Germination and Resistance Defects in Spores of a *Bacillus subtilis* Mutant Lacking a Coat Polypeptide

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(Received 13 March 1981)

Spores produced by a mutant of *Bacillus subtilis* germinate slowly and are sensitive to lysozyme. The spore coat of the mutant appears to lack a single polypeptide (mol. wt 36 000) which normally is deposited in the outer layers of the spore during stage V of sporulation. The mutation maps between *leuA* and *argA* on the *B. subtilis* chromosome and is genotypically and phenotypically distinct from another mutation *ger-36* which maps in the same region: consequently it has been assigned a new locus *spoVIA*. The phenotype of the mutant confirms a previous suggestion that lysozyme resistance is conferred by the outermost layers of the spore coat.

INTRODUCTION

The spore coat in *Bacillus subtilis* contains about 12 polypeptides (Pandey & Aronson, 1979; Jenkinson *et al.*, 1981). These are synthesized at different times during sporulation and are incorporated into the spore structure during stages V and VI (Jenkinson *et al.*, 1981). One component of the *B. subtilis* spore coat, a polypeptide with a molecular weight of approximately 36 000 (36K), begins to be formed particularly late in sporulation (after t_6) and is deposited in the outermost layers of the maturing spore. Its appearance is inhibited by the late addition of chloramphenicol during sporulation, and spores produced in the presence of chloramphenicol are sensitive to lysozyme, though heat-resistant (Jenkinson *et al.*, 1980). This suggested a direct role for the 36K polypeptide in conferring lysozyme resistance upon the spores.

During sporulation not only the resistance properties but also the germination properties of the spores develop in parallel with the laying down of the coat proteins (Dion & Mandelstam, 1980; Jenkinson *et al.*, 1980). If both germination and resistance properties are determined by the proteins of the coat, it should be possible to isolate mutants which are affected both in coat structure and in these properties. In this paper a mutant of *B. subtilis* is described which produces spores that have a specific defect in the outer coat layers. The spores are fully heat-resistant but have a delayed response to germinants and are lysozyme-sensitive. The coat of the mutant (strain 513) lacks the 36K polypeptide; consequently some proteins in the spore outer layers which are normally covered, presumably by the 36K polypeptide, are exposed on the outer surface of the spores. The mutation has been located between *leuA* and *argA* on the *B. subtilis* map, appears to be separate from *ger-36* which maps close by (Moir *et al.*, 1979), and defines a new sporulation locus *spoVIA*.

METHODS

Bacteria. *Bacillus subtilis* 168 *trpC2* which requires indole or tryptophan and sporulates normally was used as the standard strain and is referred to as the wild-type. The other auxotrophic strains used are shown in Table 1; these are all *Spo*⁺.

Table 1. *Strains of Bacillus subtilis* used

Strain designation	Genotype	Source
MB21	<i>leu-8 metC3 tal-1</i>	This laboratory
MB26	<i>leu-8 phe-12 rif-2 tal-1</i>	This laboratory
MB68	<i>phe-12 lys-1 rif-2 tal-1</i>	This laboratory
BD40	<i>phe-12 argA3 ery</i>	D. Dubnau
BD54	<i>leu-8 metB5 ile-1</i>	D. Dubnau
BD163	<i>hisA1 argC4</i>	D. Dubnau
87.2	<i>leu-8 tal-1 spoOJ</i>	Hranueli <i>et al.</i> (1974)
E36	<i>leu-2 ger-36</i>	Moir <i>et al.</i> (1979)

Growth and sporulation. Sporulation was induced by transferring exponentially growing cells at 37 °C to resuspension medium (Sterlini & Mandelstam, 1969). Times after resuspension (h) are denoted t_1 , t_2 , etc.; at t_8 , 80 to 90% of the cells contained refractile spores. Spore incidence was determined by phase-contrast microscopy.

Measurement of resistances to organic solvents, heat and lysozyme. Resistances to toluene and octan-1-ol (0.02 vol.), heat (80 °C, 15 min) and lysozyme (250 µg ml⁻¹, 37 °C, 30 min) were determined as previously described (Jenkinson *et al.*, 1980).

Measurement of resistances to acetone, phenol and trichloroacetic acid. A portion of spore suspension (0.5 ml) containing about 10⁸ spores was added to 4.5 ml of acetone (20 °C), phenol (5%, w/v, 4 °C) or trichloroacetic acid (5%, w/v, 4 °C), left for 15 min with occasional vortex mixing, and then suitable dilutions were plated on nutrient agar (Oxoid).

Assays for sporulation marker events. Extracellular serine protease was measured as described by Dancer & Mandelstam (1975). Alkaline phosphatase was measured by the method of Glenn & Mandelstam (1971). Manganese-dependent deoxyribonuclease was assayed by the method of Akrigg (1978) and glucose dehydrogenase was determined by the method of Sadoff (1966). Dipicolinic acid was determined by the method of Janssen *et al.* (1958).

Isolation of lysozyme-sensitive mutants. The initial procedure used was to select germination mutants as described by Trowsdale & Smith (1975). An exponentially growing culture of the wild-type in 50 ml hydrolysed casein medium (Sterlini & Mandelstam, 1969) was centrifuged and the cells were suspended to a density of about 2×10^9 cells ml⁻¹ in glutamate minimal (resuspension) medium and shaken at 37 °C. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine was added (final concn 100 µg ml⁻¹) and samples (2 ml) were removed after 15, 30 and 45 min. The bacteria in each sample were washed, resuspended to 5 ml, and 1 ml portions were inoculated into 10 ml portions of hydrolysed casein medium. The flasks were shaken overnight at 37 °C, and dilutions were plated on lactate/glutamate minimal agar (Piggot, 1973) to give about 100 colonies per plate. The spores were collected from each plate after 4 d at 37 °C, suspended in Difco Penassay broth (20 ml) to a density of about 4×10^8 spores ml⁻¹ and heated at 80 °C for 10 min. The spores were allowed to germinate at 42 °C for 2 h and then each suspension was heated again at 80 °C for 30 min to kill spores that had germinated normally. The suspensions were left shaking at 37 °C for 16 h, then heated (80 °C, 15 min) to kill vegetative cells, and suitable dilutions were plated on Difco tryptose blood agar base containing Oxoid nutrient broth (0.8%, w/v). After 24 h at 37 °C about 100 colonies which were more translucent than the others, and which appeared at a frequency of about 5%, were picked from the plates and streaked on lactate/glutamate minimal agar. After 4 d at 37 °C to allow sporulation to occur on the plates, a single colony from each isolate was suspended in 2 ml resuspension medium. The suspensions were heated (80 °C, 30 min), cooled and samples were removed for determination of the numbers of heat-resistant spores. To each suspension was then added 0.2 ml of a lysozyme solution (2 mg ml⁻¹), and after incubation for 1 h at 37 °C the number of survivors in each was determined by plating samples on nutrient agar. In this way, five clones were picked on the basis of their forming heat-resistant and lysozyme-sensitive spores. All these strains grew normally at 37 °C in hydrolysed casein medium and in glucose/glutamate minimal medium (Coote, 1972a).

Genetic mapping. Genetic mapping was done by transduction with phage PBS1 (Takahashi, 1963) using lysates, prepared as described by Karamata & Gross (1970) from the lysozyme-sensitive strain, as donor in crosses with a set of recipient strains (see Table 1). For the determination of recombination indices (Coote, 1972b), DNA from the donor strains was prepared by the method of Marmur (1961) from cells grown in Penassay broth and used at a concentration of about 5 µg ml⁻¹ to transform competent cells (Ayad & Barker, 1969). Transductants or transformants were selected on lactate/glutamate minimal plates with appropriate supplements. Plates were incubated for 4 d at 37 °C to allow the development of the brown pigment characteristically associated with sporulation. Colonies that were brown and opaque were recorded as Spo⁺ (sporogenous) and those which were less brown and more transparent were recorded as Spo⁻/Ger⁻ (sporulation/germination defective). However, distinguishing between Spo⁺ and Spo⁻ in this way alone was unreliable because the auxotrophic strains used for

mapping varied sufficiently in their degree of pigment formation and colony opacity to interfere with the scoring of colonies. Therefore, single-colony isolates were obtained from the transductant colonies and scored for the cotransfer of the germination defect by the tetrazolium overlay method (Lafferty & Moir, 1977). Although this method of scoring was more reliable, for some crosses it was also unsatisfactory because the recipient strains themselves varied in their tetrazolium reaction. Accordingly, for each cross where linkage was suspected, at least 20 single-colony isolates of the transductants were further tested by (a) suspending a sporulating colony in resuspension medium and measuring the number of survivors after treatment with heat (80 °C, 15 min) and then lysozyme and (b) growing and resuspending colonies by the Sterlini & Mandelstam (1969) method and determining heat and lysozyme resistances at t_{10} . Lysozyme sensitivity and germination deficiency were 100% cotransducible.

Genetic linkage is recorded as the percentage cotransduction of the mutation with the marker tested. The relatively low numbers of transductants tested were due to these difficulties in scoring (see above).

Preparation of spores. Spores from cultures at t_{20} were harvested by centrifugation (8000 g, 20 min, 4 °C), washed twice with 1 M-KCl, once with deionized water and then pelleted by centrifugation through 40% (w/v) Urografin. After three further washings with deionized water containing 0.01% (v/v) Tween 80, the final preparations contained about 98% phase-bright spores. Spores from cultures at t_9 or t_{10} were prepared by passing the sporangia through a French pressure cell and then washing the spores as previously described (Jenkinson *et al.*, 1981).

Germination characteristics of spores. Cleaned spores (see above) from cultures at t_{20} were heat-activated (75 °C, 15 min) and suspended to a density of about 5×10^7 spores ml⁻¹ in appropriate germination medium at 30 °C. The course of germination was followed by measuring (a) the loss in absorbance of the suspension at 600 nm, (b) the loss in the numbers of spores surviving treatment with toluene and (c) the approximate increase in the proportion of phase-dark to phase-bright spores.

Extraction of spore coat proteins. Cleaned and washed spores were suspended to a density of about 2×10^9 spores ml⁻¹ in 10 ml Tris/HCl buffer (50 mM, pH 7.5) containing EDTA (10 mM) and phenylmethylsulphonyl fluoride (PMSF; 2 mM), mixed with 7 ml glass beads, broken in a Braun cell homogenizer, and the insoluble fraction thus generated was extensively washed (Jenkinson *et al.*, 1981). The spore coat proteins were extracted with DS buffer [cyclohexylaminoethanesulphonic acid (CHES)/NaOH (5 mM, pH 9.8) containing sodium dodecyl sulphate (SDS; 2%, w/v) and dithioerythritol (DTE; 50 mM)] at 70 °C for 30 min. Protein solubilized was measured by the Lowry method. Extracts were centrifuged (2000 g, 4 min) before being subjected to SDS-PAGE (see below).

Radioactive labelling. Cell proteins of the sporangia were labelled and extracted as follows. A portion (0.5 ml) of sporulating culture was incubated with shaking with L-[U-¹⁴C]tyrosine [0.3 µCi (11.1 kBq), 3 µg] for 15 min at 37 °C. The cells were harvested by centrifugation (Beckman Microfuge, 30 s), washed once with NaCl (1 M) and then resuspended at 40 °C for 20 min in 0.02 ml 'lysis buffer' [Tris/HCl (50 mM, pH 8.2) containing EDTA (2 mM), PMSF (0.3 mg ml⁻¹), lysozyme (2 mg ml⁻¹) and DNAase I (Worthington; 100 µg ml⁻¹)]. The suspension was then heated at 80 °C for 15 min with 0.05 ml 'cracking buffer' [CHES/NaOH (5 mM, pH 9.8) containing SDS (2%, w/v), DTE (60 mM), glycerol (15%, v/v) and bromophenol blue (0.005%, w/v)], centrifuged (2000 g, 4 min), and a portion of the supernatant was counted for radioactivity before subjecting the extract to SDS-PAGE.

Measurement of radioactivity. Aqueous samples (0.01–0.5 ml) were treated with ethanol (6 ml) and Omnifluor (New England Nuclear) in toluene (0.5%, w/v; 8 ml) and counted in a liquid scintillation counter (LKB Wallac 81000).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were fractionated by electrophoresis on SDS-acrylamide slab gels (1.5 mm thick) prepared as described by Laemmli & Favre (1973). The separating gel contained 14.5% (w/v) acrylamide and 0.25% (w/v) *N,N'*-methylenebisacrylamide; the corresponding concentrations in the stacking gel were 5% and 0.38%. Samples to be electrophoresed were made, when necessary, to 0.002% (w/v) in bromophenol blue and 10% (v/v) in glycerol and heated to 100 °C for 5 min before loading. Electrophoresis and staining were done as previously described (Jenkinson *et al.*, 1981). Stained gels were scanned with a double-beam recording microdensitometer (Joyce, Loebel & Co., Gateshead, U.K.). Molecular weights of proteins were estimated from their distances of migration by reference to a graph relating migration distances of six marker proteins to log molecular weight. The markers used were phosphorylase b (mol. wt 94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100) and α-lactalbumin (14 400) (Pharmacia). Protein bands labelled with ¹⁴C were visualized by fluorography (Bonner & Laskey, 1974; Laskey & Mills, 1975).

Surface protein iodination. Cleaned spores (3–5 mg dry wt) were surface-labelled with ¹²⁵I [0.15 mCi (5.55 MBq)] using lactoperoxidase (EC 1.11.1.7; Sigma) as previously described (Jenkinson *et al.*, 1981). The spore coat proteins were fractionated by SDS-PAGE and the ¹²⁵I associated with each band was determined by counting 1 mm slices of the excised lane in a LKB Wallac 1270 Rackgamma counter.

Chemicals. Acrylamide and *N,N'*-methylenebisacrylamide were obtained from BDH, L-[U-¹⁴C]tyrosine was purchased from Amersham, and iodine-125 was from New England Nuclear. All other chemicals were from Sigma unless otherwise stated.

RESULTS

Sporulation and resistance properties of strain 513. After mutagenesis and isolation of colonies in which the spores were sensitive to lysozyme (see Methods), five mutants were selected for further study. Spores formed by one of these (strain 513) appeared to be the most sensitive to lysozyme. This strain sporulated normally, producing amounts of extracellular serine protease, alkaline phosphatase, endonuclease, glucose dehydrogenase and dipicolinic acid similar to those of the wild-type strain, and the spores were released from the sporangia after 12 to 15 h incubation at 37 °C.

A spore suspension of the mutant harvested and cleaned at t_{10} was tested for resistance to toluene, octan-1-ol, acetone, trichloroacetic acid, phenol, heat (80 °C, 15 min) and lysozyme. The spores resisted all these treatments except the last which gave about a fivefold reduction in viable count in 30 min. In Tris/HCl buffer (50 mM, pH 7.4) containing lysozyme (250 µg ml⁻¹), 90% or more of the spores of strain 513 lost viability in 2 h (Fig. 1); during this time there was no loss in viability of wild-type spores.

Germination properties. Since this strain was isolated by a procedure that was designed to select slow germinators (see Methods), it was expected that the spores would show some germination defect. The germination of strain 513 in L-alanine (10 mM) was severely delayed and, as measured by toluene resistance, only about 35% of the spores had germinated after

Fig. 1

Fig. 2

Fig. 1. Effect of prolonged lysozyme treatment on viability of spores of strain 168 (○) and strain 513 (●). Cleaned spores at t_{10} were suspended to a density of 5×10^7 colony-forming units (c.f.u.) ml⁻¹ in Tris/HCl buffer (50 mM, pH 7.4) containing lysozyme (250 µg ml⁻¹) at 37 °C and samples were removed at intervals for the determination of viable count.

Fig. 2. Germination of spores of strain 168 and strain 513. Cleaned spores from cultures at t_{20} were heat-activated (75 °C, 15 min) and then suspended to a density of 5×10^7 c.f.u. ml⁻¹ in Tris/HCl buffer (10 mM, pH 8.2) containing L-alanine (10 mM) at 30 °C. Samples were withdrawn at intervals and germination was determined by toluene resistance and absorbance (see Methods). Toluene resistance: 168 (○); 513 (□). Absorbance: 168 (●); 513 (■).

Fig. 3. SDS-PAGE patterns (stained with Coomassie blue) of proteins in spore coat fractions from t_6 spores of strain 168 (lane 1) and strain 513 (lane 2). The spore coat fractions were prepared and the proteins were solubilized as described in Methods. The coat protein bands are referred to by their apparent molecular weights. The same amount of protein (40 μ g) was applied to each lane. The arrow marks the region of the missing 36K polypeptide.

3 h, compared with 95 % in the wild-type (Fig. 2). Loss of toluene resistance was reflected by loss of absorbance in both strain 513 and strain 168 (Fig. 2) and in both strains the spores which germinated became phase-dark.

Germination of strain 513 spores was also delayed in Penassay broth (results not shown). Neither spores from strain 513 nor those from strain 168 germinated in a mixture of KCl/glucose/fructose (Dion & Mandelstam, 1980).

Spore coat proteins. The altered lysozyme sensitivity and germination properties might have been due to an alteration in spore coat structure. Therefore, spores of the mutant strain were purified (see Methods) and broken, and the proteins of the spore coat fraction were solubilized with DS buffer and fractionated by SDS-PAGE. Coomassie blue staining showed that the spore coat of the mutant lacks a single polypeptide (Fig. 3) with a molecular weight of about 36 000 (36 K). The distribution and relative intensities of the other polypeptides in the coat extract from strain 513 were the same as in the wild-type.

Radioactive labelling of sporangia. Two explanations for the missing 36K polypeptide were considered: (a) the protein was not synthesized, or (b) it was made but not incorporated into the coat. In an attempt to distinguish between these two possibilities, sporulating cells were pulse-labelled at intervals during sporulation with [14 C]tyrosine (see Methods). The sporangia were then lysed and extracted, and the proteins were separated by SDS-PAGE. If the 36K polypeptide, which is known to be made late in sporulation (Jenkinson *et al.*, 1981), was formed but not incorporated into the spore structure, then it should be detectable in extracts from whole cells labelled after t_6 , but not in those labelled earlier. The pattern of

Fig. 4. SDS-PAGE patterns (revealed by fluorography) of proteins extracted from sporulating cells at various times during sporulation after labelling with [^{14}C]tyrosine for 15 min (see Methods). Lanes 1-4: sporulating cells of strain 513 labelled at t_4 , t_5 , t_6 and t_7 , respectively. Lanes 5-8: sporulating cells of strain 168 labelled at t_4 , t_5 , t_6 and t_7 , respectively. The spore coat proteins are indicated (see Fig. 3).

labelled proteins in strain 513 at t_4 , t_5 and t_6 was similar to that of strain 168 at the same times (Fig. 4). Several of the coat polypeptides which contain a high amount of tyrosine and which began to appear at t_4 could be clearly identified in the whole cell extracts (Fig. 4). At t_7 , however, the 36K polypeptide was apparent in extracts from the cells of strain 168 but not in those from strain 513 (Fig. 4). Thus the mutant appears not to make this polypeptide.

In vitro labelling of surface proteins with ^{125}I . The 36K, 12K, 9K and 8K polypeptides have been identified by surface labelling with ^{125}I as being the major surface proteins in mature spores (Jenkinson *et al.*, 1981). The same technique was used to determine which proteins were exposed on the surface of spores of strain 513. The pattern in Fig. 5 shows labelling of the following polypeptides: 65K, 33K, 30K, 24K, 20K, 19K, 15K, 12K, 9K and 8K. This is very different to the pattern of labelling in the wild-type where the 30K and 15K polypeptides are completely covered by the overlying proteins (see Fig. 5 inset), and three others (24K, 20K and 19K) are largely covered.

Fig. 5. Proteins exposed on the surface of t_0 spores of strain 513. The spores were surface-labelled with ^{125}I using lactoperoxidase, washed and broken with glass beads, and the proteins of the spore coat fraction 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}I radioactivity. The inset shows the corresponding scan for spores of strain 168 surface-labelled at t_0 (see Jenkinson *et al.*, 1981).

Construction of an isogenic strain. It was important to try to rule out the possibility that strain 513 contained multiple genetic defects. This was done by transferring the mutation to strain MB21 *leu-8 metC3* by transformation with a saturating amount ($5 \mu\text{g ml}^{-1}$) of purified DNA from strain 513. Seven out of 100 Met^+ transformants tested were Ger^- by the tetrazolium overlay method, and showed the heat-resistant, lysozyme-sensitive phenotype. This is approximately the number that would be expected from transformation by congression. Three of these transformants were further characterized: each carried the germination defect of the original mutant (see Fig. 2) and the coat polypeptide profile of each was identical to that shown for strain 513 in Fig. 3. Thus, the phenotype of strain 513 is unlikely to have been caused by more than one distinct mutational event. Reversion tests may help to establish that the altered spore properties are the result of a point mutation.

Genetic mapping. The mutation was not cotransduced with *argC*, *metC*, *ilvA*, *metB*, *lys* or *hisA*. It was 45% (34/75) cotransducible with *argA* (map position 260), 67% (56/84) with *leuA* (map position 250) and 23% (23/100) with *pheA* (map position 245). The gene order suggested from three-point transduction crosses was *pheA-leuA-spo-argA* (Table 2).

The *ger-36* mutation also maps in this region (Moir *et al.*, 1979) but is highly cotransducible with *leuA* (92%). The phenotype of strain E36 which carries this mutation is similar to that of strain 513 in that it forms spores that are germination-defective and lysozyme-sensitive. However, the two strains differ in two respects: (a) electron micrographs of thin sections of strain E36 spores show the coat to be incomplete (Moir *et al.*, 1979) but in strain 513 there is no obvious abnormality (results not shown); (b) the SDS-PAGE profile of the spore coat proteins of strain E36 is different from that of strains 513 and 168 (results not shown). These differences indicate that the mutation in strain E36 was not the same as that in strain 513 and that the mutations were unlikely to be in the same gene. To check this, the

Table 2. *Three-factor transduction crosses with strains MB26 and BD40 as recipients*The donor was strain 513 (*trpC2 spo-513*).

Recipient	Selection	Recombinant class	No. in class
MB26 (<i>phe-12 leu-8 spo+</i>)	Phe ⁺	Leu ⁺ Spo ⁺	39
		Leu ⁺ Spo ⁻	20
		Leu ⁻ Spo ⁺	38
		Leu ⁻ Spo ⁻	3*
BD40 (<i>phe-12 argA3 spo+</i>)	Arg ⁺	Phe ⁺ Spo ⁺	5†
		Phe ⁺ Spo ⁻	18
		Phe ⁻ Spo ⁺	52
		Phe ⁻ Spo ⁻	25

* Rare class (four crossovers) suggesting gene order is *phe-leu-spo*.† Rare class suggesting gene order is *phe-spo-arg*.Table 3. *Determination of recombination index (R.I.) between spo-513 and ger-36*

A transformation cross was carried out with strain 513 (Trp⁻) as recipient and with a saturating amount of DNA (about 5 µg ml⁻¹) from strain E36 (Trp⁺). Trp⁺ transformants were selected and scored for wild-type (recombinant) resistance and germination properties (see Methods). The values were compared with those obtained using a mutant (strain 87.2) with a genetically distant mutation.

	Donor DNA (<i>trp</i> ⁺)		R.I.
	E36 (<i>ger-36</i>)	87.2 (<i>spoOJ</i>)	
Expt 1 (Spo ⁺ /Trp ⁺)	12/432 (0.027)	8/306 (0.026)	1.03
Expt 2 (Spo ⁺ /Trp ⁺)	11/247 (0.044)	10/248 (0.040)	1.10

recombination index (see Lacks & Hotchkiss, 1960) between the two mutations was determined as described by Coote (1972*b*). Cells of strain 513 *trpC2* were transformed to prototrophy with DNA prepared from strain E36 *leuA ger-36*, and with DNA from strain 87.2 *leuA spoOJ*. The ratio of Spo⁺ to Trp⁺ transformants was scored for both crosses (Table 3): *ger-36* and *leuA* are only about 15% cotransformed (A. Moir, personal communication) so this does not significantly affect the congression of *spo*⁺. The recombination index between *ger-36* and *spo-513* was calculated to be about 1.0, showing that the mutations are not linked and, therefore, that they lie in separate loci.

DISCUSSION

A number of germination mutants of *B. cereus* have been isolated and characterized (Aronson & Fitz-James, 1975; Stelma *et al.*, 1978, 1980). Some of these form lysozyme-sensitive spores and the defects seem to be related to an alteration in the spore coat. A large number of *B. subtilis* germination mutants have also been obtained (Trowsdale & Smith, 1975; Moir *et al.*, 1979), but in only one of these has a defect in the spore coat been demonstrated (Moir *et al.*, 1979).

The mutant described in this paper lacks a single spore coat protein which normally forms part of the outermost layer of the spore (Jenkinson *et al.*, 1981). The spores are germination-defective and lysozyme-sensitive and these phenotypes seem to fit in with the idea that the coat structure may determine both germination and resistance properties.

The properties of this mutant support a previous suggestion that lysozyme resistance is conferred primarily by the outer coat layers and requires the synthesis and deposition of the 36K polypeptide (Jenkinson *et al.*, 1981). Lysozyme-sensitive spores are also formed when

Fig. 6. Schematic diagram of the surface layers of spores of strain 168 (a) and strain 513 (b) at t_g . The diagram is based on the data in Fig. 5 and is subject to the limitations described previously (Jenkinson *et al.*, 1981); it is not meant to indicate that the proteins are in contiguous blocks or arranged in any lateral order. Each protein is represented as a unit, the area of which approximates to the amount of that protein in the coat extract. Surface exposure is indicated by the serrated edge.

Fig. 7. Map of the *pheA-leuA-argA* region of the *B. subtilis* chromosome showing the location of sporulation and germination mutations. Percentages are cotransduction frequencies; figures in parentheses are approximate corrected genetic distances between markers ($100 \times t$, where t is the fractional length of transducing fragment; see Henner & Hoch, 1980). The location of *spoIVF* is from Hranueli *et al.* (1974) and *spoIIB* and *spoIVF* are apparently inseparable by phage PBS1 transduction (I. Lamont, unpublished results).

the other major component of the outer layer, a 12K polypeptide, fails to be deposited as a result of including PMSF in the sporulation medium (Jenkinson *et al.*, 1981). Since the 12K polypeptide is laid down apparently normally in the absence of the 36K polypeptide in this mutant, it appears that these proteins can be deposited independently. This conclusion is supported by the earlier finding that the 12K protein can be laid down in the presence of chloramphenicol, and in the absence of synthesis of the 36K polypeptide (Jenkinson *et al.*, 1981). The results in this paper and those previously described also show that the 36K polypeptide is not necessary for any of the resistance properties of the spores, except lysozyme resistance.

A schematic diagram of the spore outer layers of this mutant at t_g is shown in Fig. 6, with a similar representation for the wild-type. This latter diagram has been adapted from that originally devised for wild-type spores (Jenkinson *et al.*, 1981) in which it was not possible to

state that any one polypeptide specifically covered any other. The results in this paper, however, suggest that the 30K, 24K, 20K, 19K and 15K polypeptides are covered specifically by the 36K polypeptide in the wild-type, since all are more exposed in the mutant. It is expected that the diagrammatic representation may have to be further modified in the light of more data.

The mutation in strain 513 seems separate from other sporulation and germination loci in the *pheA-argA* region (Fig. 7) and causes a specific defect late in sporulation. Hence, in keeping with the classification of Piggot & Coote (1976), *spo-513* can be provisionally assigned to a new locus *spoVIA*. Although the exact nature of the mutation in strain 513 is not clear, e.g. whether it is in the structural gene for the 36K polypeptide or in a preceding operon, further study of late-blocked mutants should enable the genetic loci controlling the synthesis of this and other coat polypeptides to be more clearly defined.

I am grateful to Professor J. Mandelstam for his continuous guidance and to Angela Maunder for technical assistance. I thank Dr Susan Hill for helpful discussions and R. Warburg for information on unpublished results. This work was supported by the Science Research Council.

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