<5\%$ of total spore protein and preferentially solubilized the 12K protein (Fig. 1, lane 4), as shown by Wood (1972). Treatment of spores with DS buffer with or without urea removed 15 to 20% of total protein. The inclusion of urea did not noticeably alter the profile of proteins extracted (Fig. 1, lanes 5 and 6). The SDS-PAGE pattern of proteins extracted from the coat fraction of t_{30} spores was similar to that for t_9 spores (Fig. 1, lane 7). However, in all the extracts from t_{30} spores the amount of 36K protein was considerably reduced and this may indicate either that it is altered during prolonged maturation, or that it is not extractable.

Appearance of coat proteins during sporulation

To determine the times of appearance of the proteins in the spore coat fraction, portions (70 ml) of sporulating culture were removed at intervals after resuspension and the sporangia were harvested by centrifugation. The sporangia were broken with glass beads, centrifuged, washed and the insoluble fractions were extracted with DS buffer. In Fig. 2, lane 1, some of the coat proteins in the extract of whole sporangia can be identified by comparing their positions with those in the coat fraction from isolated spores at t_9 (Fig. 2, lane 6). Some of the high molecular weight polypeptide bands (arrowed in Fig. 2, lane 1) appear only in the insoluble fractions obtained from broken sporangia. Since preparations from broken sporangia at t_2 also contain these proteins (results not shown) they are probably membrane proteins

Table 2. *Deposition of spore proteins in the presence of chloramphenicol (CAM) and phenylmethanesulphonyl fluoride (PMSF)*

Sporulating cultures were labelled with [^{14}C]tyrosine from t_0 . At t_5 portions (70 ml) of culture were removed and incubated with CAM (100 $\mu\text{g ml}^{-1}$), PMSF (4 mg ml^{-1}), both inhibitors, or left untreated. The radioactivities in the spores and sporangia from the cultures at t_9 were compared with those in the spores and sporangia at t_5 . The numbers of viable spores isolated at each time were similar: $2.1 \pm 0.2 \times 10^8$ colony-forming units (c.f.u.) ml^{-1} .

Sample	Radioactivity in sporangia (c.p.m. ml^{-1})	Radioactivity in isolated spores (c.p.m. ml^{-1})	Heat-resistant (80 °C, 15 min) spores (c.f.u. ml^{-1})	Specific radioactivity [c.p.m. (10 ⁸ Heat ^R spores) ⁻¹]
t_5 , control	39 760	6 800	1.2×10^7	—
t_9 , control	59 000	36 500	2.5×10^8	14 600
t_9 , CAM added at t_5	32 760	12 700	1.7×10^8	7470
t_9 , PMSF added at t_5	65 230	33 370	2.4×10^8	13 904
t_9 , CAM + PMSF added at t_5	41 130	21 800	2.4×10^8	9083

from the mother cells and prespores. The results in Fig. 2 show that the 12K and 20K bands had appeared by t_5 , the 11K protein was evident at t_5 to t_6 , and the 36K band by t_7 .

The late appearance of the 36K protein suggested that this component of the spore coat might be synthesized late in sporulation, i.e. during stage V. The alternative explanation is that the protein was synthesized earlier, but incorporated into the insoluble fraction only late during stage V. To distinguish between these possibilities, chloramphenicol (CAM; 100 $\mu\text{g ml}^{-1}$) was added to portions of a sporulating culture at t_5 and at t_6 . Addition of the inhibitor at these times inhibited the formation of the 36K protein (Fig. 2, lanes 7 to 9). Thus, the late appearance of this protein in the spore coat requires *de novo* protein synthesis.

Comparison of lanes 6 and 7 in Fig. 2 shows that most of the components of the coat have already appeared at t_5 , though some of them increased in intensity in the presence of CAM. This must represent the incorporation into the insoluble fraction of pre-formed proteins because earlier work has shown that the concentration of CAM used was sufficient to inhibit protein synthesis completely (Jenkinson *et al.*, 1980).

Deposition of spore coat proteins in the presence of chloramphenicol and phenylmethanesulphonyl fluoride

Earlier experiments showed that when CAM was added to a sporulating culture at t_5 , about 60% of the developing spores subsequently acquired heat resistance. The addition at t_5 of PMSF (4 mg ml^{-1}), a serine protease inhibitor, together with CAM allowed all the spores to develop heat resistance. This effect was attributed to the inhibition by PMSF of intracellular proteolysis (Jenkinson *et al.*, 1980). These observations, together with that which showed that spore coat material was deposited in the presence of CAM, suggested that self-assembly of proteins was occurring during the later stages of sporulation (Jenkinson *et al.*, 1980).

To obtain a more precise measurement of the amount of coat protein deposited in the presence of CAM, a sporulating culture was incubated with [^{14}C]tyrosine from t_0 (see Methods). At t_5 , spores were isolated from a portion of the culture (70 ml) and radioactivity was measured. Three further portions (each 70 ml) were removed and incubated with CAM, or PMSF, or both inhibitors. At t_9 , the spores were isolated and their radioactivity was measured. These spores, and those isolated at t_5 , were then broken and the coat fractions were prepared.

The radioactivity values showed that CAM prevented further incorporation into the sporangia; in fact, there was a slight loss of counts from 39 760 to 32 760 c.p.m. ml^{-1} between t_5 and t_9 (Table 2). Nevertheless, the radioactivity in the proteins deposited in the spores increased from 6800 to 12 700 c.p.m. ml^{-1} ; the spores in the control (untreated) culture had a

Fig. 3. SDS-PAGE patterns (stained with Coomassie blue) of coat proteins deposited in the presence of chloramphenicol (CAM) or phenylmethylsulphonyl fluoride (PMSF). Sporulating cultures were labelled with [^{14}C]tyrosine as described in the legend to Table 2. The spores were prepared from 70 ml portions of culture and the amounts of protein in each lane are roughly equivalent to the amounts of protein in spore coat fractions.

Lane 1: t_5 , spore coat fraction (15 μg protein applied). Lane 2: t_9 , control (55 μg protein). Lane 3: t_9 , CAM added at t_5 (21 μg protein). Lane 4: t_9 , CAM + PMSF added at t_5 (22 μg protein). Lane 5: t_9 , PMSF added at t_5 (48 μg protein).

much higher count (36 500 c.p.m. ml^{-1}) (Table 2). Allowing for the lower numbers of heat-resistant spores formed in the culture treated with CAM, the results showed that the spores produced in its presence had only about 50% of the protein content of normal spores (Table 2). The spores produced in the presence of CAM and PMSF had about 60% of the normal protein content.

Comparison of the SDS-PAGE patterns of proteins in the coat fractions of these spores showed that in the presence of CAM or CAM plus PMSF the 36K protein was not synthesized and no new protein bands appeared. However, a number of the bands were intensified (Fig. 3, compare lanes 1, 3 and 4) especially the 26K, 24K and 11K proteins. The 12K protein was deposited in the presence of CAM (Fig. 3, lane 3), but did not appear in significant amounts when PMSF was added either alone (Fig. 3, lane 5) or with CAM (Fig. 3, lane 4).

Times of synthesis of spore coat proteins

To measure the periods in sporulation during which the coat proteins are synthesized, portions (70 ml) of sporulating culture were removed at intervals and incubated for 1 h with [^{14}C]tyrosine (0.1 $\mu\text{Ci ml}^{-1}$, 20 $\mu\text{g L}$ -tyrosine ml^{-1}) and [^{35}S]methionine (0.1 $\mu\text{Ci ml}^{-1}$, 20 $\mu\text{g L}$ -methionine ml^{-1}). Two amino acids were used to allow for the fact that an individual protein might contain very little of one of the amino acids and thus be poorly labelled. After 1 h the cells were harvested by centrifugation and resuspended in their original volumes in supernatants from parallel cultures incubated without radioactive amino acids. An excess of both L-tyrosine and L-methionine carrier was then added (500 $\mu\text{g ml}^{-1}$ each) and the cultures were incubated to t_9 . The distribution of radioactivity in the proteins of the spore coat fractions was visualized by fluorography and compared with the distribution of radioactivity in the

Fig. 4. SDS-PAGE patterns (revealed by fluorography) of spore coat proteins extracted with DS buffer from spores at t_0 in cultures radioactively labelled with [^{14}C]tyrosine and [^{35}S]methionine for hourly periods during sporulation. The same amount of protein (40 μg) was applied to each lane. Lanes 1 to 3 were exposed for a longer period (5 d) to emphasize some of the fainter bands.

Lanes 1 to 3: labelled from t_2 to t_3 , t_3 to t_4 and t_4 to t_5 , respectively. Lanes 4 to 8: labelled from t_2 to t_3 , t_3 to t_4 , t_4 to t_5 , t_5 to t_6 and t_6 to t_7 , respectively. Lane 9: labelled from t_0 to t_9 . Lane 10: labelled from t_0 to t_9 with [^{35}S]sulphate instead of radioactive amino acids.

proteins of the spore coat fractions from spores labelled from t_0 to t_9 with [^{14}C]tyrosine and [^{35}S]methionine.

Before considering the times of synthesis, the protein patterns shown up by different methods should be compared. Comparison of Fig. 4, lane 9, with Fig. 3, lane 2, shows that virtually all the coat proteins which stain with Coomassie blue can be identified by fluorography. However, the 20K group of proteins had comparatively little radioactivity associated with them. An alternative method of labelling from t_0 to t_9 with [^{35}S]sulphate revealed that this 20K group, the 65K protein and the 11K protein were all heavily labelled (Fig. 4, lane 10). The 11K protein may be that identified by Linn & Losick (1976) as being predominant in the spore coat fraction at t_4 . These proteins are probably therefore cysteine-rich and can be contrasted with the 36K, 30K, 12K, 9K and 8K proteins which were poorly labelled with [^{35}S]sulphate (Fig. 4, lane 10). The 12K band probably includes the tyrosine-rich polypeptide purified by Goldman & Tipper (1978).

Incubation of a sporulating culture from t_1 to t_2 with radioactive amino acids did not significantly label any of the coat proteins subsequently extracted from t_0 spores (data not shown). Pulse-labelling from t_2 to t_3 and from t_3 to t_4 (during stages II to III) showed that during these periods the 33K, 30K, 26K, 24K and 12K proteins were being synthesized (Fig. 4, lanes 1, 2, 4 and 5). Pulse-labelling between t_4 and t_5 showed that the 65K, 59K, 20K, 12K, 11K and 8K proteins were being synthesized (Fig. 4, lanes 3 and 6). The late appearance of the 36K protein is confirmed by this experiment which shows it to be labelled only at later times, i.e. after t_6 (Fig. 4, lanes 7 and 8). The labelling patterns also show that the

Fig. 5. Proteins exposed on the surface of spores isolated at various times during the late stages of sporulation. At $t_{5.3}$ (a; when about 30% of the cells contained phase-bright spores), at $t_{6.3}$ (b) and at t_9 (c) the developing spores were isolated from their sporangia and surface-labelled with ${}^{125}\text{I}$ using lactoperoxidase. The spores were then washed, broken with glass beads and the proteins of the spore coat fractions were separated by SDS-PAGE. The gels were stained with Coomassie blue, scanned with a densitometer and then sliced into 1 mm sections which were counted for radioactivity in a gamma-counter. —, Densitometer scan (absorbance); ----, ${}^{125}\text{I}$ radioactivity.

alkali-extractable protein (12K) is synthesized from about t_2 , and onwards (see also Wood, 1972).

Identification of surface proteins by in vitro labelling with ${}^{125}\text{I}$

To confirm the location of the coat proteins and to determine which of them are exposed on the spore surface, intact spores were isolated at various times during sporulation and

Fig. 6. SDS-PAGE of proteins extracted from isolated t_9 spore coat fractions labelled with ^{125}I using lactoperoxidase. —, Densitometer scan (absorbance) of gel stained with Coomassie blue; ----, ^{125}I radioactivity in gel slices.

surface-labelled with ^{125}I using a lactoperoxidase-catalysed reaction. The enzyme is too large to penetrate lipid bilayers or known membrane pores and therefore it is reasonable to suppose that it will not penetrate the spore coat (see Warth, 1978).

A comparison of the labelling patterns at the different times appears to demonstrate an orderly sequence of deposition of the coat proteins. Early on, e.g. at $t_{5.3}$, the 30K, 24K and 15K proteins were the most prominent surface components (Fig. 5a), whereas at $t_{6.5}$ the 20K group of proteins became predominantly labelled (Fig. 5b). Later, as the 36K protein was synthesized, it appeared on the surface of the spores, together with the 12K, 9K and 8K proteins (Fig. 5c). Concomitant with the appearance of these proteins on the surface, the labelling in the 30K, 24K and 15K proteins and in the 20K group was reduced. Surface-iodination using a sparingly soluble chloramide as iodogen (Fraker & Speck, 1978) gave similar results.

Some proteins, e.g. 11K, remained virtually unlabelled. This could be explained in either of two ways: (i) they might not be prominent surface proteins at any time or (ii) they were not iodinated because they lack tyrosine, or for some other reason. To test the iodination potential of the coat proteins, t_9 spores were broken to make the proteins more accessible and then the washed coat fraction was labelled with ^{125}I using lactoperoxidase. Virtually all the proteins in the coat fraction were labelled (Fig. 6). Although some of the proteins (those in the 20K group, and 11K) were iodinated less heavily than others, the results showed that these proteins could have been labelled if they had been accessible to the labelling reagents.

Surface-labelling of spores with CNBr-activated dextran

Confirmation of the surface locations of the 36K and 12K proteins was obtained with CNBr-activated dextran. This agent when polymerized and activated forms covalent linkages with free amino groups, and it does not penetrate cells (Kamio & Nikaido, 1977). Thus, proteins exposed on the spore surface and labelled with CNBr-activated dextran would be greatly increased in molecular weight: displacement of a protein band, after SDS-PAGE, from its usual position towards the top of the gel would indicate reaction of that protein with the CNBr-activated dextran.

Fig. 7. SDS-PAGE patterns (stained with Coomassie blue) of proteins extracted from spore coat fractions of t_9 spores untreated (lane 1) or treated (lane 2) with CNBr-activated dextran. The same amount of protein (41 μ g) was applied to each lane.

Analysis of the spore coat proteins of t_9 spores treated with CNBr-activated dextran showed an almost complete (93%) loss of the 36K protein (Fig. 7). A lesser proportion (about 15%) of the 12K protein was lost. Compared with the untreated control, some protein was shifted to higher molecular weight as indicated by smearing of substances stained with Coomassie blue at the top of the separating gel (Fig. 7). There were little differences in density and radioactivity of the other protein bands in the samples treated with CNBr-activated dextran.

DISCUSSION

Most of the events associated with stages V and VI of sporulation, including the development of the physical resistance properties and germination characteristics of the spores, involve the incorporation, presumably into the spore coat, of proteins synthesized during the earlier stages of sporulation (Dion & Mandelstam, 1980; Jenkinson *et al.*, 1980). The object of the work described here was to characterize some of these proteins and to determine the times at which they were synthesized and their order of deposition. It was first necessary to establish a reliable method for extracting and recognizing the constituents of the spore coat. Extraction with SDS/dithioerythritol at pH 9.8 of the washed spore integument fraction, followed by electrophoresis (SDS-PAGE), gave reproducible protein patterns resembling those described by Goldman & Tipper (1978) and by Pandey & Aronson (1979).

We will first consider the times of synthesis of the spore coat proteins. Pulse-labelling experiments (Fig. 4) show that these times spread over several hours. Some of the proteins, e.g. 33K, 30K, 26K, 24K and 12K, begin to be formed early between t_2 and t_4 ; others, e.g. 11K, 9K and 8K, are formed later. The durations of synthesis of these proteins also differ. For example, the 12K protein begins to be synthesized in quantity after t_2 and continues to be made throughout sporulation. By contrast, the 24K, 26K and 33K polypeptides that appear

in the spore are not made after t_4 . One component of the spore coat, the 36K protein, appears to be synthesized only late (after t_6), and this conclusion is supported by the experiments with chloramphenicol (see below). The beginning and approximate duration of synthesis for each of the major proteins are depicted in Fig. 8.

Earlier experiments with chloramphenicol had suggested that a considerable fraction of the bulk protein could be deposited in the spore structure without *de novo* synthesis (Jenkinson *et al.*, 1980). The results in Table 2 extend these observations and show that in the presence of chloramphenicol the protein content of the spore almost doubles between t_5 and t_9 . The corresponding protein patterns (Fig. 3, lanes 1 and 3) show that some bands, e.g. 12K, which are not apparent at all at t_5 become quite distinct by t_9 in spite of the presence of chloramphenicol. Most of the other bands show a noticeable intensification between t_5 and t_9 . However, deposition of the 36K protein seems to be totally prevented if chloramphenicol is added at t_5 . This result fits with the pulse-labelling data already discussed. Since, moreover, chloramphenicol prevents the development of lysozyme resistance – but not of the other forms of resistance (Jenkinson *et al.*, 1980) – it seems that this particular property depends, in part, on the synthesis and incorporation of the 36K protein into the spore structure.

When PMSF was present, either alone or with chloramphenicol, the deposition of the 12K protein was either partially or totally prevented (Fig. 3, lanes 4 and 5). Since the 12K protein is one of those that is made fairly early in sporulation (Fig. 4), the observation that PMSF prevents its deposition suggests that proteolytic cleavage may be an essential pre-requisite for its inclusion in the spore structure. It is also significant that PMSF, like chloramphenicol, prevents the acquisition of lysozyme resistance. The tentative conclusion is that the 12K and 36K proteins, which together constitute a large part of the outer layer of the spore coat (see below), are both necessary for lysozyme resistance, i.e. that this property is conferred primarily by the outermost layers (Fig. 8).

The results so far discussed indicate that there is an order of assembly of proteins during stages V and VI. An attempt was made to follow this by using ^{125}I to label the outer proteins of the spore surface at $t_{5.3}$, $t_{6.5}$ and t_9 (mature spores). The data from Fig. 5 is shown schematically in Fig. 8. This diagram implies the existence of three distinct layers in the mature spore; however, there may be multiple layers and some of the individual proteins may be present in several of them. The diagram (Fig. 8) shows that at $t_{5.3}$ some proteins – 16K, 26K, 33K and some of the 59K – are already below the surface, and that the 30K, 24K, 20K and 11K proteins are uppermost. An hour later the 59K protein is completely embedded and the 30K protein partially so. In the mature spore, most of the proteins just mentioned have become overlaid and the outside layer consists mainly of four proteins (36K, 12K, 9K and 8K). Of these, the first two, at least, appear to be essential for the development of lysozyme resistance (see above). The deposition of the 36K protein requires *de novo* protein synthesis while the deposition of the 12K protein appears to require processing by partial proteolysis. We have no evidence to suggest that the 36K or 12K polypeptides have, or are, larger precursors.

The way in which various polypeptides are deposited on the spore surface and then progressively overlaid by others as the spore matures provides a molecular basis for the layered structure of the spore coat that is commonly observed in electron micrographs (Aronson & Fitz-James, 1976). In Fig. 8, the deposition of these layers is correlated with, though not necessarily responsible for, the changes in resistance and germination properties of the spores associated with stage V to VI. Thus, at $t_{5.3}$ the spores are toluene-resistant and, if cold-shocked, will germinate spontaneously (Dion & Mandelstam, 1980; Jenkinson *et al.*, 1980). About an hour later heat resistance has developed and the spores no longer germinate spontaneously. By t_9 the spores are lysozyme-resistant and germination now occurs only in response to L-alanine.

The lactoperoxidase-catalysed method of surface-labelling, on which these conclusions depend, specifically iodinated tyrosine residues. It was therefore necessary to ensure that the

Fig. 8. Diagrammatic representation of the times of synthesis and order of deposition of some of the spore coat proteins. The times of synthesis have been compiled mainly from the results of pulse-labelling experiments (Fig. 4). The proposed order of assembly of the various proteins is based on the relative amounts of each protein in the spore coats at each time and the distribution of ^{125}I in the proteins after surface-labelling (Fig. 5).

Each protein is represented as a unit the area of which approximates to the proportion of that protein in the total extract. The outer surface is indicated as a serrated edge. The diagram is not meant to show: (i) that any specific proteins overlay others (thus at $t_{5.3}$, the 20K group does not necessarily overlay 16K and 26K specifically) or (ii) that because a protein is surface-labelled all molecules of that protein are exposed. Shading of the units indicates the times at which the synthesis of the polypeptides begin: t_2 to t_3 , t_3 to t_4 , t_4 to t_5 , t_5 to t_6 , t_6 to t_7 , t_7 to t_8 , t_8 to t_9 . The time of synthesis of 16K is uncertain and is omitted.

The resistance properties (Jenkinson *et al.*, 1980) and germination properties (Dion & Mandelstam, 1980) of the spores associated with the deposition of the layers are also shown. 'Spontaneous' germination refers to germination of prespores in phosphate buffer following cold-shock; KGF is a mixture of KCl, glucose and fructose.

proteins contained enough tyrosine to have been labelled if they had been accessible. This was done by iodinating isolated spore coats and showing that all the proteins in question were labelled. This seems to justify the conclusion that, in the intact spore, if a protein is labelled it is on the surface, and if it is unlabelled it is embedded. The results with CNBr-activated dextran, which do not depend on tyrosine content, confirm that the 36K and 12K proteins are exposed on the surface of t_0 spores.

The sequence of synthesis and assembly summarized in Fig. 8 has implications for the classification of sporulation events and for the nomenclature of sporulation loci. The stages of sporulation are conveniently defined by morphology as seen in electron micrographs. Thus, the formation of the spore coat defines stage V, and the various resistance properties that develop concomitantly are accordingly classified as stage V events. However, of the 14 polypeptides which together account for most of the coat structure, nine begin to be synthesized between t_2 and t_4 , i.e. they are stage II or stage III events and the corresponding genes must be in stage II or stage III operons.

It is thus apparent that a defective protein produced during stage II, for example 12K polypeptide, may give rise to a lesion at stage V to VI, namely sensitivity to lysozyme. It is probably significant that several of the genetic loci designated as 'spoV' are very close to known stage II or stage III loci. It might be expected that more detailed mapping would show the identity of the loci concerned.

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