Altered Arrangement of Proteins in the Spore Coat of a Germination Mutant of *Bacillus subtilis*

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Spores produced by a mutant of *Bacillus subtilis* were slow to develop their resistance properties during sporulation, and were slower to germinate than were wild-type spores. The coat protein composition of the mutant spores, as analysed by SDS-PAGE, was similar to that of the wild-type spores. However, one of the proteins (mol. wt 12000) which is normally present in the outermost layers of mature wild-type spores and which is surface-exposed, was assembled abnormally into the coat of the mutant spores and not surface-exposed. The mutation responsible for this phenotype (*spo-520*) has been mapped between *pheA* and *leuB* on the *B. subtilis* chromosome, and was 47% cotransformable with *leuB16*. This mutation, and three others closely linked to it, define a new sporulation locus, *spoVIB*, which is involved in spore coat assembly. The phenotype of the mutant(s) supports the contention that spore germination and resistance properties may be determined by the assembly of the coat.

INTRODUCTION

The spore coat proteins in *Bacillus subtilis* are a class of sporulation-specific gene products that are assembled on the developing spore during stages V to VI of sporulation (Jenkinson *et al.*, 1981). The formation of the coat occurs in parallel with the development by the spores of their germination properties (Dion & Mandelstam, 1980) and their resistance properties (Jenkinson *et al.*, 1980) and there is evidence that the coat plays a role in determining these properties (Aronson & Fitz-James, 1976; Stelma *et al.*, 1978, 1980; Moir, 1981; Jenkinson, 1981).

Recently, two mutations in separate genetic loci have been shown to affect coat formation in *B. subtilis* (Moir, 1981; Jenkinson, 1981). The expression of one of these loci (*spoVIA*) is known to be required for the synthesis at stage V of one of the major outer-coat proteins (Jenkinson, 1981); spores lacking this protein are sensitive to lysozyme (Jenkinson *et al.*, 1981; Jenkinson, 1981).

This paper describes the isolation and phenotypic properties of four new sporulation mutants. The mutants are slower to sporulate than normal, and form spores that are slow to germinate and in which the coat proteins are misarranged. The mutations map close to *leuB16* (41–50% cotransformation) and together they define a new sporulation locus, *spoVIB*, involved in coat assembly.

METHODS

Bacteria. *Bacillus subtilis* 168 *trpC2* which sporulates normally was used and is referred to as the wild-type strain. Stocks were kept at 4 °C as dilute spore suspensions in distilled water. Other strains used are listed in Table 1.

Induction of sporulation. Cells were grown in hydrolysed casein medium to an A_{600} of 0.80 (~0.25 mg dry wt ml⁻¹) and induced to sporulate by resuspension at the same density in glutamate/inorganic salts medium containing tryptophan (20 µg ml⁻¹) or other appropriate amino acid supplement (200 µg ml⁻¹) (Sterlini &

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Abbreviation: PGYE agar, potato extract/glucose/yeast extract agar.

Table 1. *Strains of B. subtilis used*

Laboratory stock strains			
Strain designation	Genotype	Source	Reference
168	<i>trpC2</i>	This laboratory	
MB8	<i>hisA1</i>	This laboratory	
MB21	<i>metC3 leuA8 tal-1</i>	This laboratory	
MB26	<i>pheA12 leuA8 rfm tal-1</i>	This laboratory	
MB68	<i>pheA12 lys-1 rfm tal-1</i>	This laboratory	
MB79	<i>pheA12 metC3 tal-1</i>	This laboratory	
MY2016	<i>hisH2 lys-1 rfm Sul^R</i>	This laboratory	Yudkin & Turley (1980)
BD112	<i>cysA14</i>	D. Dubnau	
CU267	<i>trpC2 ilvB2 leuB16</i>	S. Zahler	
JH326	<i>leu-2 citF78</i>	J. Hoch	Ohné <i>et al.</i> (1973)
4823	<i>leu-2 citF2</i>	L. Rutberg	Ohné <i>et al.</i> (1973)
4673	<i>gerE36</i>	A. Moir	Moir (1981)
513	<i>trpC2 spoVIA513</i>	This laboratory	Jenkinson (1981)
513.1	<i>leuA8 spoVIA513</i>	This laboratory	Jenkinson (1981)
Constructed strains			
Strain designation	Genotype	Construction	
520	<i>trpC2 spo-520</i>	NTG mutagenesis of strain 168 (Jenkinson, 1981)	
520.1	<i>metC3 spo-520</i>	520 (DNA) → MB79; Phe ⁺ recombinants scored for congression of <i>spo-520</i>	
520.3	<i>trpC2 spo-520</i>	520 (DNA) → CU267; Ilv ⁺ Leu ⁺ recombinants scored for cotransformation of <i>spo-520</i>	
524	<i>spo-513 spo-520</i>	520 (DNA) → 513.1; Leu ⁺ recombinants scored for cotransformation of <i>spo-520</i>	
541	<i>trpC2 spo-541</i>	Localized NTG-mutagenesis (this paper)	
547	<i>trpC2 spo-547</i>		
552	<i>trpC2 spo-552</i>		
541.1	<i>leuB16 trpC2 spo-541</i>		
547.1	<i>leuB16 trpC2 spo-547</i>	541 (DNA) → CU267; Ilv ⁺ recombinants scored for cotransformation of <i>spo-541</i>	
552.1	<i>leuB16 trpC2 spo-552</i>	547 (DNA) → CU267; Ilv ⁺ recombinants scored for cotransformation of <i>spo-547</i>	
		552 (DNA) → CU267; Ilv ⁺ recombinants scored for cotransformation of <i>spo-552</i>	

Mandelstam, 1969). After incubation with shaking at 37 °C for 8 h, 80–90% of the cells contained phase-bright, fully refractile spores as visualized by phase-contrast microscopy. The terms 'phase-grey' and 'phase-dark' refer respectively to spores that are not fully refractile, and to spores that have no refractility, e.g. stage III pre-spores. Times of resuspension (h) are denoted by t_1 , t_2 , etc.

Measurement of sporulation marker events. Extracellular serine protease was measured by the method of Dancer & Mandelstam (1975); alkaline phosphatase was determined as described by Glenn & Mandelstam (1971), and extracellular sporulation endonuclease estimated by the method of Akrigg (1978). Glucose dehydrogenase was measured by the method of Sadoff (1966), and 2,6-dipicolinic acid was determined as described by Janssen *et al.* (1958).

Measurement of resistance of spores to toluene, heat and lysozyme. Resistance to toluene and heat was determined as described by Jenkinson *et al.* (1980). The numbers of spores surviving incubation with lysozyme (EC 3.2.1.17; 250 µg ml⁻¹, 37 °C, 30 min) were determined by plating suitable dilutions on nutrient agar (Oxoid).

Preparation of spores. Spores and sporangia were harvested from cultures by centrifugation (8000 g, 10 min, 4 °C), washed with KCl (1 M) and then with NaCl (0.5 M). The pellets were suspended to a density of about 20 mg dry wt ml⁻¹ in TEP buffer (50 mM-Tris/HCl, pH 7.2 containing 5 mM-EDTA and 2 mM-phenylmethylsulphonyl fluoride) and passed twice through an ice-cold French pressure cell at 83 MPa. This released > 90% of the spores from sporangia. The French-press treatment was omitted for the preparation of spores from cultures at t_{20} because these were kept at 4 °C for 48 h to allow complete cell lysis to occur, before the spores were harvested and washed as described above. The suspension was then diluted to 30 ml in TEP buffer containing 0.001% Tween 80, centrifuged (2000 g, 5 min, 4 °C), and the spores were washed twice more with 30 ml TEP. In some cases the spore

coat proteins were extracted as described below without further cleaning of the spores. For germination experiments the spores were further purified by centrifugation through 50% (w/v) Urografin (Jenkinson, 1981).

Spore coat proteins. For extraction of the coat proteins, spores were suspended at a density of about 10 mg dry wt ml⁻¹ in TEP buffer (10 ml) and broken with glass beads in a Braun Model MSK cell homogenizer (4 min, full speed). The insoluble fraction after breakage was treated with lysozyme (50 µg ml⁻¹, 40 °C, 30 min) and then extensively washed as previously described (Jenkinson *et al.*, 1981). The residue was termed the 'spore coat fraction' and the coat proteins were solubilized from this with DS buffer [0.5 ml cyclohexylaminoethanesulphonic acid/NaOH buffer (5 mM, pH 9.8) containing SDS (1.5%, w/v) and dithioerythritol (50 mM)], and prepared for electrophoresis as described by Jenkinson *et al.* (1981).

Solubilization of coat proteins with DTT/urea followed by NaOH. Cleaned spores from *t*₂₀ cultures were suspended at a density of 1 mg ml⁻¹ in 50 mM-DTT/8 M-urea pH 3 (0.5 ml) and incubated for 1 h at 37 °C. The suspension was centrifuged, the spores were washed twice with water (0.5 ml) and then treated with 0.1 M-NaOH (0.5 ml) at 4 °C for 15 min (Gould *et al.*, 1970). The spores were then pelleted by centrifugation, washed once with water and resuspended to an *A*₅₅₀ of 0.50 in NaH₂PO₄/NaOH buffer (100 mM, pH 7). All solutions contained Tween 80 (0.0002%) to minimize clumping of spores.

Germination properties. Spores from cultures at *t*₂₀ were harvested and washed as described above. They were then suspended at a density of 5 × 10⁷ spores ml⁻¹ at 25 °C in the germinant being tested, and germination was followed either by measuring loss of absorbance at 550 nm or by removing samples (0.1 ml) at intervals and measuring the numbers of spores surviving toluene treatment.

SDS-PAGE. Proteins were fractionated by electrophoresis in 15% (w/v) acrylamide slab gels as described by Jenkinson *et al.* (1981) using the system of Laemmli & Favre (1973).

Surface-protein iodination. Cleaned spores (5–10 mg dry wt) were surface-labelled with ¹²⁵I (New England Nuclear) using lactoperoxidase (EC 1.11.1.7; Sigma) (Jenkinson *et al.*, 1981). The spore coat proteins were then fractionated by SDS-PAGE and the ¹²⁵I associated with each band was determined by counting 1 mm slices in an LKB Wallac 1270 Rackgamma counter.

Mapping by PBS1 transduction. Transducing lysates were prepared from the donor strain as described by Karamata & Gross (1970). For transduction, the recipient strain was grown at 37 °C in Brain Heart Infusion Broth (Oxoid) containing 0.5% (w/v) Yeast Extract (Oxoid) until the cells were motile, and then 1 ml was incubated with 0.1 ml phage lysate with gentle shaking at 37 °C for 30 min. The cells were harvested by centrifugation, washed twice in minimal salts medium (10 ml) (Anagnostopoulos & Spizizen, 1961) containing 0.5% (w/v) glucose, suspended in 1 ml fresh salts medium and 0.1 ml portions were plated on lactate/glutamate minimal agar (Piggot, 1973) with appropriate supplements. After incubation of the plates for 48 h at 37 °C, colonies were picked for scoring as described below.

Preparation of transforming DNA. DNA for transformation was prepared by one of two methods. For the construction of strains, DNA was prepared by the method of Ward & Zahler (1973). For mapping of linked genes, DNA was prepared by the method of Marmur (1961) and its concentration was estimated from the *A*₂₆₀ of a solution in saline-sodium citrate (0.15 M-NaCl in 0.015 M-sodium citrate, pH 7.0).

Preparation of competent cells. Cells were made competent as follows. Pre-transformation medium (5 ml) was inoculated with a single colony isolate of the recipient strain previously grown for 18 h at 37 °C on nutrient agar [or on Tryptone Blood Agar base (Difco) containing 0.5% glucose for strains harbouring the *citF* mutation]. The composition of pre-transformation medium was essentially the same as that described by Anagnostopoulos & Spizizen (1961) except that the casein hydrolysate (Oxoid) concentration was increased to 0.2% (w/v), and MnSO₄·4H₂O (0.5 µM) and CaCl₂ (50 µM) were included (M. Young, personal communication). The cells were grown in pre-transformation medium at 37 °C, with shaking, to stationary phase (*A*₆₀₀ ~ 3.0) and then diluted 10-fold into warm transformation medium (Young & Spizizen, 1961).

Transformation. Transforming DNA was added to competent cells (1 ml) at a concentration of either 0.05 µg ml⁻¹ (for mapping) or at a saturating concentration (~1 µg ml⁻¹) for congression of unlinked genes. The cultures were shaken for 90 min at 37 °C, diluted 10- and 100-fold in transformation medium, and 0.1 ml samples were plated on to lactate/glutamate minimal agar with appropriate supplements.

Preparation of heavily mutagenized DNA for localized mutagenesis. An overnight culture of strain 168 *trpC2* in Penassay broth (Antibiotic medium no. 3, Difco) was diluted in fresh, warm Penassay broth to a cell density of about 1 × 10⁸ c.f.u. ml⁻¹ and grown with shaking at 37 °C to a density of about 4 × 10⁸ c.f.u. ml⁻¹. The cells were harvested by centrifugation, suspended in 10 ml resuspension medium (see above) and treated with NTG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, final concentration 100 µg ml⁻¹) for 30 min at 37 °C with gentle shaking. The cells were then harvested by centrifugation, washed twice with fresh Penassay broth (40 ml each time) and suspended in 200 ml warm, aerated Penassay broth and incubated with shaking for a further 1 h at 37 °C. The cells were then pelleted by centrifugation and the DNA was extracted by the method of Marmur (1961).

Scoring of recombinants. Single colony isolates of transductants and transformants were picked on to lactate/glutamate minimal agar containing appropriate supplements and on to potato extract/glucose/yeast extract

(PGYE) agar and scored for germination deficiency by the tetrazolium overlayer method (Lafferty & Moir, 1977). In some cases, colonies of recombinants carrying the sporulation mutation could be easily distinguished on PGYE agar by their translucence after chloroform-treatment and the tetrazolium overlay was not necessary. However, they could only occasionally be distinguished from colonies of the recipient strains on the basis of pigment production or opacity on lactate/glutamate minimal agar.

Chemicals. Acrylamide, *N,N'*-methylenebisacrylamide and SDS were from BDH. All other chemicals, unless otherwise stated, were from Sigma.

RESULTS

Times of appearance of late spore properties in strain 520

Initially strain 520 was isolated after mutagenesis of strain 168 with NTG and a procedure for selecting mutant spores that germinate slowly and are also lysozyme-sensitive (Jenkinson, 1981). Colonies formed by this strain on PGYE agar (see Methods) were more translucent than those of the wild-type strain and, unlike colonies of the wild-type, they did not turn red when overlaid with tetrazolium-containing agar (see Methods) and incubated for 6–8 h at 37 °C. Furthermore, after sporulation in resuspension medium, about 20% of the spores at t_{20} were sensitive to lysozyme. The remainder were as resistant to lysozyme as were wild-type spores and survived more than 3 h incubation with lysozyme (250 $\mu\text{g ml}^{-1}$, 37 °C). Since lysozyme resistance is the last of the resistance properties to develop (Jenkinson *et al.*, 1980) this suggested that a proportion of the cells of the mutant might have failed to complete stage VI of sporulation.

To test this, the cells were induced to sporulate and, at various times after resuspension, the numbers of spores surviving treatment with toluene, with heat and with lysozyme were measured. In cultures of the wild-type, toluene resistance began to develop at about t_4 , and spores resistant to heating (80 °C, 15 min) and resistant to lysozyme (250 $\mu\text{g ml}^{-1}$, 37 °C, 30 min) appeared at about $t_{5.5}$ and t_6 , respectively (Fig. 1). The appearance of each of these resistance properties in strain 520 was delayed by about 40 to 60 min (Fig. 1). The spores, nevertheless, achieved full resistance to toluene and heat, but lysozyme resistance was particularly slow to develop, and even at t_{10} , when a plateau was reached, 30% of the spores remained sensitive to lysozyme (Fig. 1).

Amounts of extracellular serine protease, alkaline phosphatase, extracellular sporulation endonuclease, glucose dehydrogenase and dipicolinic acid produced by strain 520 were similar to those produced by strain 168, and these marker events appeared at the same times during sporulation of the mutant as they did in the wild-type (results not shown).

Germination properties

To test the germination characteristics of the spores produced by strain 520, cleaned t_{20} spores were suspended in either L-alanine (10 mM), Penassay broth, or glucose/fructose/asparagine/KCl (10 mM each) and the numbers of spores remaining ungerminated (tested by their ability to survive treatment with toluene) were measured at intervals. Over 80% of the spores of the wild-type germinated in L-alanine in 90 min (Fig. 2), whereas only about 30% of the mutant spores had germinated, even after 5 h (Fig. 2). A comparable delay in germination of mutant spores was observed in Penassay broth and in glucose/fructose/asparagine/KCl (results not shown). Similar germination deficiencies were observed in spores of strain 520.3, a derivative of strain CU267 into which the *spo-520* mutation had been transferred by DNA-mediated transformation (see Table 1).

Spore coat proteins

To see if the germination defects of the mutant were associated with an altered spore coat (see Introduction), spores were harvested from cultures of the wild-type and mutant strains at t_{10} and their coat protein profiles were compared after SDS-PAGE. The gel patterns were similar, although the relative amount of one of the major components (the 36K polypeptide) was reduced in the mutant (Fig. 3).

Fig. 1

Fig. 2

Fig. 1. Development of resistance to toluene (○, ●), heating (□, ■) and lysozyme (△, ▲) during sporulation of the wild-type (open symbols) and strain 520 (filled symbols).

Fig. 2. Germination of spores of the wild-type (○) and strain 520 (●) in 10 mM-L-alanine. Cleaned spores were suspended to a density of 5×10^7 spores ml⁻¹ in Tris/HCl buffer (10 mM, pH 8.2) containing L-alanine (10 mM) and germination was followed by measurement of the numbers of spores surviving treatment with toluene (see Methods).

Fig. 3. SDS-PAGE profiles of proteins stained with Coomassie blue from the spore coat fractions of spores of strain 168 (lane A) and strain 520 (lane B). Polypeptide bands are labelled with their approximate molecular weights (K = kDal).

Fig. 4. Surface iodination of proteins on spores of (a) strain 520 at $t_{6.8}$, (b) strain 520 at $t_{1.0}$, and (c) wild-type at $t_{1.0}$. Spores were isolated from sporangia and surface-labelled with ^{125}I as described in Methods. The spore coat proteins were fractionated by SDS-PAGE and the gels stained with Coomassie blue. They were 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.

Surface-protein iodination

Four of the coat proteins (36K, 12K, 9K and 8K) have been shown to be prominent components on the surface of mature (t_9) spores of the wild-type (Jenkinson *et al.*, 1981). Several of the other proteins in the outer spore layers are not surface-exposed in mature spores and are presumed to be partially or completely overlaid. Although the coat proteins of the mutant spores were qualitatively the same as those of the wild-type, it was possible that their arrangement was different. Accordingly, spores of strain 520, allowed to develop to maturity ($t_{1.0}$) were surface-labelled with ^{125}I using lactoperoxidase (see Methods). The coat proteins were extracted, fractionated by SDS-PAGE, and the radioactivity associated with each band was determined.

The pattern of surface-labelling in the proteins of the coat of the mutant spores was different from the pattern in the wild-type. Thus, in the latter, most of the radioactivity was found in four

Fig. 5. Surface iodination of proteins of mature spores (t_{10}) from (a) double mutant strain 524, and (b) strain 513. Spores were isolated from sporangia, labelled, and the coat proteins separated by SDS-PAGE and scanned as described in the legend to Fig. 4. —, Densitometer scan (absorbance); ----, ^{125}I radioactivity. The asterisk marks the position of the missing 36K polypeptide.

proteins (36K, 12K, 9K and 8K), with some labelling in the 20K, 19K and 15K bands (Fig. 4c). In the mutant, the 36K, 9K and 8K bands were also surface-labelled but the 12K band was virtually unlabelled (Fig. 4b); in addition the 30K and 24K polypeptides not normally exposed on wild-type spores were labelled (Fig. 4b). The same abnormal pattern of labelling was observed when spores of the isogenic strain 520.3 were surface-iodinated at t_{10} .

Further experiments done by isolating spores of strain 520 at earlier times ($t_{5.5}$ and $t_{6.8}$) and surface-labelling them, showed that the 12K polypeptide begins to be deposited in the coat of the mutant spores at the same time (after t_6) as it does in the wild-type. At $t_{6.8}$ the 12K protein was surface-labelled (Fig. 4a) and the labelling pattern in the proteins was virtually identical to that in the wild-type at $t_{6.5}$ (Jenkinson *et al.*, 1981).

Construction of a double mutant

The results above appear to show that between $t_{6.8}$ and t_{10} in the mutant, the 12K protein was assembled differently into the spore outer layers. Since the 36K polypeptide of the coat in the wild-type is made late in sporulation and is deposited together with the 12K protein (Jenkinson *et al.*, 1981), it was possible that in the mutant strain 520 the 36K protein was deposited abnormally so that it covered the 12K polypeptide. To test this possibility, the mutation *spo-513* which causes loss of the 36K polypeptide (Jenkinson, 1981) was introduced into strain 520 by transformation (see Table 1).

To show genetically that this double mutant, strain 524, contained both mutations, DNA was prepared from it and used at saturating concentration to transform strain MB79 *metC3 pheA12* to *Met*⁺. Under these conditions approximately 5% congression of unlinked genes should occur, and in fact out of 660 transformants 31 had the *spo-513* phenotype and 36 had the *spo-520* phenotype, confirming that strain 524 carried both mutations.

The spores of the double mutant (which lacked the 36K polypeptide, Fig. 5a) were then surface-iodinated as described above, and the distribution of ^{125}I in the coat proteins compared

Table 2. *Comparison of germination rate and lysozyme sensitivity of spores from strains 513, 520, 524 and 168 (wild-type)*

Spores were germinated in 10 mM-L-alanine, or treated with lysozyme (250 µg ml⁻¹, 37 °C, 30 min) as described in Methods.

Strain	Germination (percentage loss in A_{550} after 3 h)	Lysozyme sensitivity (percentage loss in viability)*
513	30†	80†
520	25	28
524	8	91
168	55	3

* The initial viable count was 5×10^7 spores ml⁻¹.

† Data from Jenkinson (1981).

with that in the coat proteins of each of the parent strains (Figs 4*b* and 5*b*). In the double mutant the 30K, 24K, 19K and 15K proteins were more exposed than in either parent, these being the polypeptides which have been shown previously to be covered by the 36K protein (Jenkinson, 1981). More significantly, however, the relative amount of the 12K polypeptide in the coat of the double mutant was only about 20% of that found in the coat extracts of either parent. Also, in the double mutant this protein was surface-iodinated (Fig. 5*a*). From these results it was not possible to conclude that in strain 520 the 36K protein covered the 12K protein.

The spores of the double mutant were slower to germinate than the spores of either parent (Table 2) and they were more sensitive to lysozyme than spores of strain 513 (Table 2).

Mapping of spo-520

Mapping of the mutation in strain 520 was first carried out by phage PBS1-mediated transduction. The mutation (*spo-520*) was 94% (183/193) cotransducible with *leuA8* (strain MB21 as recipient), 86% (260/300) cotransducible with *ilvB2* (strain CU267 as recipient) and 34% (17/50) cotransducible with *pheA12* (MB68 as recipient). It was not cotransducible with *cysA14*, *hisA1*, *metC3* or *lys-1*.

Further mapping by transformation showed that *spo-520* was 38% cotransformable with *leuA8* (see Table 3). Since the cotransduction and cotransformation frequencies observed for *spo-520* were similar to those for *gerE36* (Moir, 1981), and this latter mutation, which also affects spore coat deposition, maps between *citF2* and *leu-2* (Moir, 1981), it was worth checking whether *spo-520* and *gerE36* were closely linked. To do this, strain 4823 *leu-2 citF2* was transformed with DNA from strain 520 and Cit⁺ Leu⁺ recombinants were selected and scored for co-transformation of *spo-520*. In this cross, 54% of the recombinants carried the sporulation and germination defect (Table 3) while in a corresponding cross of strain 4823 with 4673 *gerE36*, 96% of the Cit⁺ Leu⁺ recombinants were germination-defective (Table 3), suggesting the order *citF2-gerE36-leu-2*. Since in other crosses, *spo-520* was more closely linked to *leuA8* (38%) than to *citF78* (8%) (results not shown), and since it seems not to map between the two outside markers, the results suggested that the relative gene order is *citF78-citF2-leu-2-spo-520*.

A three factor cross was then carried out in which strain CU267 *trpC2 ilvB2 leuB16* was transformed with DNA from strain 520 *trpC2 spo-520*. When Leu⁺ recombinants were selected and scored for transformation of *ilv* and *spo*, 47% of the Leu⁺ transformants were Spo⁻ and no rare class of recombinants was generated. This suggests that *leuB* lies between the other two markers. This was checked by selecting for Ilv⁺ recombinants. Of these only 3/188 were Leu⁻ Spo⁻, thus confirming that the gene order was *ilvB2-leuB16-spo-520* (Table 3). DNA from strain 4673 *gerE36* was also used to transform strain CU267, and in this case the rare class of recombinants was generated when Leu⁺ transformants were selected (Table 3) confirming the order *gerE36-ilvB2-leuB16* determined by Moir (1981) using phage SPP1 transduction. Thus *gerE36* and *spo-520* are separated by the *leu* genes, and both are separate from *spo-513*, a recently described mutation affecting spore coat production (Jenkinson, 1981) which is unlinked to *leuA8* by transformation (unpublished results).

Table 3. Mapping of *spo-520* and *gerE36* by 2- and 3-factor transformation crosses

Recipient	Donor DNA	Selected marker	Number tested	Unselected marker	Number in class	Linkage	Suggested order
MB21 <i>metC3 leuA8</i>	<i>trpC2 spo-520</i>	Leu ⁺	50	Spo ⁺ Spo ⁻	31 19	38% of <i>spo-520</i> with <i>leuA8</i>	---
4823 <i>citF2 leu-2</i>	<i>trpC2 spo-520</i>	Cit ⁺ Leu ⁺	100	Spo ⁺ Spo ⁻	46 54	---	---
CU267 <i>trpC2 ilvB2 leuB16</i>	<i>trpC2 spo-520</i>	Ilv ⁺	188	Leu ⁺ Spo ⁺ Leu ⁺ Spo ⁻ Leu ⁻ Spo ⁺ Leu ⁻ Spo ⁻	72 37 76 3	21% of <i>spo-520</i> with <i>ilvB2</i> ; 58% of <i>leuB16</i> with <i>ilvB2</i>	<i>ilvB2-leuB16-spo-520</i>
4823 <i>citF2 leu-2</i>	<i>gerE36</i>	Cit ⁺ Leu ⁺	100	Ger ⁺ Ger ⁻	4 96	---	<i>citF78-gerE36-leu-2</i>
CU267 <i>trpC2 ilvB2 leuB16</i>	<i>gerE36</i>	Leu ⁺	158	Ilv ⁺ Ger ⁺ Ilv ⁺ Ger ⁻ Ilv ⁻ Ger ⁺ Ilv ⁻ Ger ⁻	73 18 66 1	12% of <i>gerE36</i> with <i>leuB16</i> ; 57 of <i>ilvB2</i> with <i>leuB16</i>	<i>gerE36-ilvB2-leuB16</i>

As a final check on these results, the recombination index (Lacks & Hotchkiss, 1960) between *gerE36* and *spo-520* was determined as described by Coote (1972). From two separate experiments the R.I. was calculated to be 0.98 (data not shown) thus confirming that the mutations are not linked by transformation.

Isolation of further sporulation mutants with mutations in the same region

To try to isolate further germination mutants with mutations close to *spo-520*, a localized mutagenesis was carried out using mutagenized DNA extracted from the wild-type strain. A suitable way of doing this would have been to use the closely-linked mutation *leuB16*, select *Leu*⁺ transformants, and to look amongst these for stage VI mutants. However it is difficult to distinguish these mutants from a *Spo*⁺ background, so to circumvent this problem an uncharacterized sporulation mutant blocked at stage IV, recently isolated in this laboratory, and whose mutation was about 20% linked by transformation to *leuB16*, was used as recipient. After the transformation, Schaeffer's sporulation medium (3 ml; Schaeffer *et al.*, 1965) was added. The culture was allowed to sporulate for 16 h at 37 °C and then it was heated (80 °C, 15 min) to kill cells. This eliminates any *Spo*⁻ recipients or transformants, and selects for *Spo*⁺ transformants. The suspension was centrifuged, the pellet suspended in Penassay broth (5 ml) and the spores were incubated for 2 h at 37 °C with shaking to germinate them. The culture was then heated again to kill germinated spores, and thus to select for slow germinators, and portions (0.2 ml) were plated on to PGYE agar. After overnight incubation at 37 °C, colonies appeared at a frequency of about 10 per plate and a number were picked and tested for defective germination on PGYE/tetrazolium agar (see Methods). Three strains were purified on the basis of preliminary mapping data which indicated that each strain carried a separate mutation.

Phenotypic properties of strains 541, 547 and 552

The phenotypic properties of these three strains were identical to those of strain 520, i.e. they sporulated more slowly than the wild-type and the spores germinated more slowly. In Fig. 6 the germination rates of the spores from isogenic derivatives of each mutant strain (see Table 1) in 10 mM-L-alanine are compared with those of strain 520.3 and strain CU267 (*Spo*⁺ isogenic parent). The mutants had distinctly different germination rates and these always followed the order CU267 > 520.3 > 541.1 > 547.1 > 552.1. Surface iodination of *t*₁₀ spores of strains 541, 547 and 552 (and of the isogenic strains 541.1 etc.) showed the same pattern of abnormal labelling in the coat proteins as that in strain 520, i.e. the 12K protein was virtually un-iodinated, and the 24K and 19K polypeptides were heavily labelled.

Solubility of coat protein of mutant spores

Treatment of *B. subtilis* spores with 50 mM-DTT in 8 M-urea at pH 3, followed by 0.1 M-NaOH, renders them extremely sensitive to lysis by lysozyme (Gould *et al.*, 1970). Since the coat proteins of the mutant strains described here were apparently misarranged it was possible that the coat proteins would be more readily solubilized with the above agents than those of the spores of a wild-type strain. Accordingly, *t*₂₀ spores of the isogenic derivatives of the four mutants and of strain CU267 were treated with the above agents as described in Methods, and their subsequent rates of lysis upon the addition of lysozyme were measured by the fall in absorbance at 550 nm. The results in Fig. 7 show that the spores of strain CU267 (normal spores) lysed fairly rapidly in lysozyme after the treatment (50% loss of absorbance in 10 min). However, the spores of the mutant strains all lysed far more rapidly than this and the fastest of these (strain 547) lost 50% absorbance in 2 min (Fig. 7). Thus as a result of the misarrangement of the coat proteins the extracted spores were more susceptible to lysozyme treatment (i.e. the proteins were more readily solubilized by reducing agent-alkali).

Genetic mapping

The mutations *spo-541*, *spo-547* and *spo-552* were respectively 41% (170/416), 42% (175/416) and 50% (208/416) cotransformable with *leuB16*. The recombination index between each of these three mutations and *spo-520* was estimated to be < 0.05 (Table 4) but their relative order

Fig. 6

Fig. 7

Fig. 6. Germination of spores of strains CU267 (normal spores) (○), 520.3 (●), 541.1 (△), 547.1 (▲) and 552.1 (□) in 10 mM-L-alanine. Cleaned spores were suspended at an A_{550} of 0.35 in Tris/HCl buffer (10 mM, pH 8.2) containing L-alanine (10 mM) and germination followed by the fall in A_{550} .

Fig. 7. Lysis of spores of strains CU267 (A), 520.3 (B), 541.1 (C), 547.1 (D) and 552.1 (E) in lysozyme after treatment with dithiothreitol/urea and NaOH as described in Methods. Treated spores in 1 ml $\text{NaH}_2\text{PO}_4/\text{NaOH}$ buffer (100 mM, pH 7) were warmed to 37 °C in a cuvette within a heated block in a Pye Unicam SP800 spectrophotometer. Lysozyme (0.01 ml of a 20 mg ml⁻¹ solution in $\text{NaH}_2\text{PO}_4/\text{NaOH}$ buffer) was added and the fall in A_{550} was automatically recorded.

Table 4. Recombination index between *spo-541*, *spo-547*, *spo-552* and *spo-520*

Strain 520.1 *metC3* was used as recipient in a transformation cross with DNA at saturating concentration (5 µg ml⁻¹) from each of the other strains and from the wild-type 168 *trpC2* (control). *Met*⁺ transformants were selected and colonies first purified on lactate/glutamate minimal agar and then picked on to PGYE agar to score for *Spo*⁺ (*Ger*⁺).

	Donor DNA	<i>Spo</i> ⁺ / <i>Met</i> ⁺	<i>Spo</i> ⁺ (%)	Recombination index
Experiment 1	541	0/516	0	<0.042
	552	0/516	0	<0.042
	168	24/516	4.651	—
Experiment 2	541	1/936	0.107	0.018
	547	1/936	0.107	0.018
	552	2/936	0.214	0.036
	168	12/204	5.882	—

Fig. 8. Map of the *leu* region showing genetic distances of *gerE36* and *spoVIB520* mutations affecting spore coat deposition. Distances are in transformation units, $(100 - \% \text{cotransformation}) \div 100$. The transformation distance between *ilvB2* and *leuB16* is similar to that determined by Ward & Zahler (1973).

was not determined from three-point crosses because of the difficulty in scoring large numbers of transformants. These four mutations together define a new sporulation locus, *spoVIB*, and a map of the region is shown in Fig. 8.

DISCUSSION

In general, spore coat mutants of *Bacillus cereus* which have been described have germination defects (Aronson & Fitz-James, 1976; Stelma *et al.*, 1978, 1980), and the two spore-coat mutants of *B. subtilis* that have been characterized also have germination defects (Moir, 1981; Jenkinson, 1981). If the germination and resistance properties are indeed dependent on the proper assembly of the coat then aberrations in assembly should be reflected by aberrations in these properties. The coat of the mutant strain 520 exhibits a number of abnormalities. First, the 12K protein is largely overlaid in mature spores whereas in the wild-type it is prominent on the surface. Second, the assembly of the coat is delayed in relation to that in the wild-type. Third, the misarrangement of the proteins in the coats of the mutants causes the mutant spores to be unduly sensitive to lysozyme after extraction with reducing agent-alkali (Fig. 7). Finally, although the base layer of the coat (containing 26K, 33K, 65K, 30K, 15K and 24K proteins) in the mutant strain 520 is laid down in the normal way, a number of the proteins (e.g. 24K, 19K and 15K) show an abnormally high degree of surface labelling. The significance of the latter is uncertain and so is the fact that about 30% of the mutant spores at t_{10} are lysozyme-sensitive. Both of these findings may be consequential effects arising from the delayed assembly of the coat. Thus, the mother cells of a sporulating culture begin to lyse after about t_0 , and in those cells in which lysis takes place before the deposition of the 36K polypeptide the spores concerned will be lysozyme-sensitive. In addition, those proteins in them that would normally have been overlaid by deposition of the 36K protein will be more accessible to the surface-labelling agents and will exhibit an unusually high degree of iodination.

One explanation for the fact that the 12K polypeptide in the mutant spores is not iodinated significantly would be to assume that it had been covered by the 36K protein. To test this, a second mutation *spo-513*, which prevents the deposition of the 36K polypeptide (Jenkinson, 1981) was introduced into strain 520. However, the amount of 12K protein in the coat of the double mutant was considerably reduced, so the experiment provided no information about the relative arrangement of the 12K and 36K proteins in the mutant strain 520. The result does suggest though, that in this mutant, but not in the wild-type, the deposition of the 12K polypeptide is in some way dependent on the formation or deposition of the 36K component.

Recently it has been suggested (Stewart & Ellar, 1982) that some, perhaps the majority, of the proteins described as coat proteins in *B. subtilis* spores (Goldman & Tipper, 1978; Jenkinson *et al.*, 1981) are in fact components of the spore outer membrane. This supposition seems to ignore the fact that when iodination is carried out on mature spores, i.e. when labelling of membrane components is hardly likely, seven polypeptides are labelled in wild-type spores (36K, 12K, 9K and 8K heavily, and 20K, 19K and 15K partially). To these should be added the proteins labelled in strain 513 (30K and 24K) giving a total of at least nine proteins out of the 14 initially identified (Jenkinson *et al.*, 1981). It is thus apparent that most of the proteins described are components of the coat and not of the membrane. This underlines the greater complexity of the *B. subtilis* spore coat compared with that of *Bacillus megaterium*.

The most likely explanation for the phenotypic properties of strain 520 is that one of the proteins of the coat is altered in primary sequence. However, despite attempts to show this by peptide analysis, there appeared to be no obvious differences between the 36K, 24K, 20K, 19K or 12K proteins of mutant and wild-type. It is possible that one of the other coat proteins is different in the mutant, or that the mutation affects the activity of some processing enzyme or regulatory molecule required for coat assembly.

Three loci have been identified as being involved in spore coat production in *B. subtilis*. Mutations in *spoVIA* and *gerE* affect in addition to coat deposition, the germination and resistance properties of the spores. The third, *spoVIB*, is involved in coat assembly, and mutations within a small region of this locus (Fig. 8) give rise to slow development of resistance

properties during sporulation, and to spores that are slow to germinate. The effects of mutation in *spoVIA*, *spoVIB* and *gerE* show that there is no fundamental distinction between *spo* and *ger* loci. Close examination of other germination mutants with mutations in other *ger* loci (Moir *et al.*, 1979) might be expected to reveal differences in coat assembly.

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