***spoVH* and *spoVJ* – New Sporulation Loci in *Bacillus subtilis* 168**

By SUSAN H. A. HILL

Microbiology Unit, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, U.K.

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Three sporulation mutants have been isolated which produce spores with an atypical resistance phenotype, i.e. they are sensitive to organic solvents and heat but resistant to lysozyme. All three mutants produced serine protease, alkaline phosphatase and glucose dehydrogenase which are biochemical marker events for stages I, II and III. Two of the three mutants produced dipicolinic acid, a late marker, but the third was defective in its production. Heat-resistance was not restored to any of the mutants by the provision of exogenous dipicolinate. Gel electrophoresis showed that the mutant spores had similar patterns of spore coat proteins to the wild-type and electron microscopy revealed no significant structural differences. The three mutations responsible for the phenotypes of the mutant spores lie in the *phe-argA* region of the *Bacillus subtilis* chromosome. Recombination index values indicate that the mutations are in three separate genes. They define at least two new sporulation loci, designated *spoVH* and *spoVJ*.

INTRODUCTION

Late events in sporulation of *Bacillus subtilis* include the deposition of spore coat proteins, the development of germination properties and the successive acquisition of resistance to organic solvents, heat and lysozyme (Dion & Mandelstam, 1980; Jenkinson *et al.*, 1980). Evidence presented by Dion & Mandelstam (1980) and Jenkinson *et al.* (1980) suggests that the late events in sporulation, occurring at stages V and VI, involve the assembly of proteins, most of which are synthesized before or during stage IV. Only one of the late events, the acquisition of complete lysozyme resistance, requires the presence of a spore coat protein (mol. wt 36000) which is not synthesized until stage VI (Jenkinson *et al.*, 1981; Jenkinson, 1981). It is therefore apparent that the 'dependent sequence of events' thought to reflect the order of expression of sporulation genes in the earlier stages of the process (Mandelstam, 1969, 1976; Piggot & Coote, 1976) does not hold during the later stages. Instead, the successive acquisition of late properties depends on the order of assembly of pre-formed proteins (Dion & Mandelstam, 1980; Jenkinson *et al.*, 1980, 1981). It should, therefore, be possible to isolate mutants in which a 'late' property is normal while an 'earlier' one is defective. We have now been able to show that mutants with this previously unexpected phenotype do exist and in this paper the isolation and characterization of three organic solvent-sensitive, heat-sensitive but lysozyme-resistant mutants are described. The mutations carried by these three mutants identify two new sporulation loci.

METHODS

Chemicals. Unless otherwise stated chemicals were obtained from Sigma.

Organisms. *Bacillus subtilis* Marburg 168 *trpC2* which requires either indole or tryptophan and which sporulates normally is referred to as the wild-type. Details of sporulation defective (Spo⁻) mutants derived from 168 and Spo⁺ auxotrophic strains used for genetic analysis are shown in Table 1.

Abbreviations: DPA, 2,6-dipicolinic acid; NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; RI, recombination index.

Table 1. *Strains of Bacillus subtilis used*

Strain	Genotype	Phenotype	Source†
168	<i>trpC2</i>	Spo ⁺ (wild-type)	Laboratory stock
516	<i>trpC2 spoVH516</i>	Spo ⁻ , heat-sensitive, lysozyme-resistant	NTG mutagenesis of 168
517	<i>trpC2 spoVJ517</i>	Spo ⁻ , heat-sensitive, lysozyme-resistant	NTG mutagenesis of 168
518	<i>trpC2 spoVH518</i>	Spo ⁻ , heat-sensitive, lysozyme-resistant	NTG mutagenesis of 168
MB21	<i>metC2 leuA8</i>	Spo ⁺	Laboratory stock
Cu448	<i>pheA2 ilvBΔ1 argA2 trpC2*</i>	Spo ⁺	S. A. Zahler
517.21	<i>metC3 leu⁺ spoVJ517</i>	Spo ⁻ , heat-sensitive, lysozyme-resistant	PBS1 (517) × MB21; Leu ⁺
516.21	<i>metC3 leu⁺ spoVH516</i>	Spo ⁻ , heat-sensitive, lysozyme-resistant	PBS1 (516) × MB21; Leu ⁺
518.21	<i>metC3 leu⁺ spoVH518</i>	Spo ⁻ , heat-sensitive, lysozyme-resistant	PBS1 (518) × MB21; Leu ⁺
516.448	<i>ilvB spoVH516 phe trpC2 argA⁺</i>	Spo ⁻ , heat-sensitive, lysozyme-resistant	PBS1 (516) × Cu448; ArgA ⁻
517.448	<i>ilvB spoVJ517 phe trpC2 argA⁺</i>	Spo ⁻ , heat-sensitive, lysozyme-resistant	PBS1 (517) × Cu448; ArgA ⁻
518.448	<i>ilvB spoVH518 phe trpC2 argA⁺</i>	Spo ⁻ , heat-sensitive, lysozyme-resistant	PBS1 (518) × Cu448; ArgA ⁻

* *ilvBΔ1* is a deletion which covers all known sites in *ilvB* but does not extend into *ilvC*.

† 'PBS1 (A) × B; Aux⁻' means that phage PBS1 grown on strain A was used to transduce strain B; Aux⁺ transductants were selected and the *spo* marker was acquired by cotransduction.

Induction of sporulation. Bacteria were grown in a hydrolysed casein medium at 37 °C with shaking until the culture contained 0.25 mg dry wt bacteria ml⁻¹, and were then resuspended to the same density in warm (37 °C) resuspension medium (Sterlini & Mandelstam, 1969). Incubation at 37 °C with shaking was continued. The time of resuspension and subsequent hourly intervals are referred to as *t*₀, *t*₁, *t*₂, etc. About 80–90% of wild-type cells contained phase bright spores by *t*₈. The stages of sporulation are those described by Ryter (1965). Culture density was determined by absorbance measurements at 600 nm in a Unicam SP600 spectrophotometer and these were converted to units of mg dry wt bacteria ml⁻¹ with a calibration curve.

Auxotrophic requirements of strains were satisfied by the inclusion of tryptophan at 20 µg ml⁻¹ and all other appropriate amino acids at 200 µg ml⁻¹ in minimal media.

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 spores are termed phase grey.

Isolation of mutants. Wild-type bacteria from hydrolysed casein medium were suspended at 0.25 mg dry wt ml⁻¹ in resuspension medium and treated with 100 µg NTG ml⁻¹ at 37 °C for 30 min in the dark. The cells were washed three times with resuspension medium. The washed cells were suspended in 10 ml resuspension medium and portions (0.1 ml) were used to inoculate 20 conical flasks (vol. 50 ml) each containing 5 ml hydrolysed casein medium. The flasks were incubated at 37 °C with shaking overnight to permit phenotypic expression of any newly induced mutations. The cultures were diluted and 0.1 ml portions were plated on lactate–glutamate minimal agar to give about 100 colonies per plate. This agar medium was resuspension medium (Sterlini & Mandelstam, 1969) containing 0.2% (v/v) sodium lactate (Fisons) and 1% (w/v) agar (Davis Gelatine, Warwick). It allows production of the brown pigment associated with sporulation in the wild-type (Iichinska, 1960; Schaeffer & Ionesco, 1960) after 2–3 d incubation at 37 °C while Spo⁻ mutants produce translucent, white or light brown colonies.

Measurement of resistance to toluene, octanol, heat and lysozyme. Resistance to organic solvents, heat and lysozyme was determined as described by Jenkinson *et al.* (1980).

Resistance properties of free spores. To ensure that the resistance properties shown by each of the mutants was attributable to defective spores *t*₁₀ cultures of each mutant were passed through an ice-cold French pressure cell at 83 MPa, which released >90% of the spores from their mother cells (Jenkinson *et al.*, 1981). The resistance properties of the free spores were similar to those shown by cells not passed through the French press.

Enzyme assays. Serine protease was determined using the method described by Dancer & Mandelstam (1975) with Remazol brilliant blue hide powder (Calbiochem) as substrate.

Alkaline phosphatase was determined by a modification (Glenn & Mandelstam, 1971) of the method of Torriani (1960).

Glucose dehydrogenase was determined by a modification of the method of Sadoff (1966). Cultures (50 ml) in resuspension medium were incubated at 37 °C with shaking to stage *t*₆. The cultures were centrifuged (5000 g, 10 min) and the pellets were resuspended in 2.5 ml 0.5 M-sodium acetate/acetic acid solution (pH 6.5) containing 0.25 ml glycerol (17.5%, v/v) and lysozyme (50 µg ml⁻¹). Samples were incubated at room temperature for 10 min and then sonicated twice at 600 W (90 s each time) in a cold stainless steel conical flask containing 2.5 ml glass beads. Sonication for 3 min in a Mullard sonicator released about 90% of spores from their mother cells and subsequently disrupted >80% of the free spores. The glass beads were allowed to settle out of suspension and the supernatants were centrifuged (Beckman Microfuge, 3 min) and used for assays. Assay mixtures contained 0.1 ml

0.1 M-Tris/HCl buffer (pH 8.0), 0.1 ml 0.02 M-NAD, 0.1 ml 1 mM-MnSO₄ and 0.1 ml cell supernatant. The reaction was started by the addition of 0.1 ml 1 M-D-glucose and followed in a Unicam SP800 spectrophotometer at 37 °C (wavelength 340 nm). One unit of enzyme is defined as the amount catalysing the reaction of 1 nmol substrate min⁻¹ and specific activity is expressed as units (mg protein)⁻¹. Soluble protein was determined using the Lowry method.

Determination of DPA. DPA was measured by the method of Janssen *et al.* (1958). When testing for the 'curing' effects of DPA, cultures in resuspension medium were provided with DPA (Koch-Light) at a final concentration of 100 µg ml⁻¹ by adding appropriate volumes of a solution of DPA (5 mg ml⁻¹, pH 7.1) at *t*₄.

Preparation of transducing lysates. PBS1 transducing lysates were prepared by the method of Coote (1972) which was based on that described by Karamata & Gross (1970).

Transduction. Transductions with PBS1 were performed as described by Coote (1972) with the minor modification that all bacteria were washed three times in resuspension medium (not supplemented with any amino acids) before plating on appropriately supplemented lactate/glutamate agar.

Transformation. DNA was extracted by the method of Marmur (1961) and stored at -20 °C in 1 ml volumes (each containing approximately 1 mg nucleic acid). DNA preparations were not treated with ribonuclease. Competent cells were prepared by the method of Ayad & Barker (1969). Saturating concentrations of DNA (> 1 µg ml⁻¹) were used for constructing isogenic strains and for determining recombination indices. For mapping experiments non-saturating concentrations of DNA (20–100 ng ml⁻¹) were used. In either case competent cells were incubated with the DNA at 37 °C for 1 h. Transformed cells and cells not treated with DNA, to serve as a recipient control, were washed three times and diluted in the minimal medium of Spizizen (1958) before plating on appropriately supplemented lactate/glutamate agar.

Preparation of isogenic strains. Saturating amounts of DNA prepared from the mutants were used to transform recipient strain MB21 Spo⁺ *leu metC*. Selection was for *metC*⁺ and generally about 5% of these transformants had also received the *spo* mutations of the donors by congression. The congressants had the same sporulation characteristics as their parent donor strains suggesting that the mutants did not carry multiple mutations.

Determination of RIs. Mutants *spo*₁ *met*⁺ and *spo*₂ *met* were crossed by transformation, with saturating concentrations of DNA. Recipient (*metC*) strains were transformed to prototrophy and the number of Spo⁺ transformants, obtained by congression, were scored. Wild-type DNA was used as the internal standard. The distance between the mutation was measured in terms of the RI (Lacks & Hotchkiss, 1960). The RI is given by the ratio:

$$\frac{\text{Spo}^+/\text{Met}^+ (\text{spo}_1 \text{ mutant as donor})}{\text{Spo}^+/\text{Met}^+ (\text{wild-type as donor})}$$

The RI is 1.0 when mutations are unlinked and decreases the more closely they are linked.

Extraction of spore coat proteins/SDS-PAGE. The methods described by Jenkinson *et al.* (1981) and Jenkinson (1981) were used.

Electron microscopy. Samples of about 10 ml were removed from sporulating cultures and prepared for examination under the electron microscope as described by Kay & Warren (1968).

RESULTS

Isolation of mutants

Out of about 200 sporulation-defective colonies obtained after NTG treatment, six, each of which contained a high proportion of phase grey spores, were chosen for more detailed examination. The growth rate of each mutant in liquid lactate minimal medium and liquid glucose/glutamate minimal medium was similar to that of the wild-type (data not shown) suggesting that they were specifically sporulation mutants and not damaged in their vegetative metabolism.

Resistant phenotypes of mutants

The resistance phenotype of each mutant was determined with *t*₁₀ cultures in resuspension medium. Two of the mutants, designated 517 and 518, exhibited the same phenotype, i.e. the spores were sensitive to treatment with organic solvents and heat but resistant to treatment with lysozyme (Table 2). In a third mutant, 516, about 5–10% of the spores were lysozyme resistant while heat-resistance and organic solvent-resistance were lower by three and one orders of magnitude, respectively. Representative values for resistances are shown in Table 2. Toluene- and octanol-resistance normally characterize stages IV and V of sporulation, respectively.

Table 2. *Resistance phenotypes of strains 516, 517 and 518*

The percentage of phase grey spores was determined microscopically at t_{10} . The resistance properties of the strains were also tested at t_{10} . Typically the wild-type strain produced a culture at t_{10} in resuspension medium containing 80% phase bright, resistant spores, i.e. in a culture containing 5.0×10^8 c.f.u. ml⁻¹, 4.0×10^8 ml⁻¹ were resistant to each of the treatments. The wild-type acquires resistance to the treatments successively in the order shown below.

	C.f.u. (ml resuspension medium) ⁻¹		
	Strain 516	Strain 517	Strain 518
Total viable count. . .	4.0×10^8	4.2×10^8	8.0×10^8
Treatment			
Toluene	1.0×10^6	1.7×10^5	1.0×10^6
Octanol	8.0×10^6	1.1×10^5	8.0×10^2
Heat	1.2×10^4	2.4×10^5	1.0×10^2
Lysozyme	3.8×10^7	4.2×10^8	7.0×10^8
Percentage of phase grey spores	90%	80%	80%

Table 3. *Sporulation characteristics of strains 516, 517 and 518*

Values, obtained in resuspension experiments (at t_6 for serine protease, alkaline phosphatase and glucose dehydrogenase; at t_7 for DPA), are expressed as a percentage of the wild-type values. These were: serine protease 8 units ml⁻¹ culture; alkaline phosphatase 45 units ml⁻¹ culture; glucose dehydrogenase 48 units (mg protein)⁻¹; and DPA 18 µg ml⁻¹ culture. One unit of enzyme is defined as the amount catalysing the reaction of 1 nmol substrate min⁻¹.

	Enzyme activity (percentage of wild-type activity)		
	Strain 516	Strain 517	Strain 518
Serine protease	48	100	65
Alkaline phosphatase	91	31	99
Glucose dehydrogenase	40	75	60
DPA	0 (<1)	31	73

Strains 516, 517 and 518 were chosen for more detailed examination because of their resistance phenotype.

Sporulation characteristics of strains 516, 517 and 518

Each of the three mutants was tested for production of serine protease, alkaline phosphatase and glucose dehydrogenase, which respectively characterize the first three stages in sporulation, and also tested for DPA which is normally associated with stage V (Waites *et al.*, 1970). All three mutants produced serine protease, alkaline phosphatase and glucose dehydrogenase, though the amounts sometimes differed appreciably from those found in the wild-type. Two of the mutants, 517 and 518, also produced DPA but 516 was defective in DPA production (Table 3).

Effect of added DPA on heat-resistance

It is known that some types of heat-sensitive spores acquire heat-resistance if they develop in the presence of added DPA (Balassa *et al.*, 1979). Accordingly, mutants 516, 517 and 518 were tested to determine to what extent external DPA would restore the normal phenotypic properties. The resistance phenotype of spores developed in the presence of DPA (100 µg ml⁻¹) was tested at t_{10} . None of the mutant spores was 'cured' by the addition of DPA (100 µg ml⁻¹) to the sporulating cultures at t_4 .

Genetic mapping of spo516, spo517 and spo518

In preliminary transduction mapping each of the three mutants was used as donor with strain MB21 Spo⁺ *metC3 leuA8* as recipient. Separate selections were made for *metC*⁺ and *leuA*⁺ transductants on appropriately supplemented lactate-glutamate minimal media. The Spo

Table 4. Three-factor transduction crosses with Cu448 as recipient [*argA* Spo⁺ *ilvB* (000)]

A total of 200 transductants were tested in each cross. Sporulation phenotypes were tested by pigment production and confirmed in cases of doubt by microscopy. Cotransduction frequencies between auxotrophic markers were determined by replica plating on appropriately supplemented lactate/glutamate agar.

Donor: <i>arg</i> ⁺ Spo ⁻ <i>ilv</i> ⁺ (111)			No. of transductants in the different classes of recombinants				Possible order	No. of transductants in the cross requiring multiple cross-overs	Suggested order
Selected marker	Unselected marker		100	101	110	111			
1--	-1-	--1							
<i>arg</i> ⁺	<i>spo516</i>	<i>ilv</i> ⁺	62	12	97	29	$\left\{ \begin{array}{l} arg\ spo\ ilv \\ arg\ ilv\ spo \\ spo\ arg\ ilv \end{array} \right.$	12 97 *	<i>arg spo ilv</i>
<i>arg</i> ⁺	<i>spo517</i>	<i>ilv</i> ⁺	70	9	78	43	$\left\{ \begin{array}{l} arg\ spo\ ilv \\ arg\ ilv\ spo \\ spo\ arg\ ilv \end{array} \right.$	9 78 *	<i>arg spo ilv</i>
<i>arg</i> ⁺	<i>spo518</i>	<i>ilv</i> ⁺	69	10	91	30	$\left\{ \begin{array}{l} arg\ spo\ ilv \\ arg\ ilv\ spo \\ spo\ arg\ ilv \end{array} \right.$	10 91 *	<i>arg spo ilv</i>

* Multiple cross-overs not detected with this order.

phenotype of the transductants was determined by pigment production and confirmed in cases of doubt by microscopy. The three mutations were not linked to *metC* but were all about 80% linked to *leuA*, the values being: *spo516*, 83% (661/792 transductants); *spo517*, 79% (575/731 transductants) and *spo518*, 83% (820/987 transductants). The approximate map positions of *spo516*, *spo517* and *spo518* were confirmed with a different recipient strain, Cu448 *phe ilvB argA trpC2*, selection being made for *ilvB*⁺ transductants (*ilvB* is adjacent to *leuA* at the 250° position on the circular *B. subtilis* linkage map (Henner & Hoch, 1980). In this series of experiments linkages to *ilvB* were as follows: *spo516*, 88% (734/831 transductants); *spo517*, 81% (1700/2091 transductants) and *spo518*, 88% (1372/1560 transductants).

In transformation experiments with Cu448 as recipient and non-saturating amounts of DNA (20 ng ml⁻¹) linkages to *ilvB* were: *spo516*, 51% (1228/2385 transformants); *spo517*, 59% (1476/2519 transformants) and *spo518*, 52% (525/1003 transformants).

Although the three mutations were cotransformable with *ilvB*, other auxotrophic markers in the region were too distant to enable more detailed mapping experiments to be made by transformation. It was therefore necessary to use PBS1-mediated transduction to order the mutations with respect to other markers in the *ilvB* region. In three-factor crosses with each of the mutants as donor and Cu448 as recipient, *argA*⁺ transductants were selected and scored for cotransduction of the unselected markers *spo* and *ilvB*. The results (Table 4) show that *spo516*, *spo517* and *spo518* are clustered together between the outside markers *ilvB* and *argA*. These markers lie between the 250° and 260° positions on the circular linkage map of *B. subtilis* (Henner & Hoch, 1980).

The relative order of *spo516*, *spo517* and *spo518* was determined by reciprocal transformation crosses with non-saturating concentrations of DNA (100 ng ml⁻¹). The recipients, 516.448, 517.448 and 518.448, were derived as shown in Table 1. *ilvB*⁺ transformants were selected and scored for their sporulation phenotypes. The order can be deduced from a consideration of the number of cross-overs needed to give Spo⁺ recombinants.

Table 5. *Relative order of spo516, spo517 and spo518 mutations examined by transformation crosses*

Non-saturating concentrations of DNA (100 ng ml⁻¹) were used for each transformation cross. Sporulation phenotypes were tested by pigment production and confirmed in cases of doubt by microscopy. The data suggest that the gene order is *ilvB spo516 spo518 spo517* (for explanation see text).

Donor strain	Recipient strain	No. of <i>ilvB</i> ⁺ transformants	No. of <i>ilvB</i> ⁺ transformants which were Spo ⁺
516 (<i>spo516 trpC2</i>)	517.448 (<i>ilvB spo517 phe trpC2 argA</i> ⁺)	3120	70 (2.2%)
517 (<i>spo517 trpC2</i>)	516.448 (<i>ilvB spo516 phe trpC2 argA</i> ⁺)	2454	410 (16.7%)
516 (<i>spo516 trpC2</i>)	518.448 (<i>ilvB spo518 phe trpC2 argA</i> ⁺)	4077	78 (1.9%)
518 (<i>spo518 trpC2</i>)	516.448 (<i>ilvB spo516 phe trpC2 argA</i> ⁺)	2740	370 (13.5%)
517 (<i>spo517 trpC2</i>)	518.448 (<i>ilvB spo518 phe trpC2 argA</i> ⁺)	1726	208 (12.1%)
518 (<i>spo518 trpC2</i>)	517.448 (<i>ilvB spo517 phe trpC2 argA</i> ⁺)	4300	83 (1.9%)

Table 6. *RI values for mutants 516, 517 and 518*

Saturating concentrations of DNA (> 1 µg ml⁻¹) were used for each cross. Sporulation phenotypes were tested by pigment production on lactate/glutamate agar and confirmed in cases of doubt by microscopy.

Donor strain	Recipient strain	No. of <i>metC</i> ⁺ transformants (selected marker)	No. of <i>metC</i> ⁺ transformants which were Spo ⁺	RI
168 (<i>spo</i> ⁺ <i>metC</i> ⁺)	516.21 (<i>spo516 metC</i>)	5916	460 (7.9%)	—
168 (<i>spo</i> ⁺ <i>metC</i> ⁺)	517.21 (<i>spo517 metC</i>)	8388	763 (9.1%)	—
168 (<i>spo</i> ⁺ <i>metC</i> ⁺)	518.21 (<i>spo518 metC</i>)	5665	425 (7.5%)	—
516 (<i>spo516 metC</i> ⁺)	517.21 (<i>spo517 metC</i>)	4356	292 (6.7%)	0.74
517 (<i>spo517 metC</i> ⁺)	516.21 (<i>spo516 metC</i>)	6259	358 (5.7%)	0.72
517 (<i>spo517 metC</i> ⁺)	518.21 (<i>spo518 metC</i>)	4207	302 (7.2%)	0.96
518 (<i>spo518 metC</i> ⁺)	517.21 (<i>spo517 metC</i>)	5228	347 (6.6%)	0.73
516 (<i>spo516 metC</i> ⁺)	518.21 (<i>spo518 metC</i>)	4828	100 (2.1%)	0.28
518 (<i>spo518 metC</i> ⁺)	516.21 (<i>spo516 metC</i>)	4352	95 (2.2%)	0.28

It can be seen that in the first cross, four cross-over events are required to yield the Spo⁺ phenotype, whereas in the reciprocal cross only two are required. The results (Table 5) showed that with *spo516* (donor) × *spo517* (recipient), Spo⁺ transformants were rare (2.2%) whereas in the reciprocal cross the value was 16.7%. This indicates that *spo516* is the nearer of the two mutations to *ilvB*. Examination of the remaining data (Table 5) in this way suggests that the relative order is *ilvB spo516 spo518 spo517*.

RIs between spo516, spo517 and spo518

To obtain a measure of the map distances between the mutations, RIs between each pair of mutants were determined. The RIs between strains 516 and 517, 516 and 518 and 517 and 518 are shown in Table 6. The RI data suggest that *spo516*, *spo517* and *spo518* lie in three separate genes but that *spo516* and *spo518* may be in the same sporulation locus; *spo517* is clearly in a different sporulation locus virtually unlinked to the other two.

Gel electrophoresis of spore coat proteins produced by strains 516, 517 and 518

Spores from strains 516, 517, 518 and 168 (wild-type) were prepared from *t*₀ resuspended cultures. The coat proteins were fractionated by SDS-PAGE (Fig. 1). The four major coat proteins identified by Jenkinson *et al.* (1981) have been indicated in Fig. 1. It can be seen that each mutant has the major proteins of the wild-type (36K, 20K, 12K and 11K, where K = kDal). Although minor quantitative differences occurred in each of the mutants they are unlikely to be significant (Jenkinson *et al.*, 1981).

Fig. 1. SDS-PAGE patterns (stained with Coomassie blue) of proteins extracted from coats of spores of strains 516, 517, 518 and 168 (wild-type). Lane 1, 168; lane 2, 516; lane 3, 517; lane 4, 518; lane 5, standards. K = kDal.

Electron microscopy of strains 516, 517 and 518

Electron micrographs were prepared from spores of *t₇* cultures of strains 516, 517, 518 and 168 (wild-type). No consistent structural differences could be seen between any of the strains and the micrographs are therefore not shown.

DISCUSSION

The mutants described in this paper were chosen for study because they possessed a previously undescribed phenotype, i.e. they were lysozyme-resistant but relatively sensitive to organic solvents and to heat. Under standard conditions of sporulation, two of the mutants, 517 and 518, gave normal numbers of lysozyme-resistant spores ($> 10^8$ ml⁻¹); the third, mutant 516, produced only 5–10% of this number. Each mutant grew as well as the wild-type under a variety of conditions, suggesting that they were blocked in functions specifically concerned with sporulation and not damaged in their vegetative metabolism. Each mutant produced a large number of phase grey spores and their properties suggest that they were blocked at stage V. Like many mutants blocked at stage V all three are oligosporogenous rather than asporogenous and also form a significant number of phase bright spores.

The fact that the mutants are oligosporogenous and that the sporulation properties of each mutant could be transferred to Spo⁺ backgrounds by single transduction and transformation events makes it highly unlikely that any of them is damaged in more than one sporulation gene. This assumption was substantiated by the fact that the three mutants reverted spontaneously to wild-type at a frequency of about 10⁻⁸ (data not shown) showing that the lesions are point mutations. Furthermore, the transformants and transductants derived from each of the mutants had the same sporulation phenotype as their parents. The transduction and transformation data favour the view that *spo516*, *spo517* and *spo518* identify three separate genes comprising two new sporulation loci since transformation data suggest that *spo516* and *spo518* are likely to be in the same locus (RI 0.28; see Piggot & Coote, 1976) whereas *spo517* seems more likely to have occurred in a separate sporulation locus from both *spo516* and *spo518* (RIs > 0.7). The new loci map in the *phe-argA* region of the *B. subtilis* chromosome. Since a mutant described by Rosenbluh *et al.* (1981) has already been designated *spoVG*, the designation *spoVH* will be used for mutants 516 and 518. To avoid typographical confusion with stage VI mutants the designation *spoVI* will not be used and 517 will be designated *spoVJ*.

The biochemical properties of the mutants with respect to production of enzymes associated with earlier stages of sporulation (Waites *et al.*, 1970) was as expected; the three mutants all produced serine protease, alkaline phosphatase and glucose dehydrogenase. However, one of the three mutants, 516, was also defective in DPA production (DPA production is a stage V event). Unlike some other DPA-negative mutants, e.g. *spoVF* (Piggot & Coote, 1976) and *dpa-1* (Balassa *et al.*, 1979), 516 was not restored to full heat-resistance ('cured') by the provision of exogenous DPA. It may be that the DPA-negative and heat-sensitive phenotype is due to the pleiotropic nature of the *spo516* mutation, e.g. the genes for the heat and DPA characters may share a common mechanism of regulation which has been altered by *spo516*. Strains 517 and 518 are DPA-positive and, as expected, the provision of exogenous DPA did not render them heat-resistant.

It now appears that the majority of events associated with the later stages of sporulation, including the development of physical resistance properties and the germination properties of the spores, involve incorporation into the spore coat of proteins synthesized during the earlier stages of the sporulation process (Dion & Mandelstam, 1980; Jenkinson *et al.*, 1981). Although 516, 517 and 518 gave similar SDS-PAGE patterns of spore coat proteins compared to the wild-type it is possible that their unusual resistance properties may be due to faulty assembly of spore coat proteins or perhaps to a failure to synthesize one or more minor coat polypeptides not normally detected by SDS-PAGE.

The failure of electron microscopy to reveal any differences between the mutant and wild-type spores is not surprising because only a gross defect in the coat or cortex would be expected to produce a recognizable change in morphology.

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