Properties of a Mutant of *Bacillus subtilis* 168 in which Spore Germination is Blocked at a Late Stage

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A mutation, *gerJ50*, causing defective spore germination has been located between *lys-1* and *trpC2* on the *Bacillus subtilis* map and is 92% cotransduced with *trpC2* by phage PBS1. Spores of strains containing *gerJ50* will respond to germinants but the fall in absorbance is only 60% of that of the wild-type. During germination, mutant spores reach only an intermediate phase-grey stage, in contrast to those of the wild-type which become dark. The germination response, measured by the release of dipicolinic acid and the loss of resistance to heat, chloroform, octanol or toluene, is normal. The release of hexosamine-containing fragments from mutant spores is incomplete but has similar kinetics to that of wild-type spores. The mutant spores are more sensitive to heating at 90 °C which may point to a structural abnormality, but there is no evidence of a spore coat deficiency from electron microscopy of thin sections or freeze fractures or from the chemical resistances of the spores. Thus, the germination of mutant spores is initiated normally, but blocked at a late stage.

INTRODUCTION

Germination of bacterial spores comprises the series of events that occur when resistant dormant spores develop into sensitive, actively metabolizing forms (Gould, 1969). In some species the spores must be activated, usually by heating, before they can respond to physical or, more commonly, chemical stimuli that will initiate germination. Germinants are thought to act by stimulating the spores such that they become irreversibly committed to losing their dormant properties (Scott *et al.*, 1978). The process of germination itself is essentially degradative because the cortical peptidoglycan is depolymerized (Hamilton & Stubbs, 1967) and dipicolinic acid (Powell & Strange, 1953) is released from the core (Hashimoto & Gerhardt, 1960) resulting in non-refractile spores which have lost about 30% of their dry weight and are fully sensitive to heat and chemicals. This process has been divided into two phases (Vary & Halvorson, 1965), namely, the microlag, which is the time from the addition of germinant until loss in refractility, and microgermination, which entails its loss. In another subdivision Hashimoto *et al.* (1969*b*) described a first stage as that which took place at high temperatures or high CaCl₂ concentrations, and the second as that which was inhibited under these conditions.

The overall aim of our work is to understand the processes that occur immediately before and during spore germination and use is made of mutants whose spores do not respond to one or more of the germinants to which wild-type spores respond (Trowsdale & Smith, 1975). In this paper we report the results of experiments on strains containing the germination mutation *gerJ50*, which differs from those previously described (Moir *et al.*, 1979) in that spores of strains containing it are blocked at a later stage in germination. Its relationship to another germination mutation, *gerE36* (Moir, 1981) which also gives rise to a block at a late stage in germination, is discussed.

METHODS

Bacterial strains. All strains were derived from *Bacillus subtilis* 168 (Table 1). Strain 1604 (*trpC2*) and its derivative 4750 (*gerJ50 trpC2*) are referred to in the text as the wild-type and mutant, respectively.

Media. Difco Penassay broth (PAB) was used as a liquid growth medium. Solid media were: Oxoid nutrient agar (NA; 1.5%, w/v); soft nutrient agar (SNA; 1%, w/v, NA, containing NaCl, 5 mg ml⁻¹); potato glucose yeast extract agar (PGYEA; Dring & Gould, 1971); Difco Tryptose blood agar base overlay containing 1% (w/v) Difco Bacto agar and 2,3,5-triphenyltetrazolium chloride (TZM, 50 µg ml⁻¹); and minimal salts (MS; Anagnostopoulos & Spizizen, 1961) solidified with 1.5% (w/v) agar to give minimal agar (MA) in which amino acid supplements were at 20 µg ml⁻¹ and glucose (5 mg ml⁻¹) was used as the primary carbon source. MMDL plates contained MA, D-alanine (10 mM), L-alanine (10 mM), glucose (5 mg ml⁻¹), L-tryptophan (20 µg ml⁻¹), CaCl₂ (1.4 mM), MnCl₂ (0.1 mM) and MgCl₂ (2 mM). MMDL overlay contained MS, TZM (50 µg ml⁻¹), L-alanine (15 mM), D-alanine (40 mM) and agar (1%, w/v).

Preparation of spore suspensions. A 4 ml portion of an exponential phase culture grown in PAB from a single colony on NA was spread on to a large (600 cm²) freshly poured area of wet PGYEA in a shallow dish and incubated for 24–28 h at 37 °C or 80–90 h at 26 °C to yield at least 80% phase-bright spores. The harvesting and washing procedure was described by Lafferty & Moir (1977) except that at the fourth washing the spore suspension was treated with lysozyme (200 µg ml⁻¹) in saline (0.085%, w/v) for 30 min at 20 °C. Spore suspensions in distilled water were stored at 4 °C at a density of 5×10^8 spores ml⁻¹.

Germination studies. The germination of spore suspensions was followed by measuring the fall in absorbance at 580 nm at 37 °C using a Vitatron U.P.M. spectrophotometer. Readings were taken at 5 min intervals over a 60 min period after the addition of heat-activated spores (30 min at 70 °C) to prewarmed germinant, and were expressed as a percentage of the initial A_{580} value (about 0.3). Germinants used were L-alanine (up to 10 mM), D-alanine (up to 10 mM), asparagine (10 mM), D-glucose (10 mM), D-fructose (10 mM), KCl (10 mM) and PAB; all except PAB were used in 10 mM-Tris/HCl buffer, adjusted to pH 8.4 at 20 °C.

The germination phenotype of stab inocula of the mutants was determined by the tetrazolium overlay method of Moir *et al.* (1979). In addition, spore-containing colonies were prepared on MMDL plates by incubation at 37 °C for 3 d; these were then exposed to chloroform vapour at 20 °C for 3 h, overlaid with MMDL and incubated at 37 °C for 6 h. In both these tests the wild-type colonies reduced the dye to give a red colour.

Release of dipicolinic acid (DPA) was determined at 5 min intervals in 3 ml samples of germinating spore suspensions. The samples were passed through Millipore filters (0.45 µm pore size) and the DPA content of the filtrates was determined by the method of Scott & Ellar (1978).

Table 1. *Strains of Bacillus subtilis* 168

Strain designation	Genotype*	Origin†
1604	<i>trpC2</i>	Moir <i>et al.</i> (1979)
4750	<i>trpC2 gerJ50</i>	Ultraviolet irradiation of 1604
MB21	<i>metC3 leuA8 tal-1</i>	P. Piggot
BD92	<i>hisA1 cysB3 trpC2</i>	C. Anagnostopoulos
BD111	<i>thr-5 trpC2 cysB3</i>	D. Dubnau
BD112	<i>cysA14</i>	D. Dubnau
GSY334	<i>leuA8</i>	C. Anagnostopoulos
1506	<i>lys-1</i>	Spontaneous Trp ⁺ revertant of GSY254 (C. Anagnostopoulos)
4756	<i>gerJ50</i>	Lys ⁺ transductant from PBS1 cross 1506 × 4750
SB455	<i>mtr</i>	E. Nester
IA132	<i>trpC2 aroC7</i>	Bacillus Genetics Stock Center. SB121 of E. Nester. Contains a TZM-white marker
9098	<i>trpC2 aroC7</i>	Lys ⁺ transductant of PBS1 cross 1506 × IA132
SB137	<i>hisB2 aroB2</i>	Nester <i>et al.</i> (1963). Contains a TZM-white marker
4783	<i>hisB2 aroB2</i>	Trp ⁺ transductant from PBS1 cross 1604 × SB137
SL646	<i>leuA8 spoIVA67 tal-1</i>	P. Piggot
1408	<i>trpC2</i>	Trowsdale & Smith (1975). Contains a TZM-white marker
1488	<i>gerC88 trpC2 wrd</i>	NTG mutagenesis of 1408
1558	<i>gerC58 trpC2 wrd</i>	NTG mutagenesis of 1408
9020	<i>trpC2 gerC88</i>	Aro ⁺ transductant from SPP1 cross 4783 × 1488
4600	<i>gerE36 trpC2 his</i>	Moir <i>et al.</i> (1979)

* The *wrd* mutation confers a temperature-sensitive growth phenotype (Trowsdale & Smith, 1975); the *mtr* mutation confers resistance to tryptophan analogues (Hoch *et al.*, 1971).

† NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; TZM, 2,3,5-triphenyltetrazolium chloride.

Spore resistance was measured for both germinating and dormant spore suspensions by adding 0.1 ml samples (containing about 5×10^7 spores) to 10 ml MS and incubating 2 ml of these dilutions either at 70 °C for 30 min to measure heat resistance or with 0.25 ml chloroform, toluene or octanol (after 1 min vortexing) for 30 min at 20 °C. Viable counts of serial dilutions of treated and untreated suspensions were determined in duplicate in 3 ml SNA layers on NA plates incubated at 37 °C for 15 h.

Germinating or dormant suspensions were observed with a Zeiss GFL phase contrast microscope, using a green filter, at 800 $\times$ magnification. Photographs were taken on Kodak-50 professional film, using a Leitz Wetzlar Ortholux II phase contrast microscope without filters.

To follow the release of hexosamine-containing fragments during germination, a concentrated suspension of spores (A_{580} about 400) was heat-activated (30 min at 70 °C) and diluted 10-fold into a mixture of L-alanine (10 mM), *O*-carbamyl-D-serine (5 mg ml⁻¹) as an alanine racemase inhibitor, and Tris/HCl buffer (10 mM, pH 8.4 at 20 °C) at 37 °C. Samples of 1 ml were taken at 5 min intervals and centrifuged for 2 min at 12000 g; hexosamine in both the supernatants and pellets was measured by the method of Warth (1971), after hydrolysis of the samples in 6 M-HCl for 2 h at 100 °C *in vacuo*, and compared with a glucosamine standard. The progress of germination was also monitored in parallel by measuring the A_{580} of a 1 in 30 dilution of the spore suspensions.

Measurement of spore heat resistance (D_{90}). A 0.1 ml portion of a spore suspension was added to 10 ml MS at 90 °C; 0.1 ml samples were taken at intervals, diluted in MS, plated in 3 ml SNA on NA plates and incubated at 37 °C for 15 h. The D_{90} value was determined as the time taken for the number of colony-forming units in samples to fall to 10 % of the original value.

Mutant isolation procedure. A 5 ml portion of a mid-exponential phase culture of strain 1604 (wild-type) in PAB was centrifuged, resuspended in 1 ml 100 mM-MgSO₄ and irradiated with ultraviolet light to give 0.1 % survival. A spore suspension was prepared in water and 0.8 ml of this suspension (A_{580} 3.0) was added to 6 ml Tris/HCl (0.1 M, pH 7.4) containing L-alanine (0.1 M), D-alanine (0.1 M) and *O*-carbamyl-D-serine (0.1 M) and incubated at 37 °C for 2 h. After centrifugation and resuspension in 5 ml water, 2 ml of the resulting suspension was layered on to 8 ml Endografin, $\rho = 1.255$ [Schering Chemicals; prepared as a 68 % (v/v) dilution in distilled water of a 70 % (w/v) solution] and centrifuged for 30 min at 4 °C at 22000 g. The upper layer, containing mainly germinated spores, was centrifuged on fresh Endografin; the upper layer was again removed, and suitable dilutions were plated on PGYEA and incubated at 37 °C. Colonies were then stab-inoculated on to fresh PGYEA in glass Petri dishes, incubated at 37 °C to allow sporulation and exposed to chloroform for 3 h; inocula were stabbed to further PGYEA and MMDL glass plates to test TZM reactions.

Transduction. Phage PBS1 was used as described by Moir *et al.* (1979). Phage SPP1 was used as described by Ferrari *et al.* (1978) except that vegetative cells were used, rather than spores, to prepare lysates.

Transformation. DNA was prepared from vegetative cells by the method of Marmur (1961) and transformation was performed by a modification of the method of Anagnostopoulos & Spizizen (1961). Cells were grown overnight on MA containing glucose, casein hydrolysate (0.02 %) and any required amino acids (50 µg ml⁻¹). MgSO₄ (5 mM) and trace elements (FeCl₃, CaCl₂, ZnCl₂ and MnCl₂, each 10 µM) were added to the transformation media.

Electron microscopy. Methods used were essentially similar to those described by Luft (1956) and Sousa *et al.* (1974). Washed spore pellets produced at 26 °C were suspended in either 2 % (w/v) KMnO₄ for 90 min at 37 °C, or in phosphate buffer (pH 7.3) containing 3 % (w/v) glutaraldehyde for 90 min at 4 °C followed by treatment with 1 % (w/v) osmium tetroxide for 2 h at 20 °C. The resulting pellets were then washed, resuspended in 1 % agar, dehydrated, embedded in the resin of Spurr (1969), and gold to silver grey sections were cut. Glutaraldehyde-treated sections were also stained with uranyl acetate and lead citrate. Other spore pellets were suspended in 30 % (v/v) glycerol overnight, and then a fracture was made at -110 °C and coated immediately with platinum and carbon.

RESULTS

Isolation of the mutant

The selection procedure was designed to enrich for spores that were able to germinate in the presence of D-alanine (a competitive inhibitor of L-alanine-stimulated germination; Wolf & Mahmoud, 1957) so that comparison with mutants thought to be blocked in the L-alanine response (Moir *et al.*, 1979) could be made. Of 240 colonies tested, 5 were TZM-white on PGYEA plates and 10 on MMDL plates; none were white on both media. For each of these 15 potential mutants, the fall in absorbance of spore suspensions in L-alanine was inhibited by D-alanine to the same extent as for the wild-type.

Spores from one colony (strain 4750, mutation *ger-50*), which was TZM-white on MMDL plates, reached only a phase-grey stage during germination in L-alanine. On closer study of

the PGYEA TZM plate test we found that when the plates were incubated for only 4 h at 37 °C the mutant clones were pale red, assuming the normal dark red colour after a further 2 h at 37 °C, although spores in these clones were phase-grey while those of the wild-type were phase-dark.

Map location of ger-50

A phage PBS1 lysate of the mutant was used to transduce a series of auxotrophs to prototrophy. The germination phenotype of the recombinants was determined using the PGYEA TZM test. No linkage to *metC3*, *leuA8*, *hisA1*, *cysB3*, *thr-5* or *cysA14* was detected but cotransduction with *trpC2* (92%) and *lys-1* (79%) was obtained (Table 2, crosses 1 and 2) and the results of a three-point cross were consistent with the order *lys-1-ger-50-trpC2* (cross 1).

Finer mapping was then carried out using phage SPP1 transduction and transformation. Crosses 3 and 6 demonstrate that *ger-50* is on the *trpC2*-distal side of both *mtr* and *aroC7*. Crosses 4 and 5 establish that *ger-50* is approximately 13% and *spoIVA67* approximately 60% linked to *aroB2* in SPP1 transduction and thus that the two mutations are well separated. The relative orders of *aroC7*, *mtr*, *spoIVA67*, *aroB2*, *trpC2* and *hisB2* have been determined previously (Nester *et al.*, 1963; Piggot, 1973) and so the following order can be deduced: *lys-1-ger-50-aroC7-mtr-spoIVA67-aroB2-trpC2-hisB2*.

The fall in absorbance of spore suspensions of at least three TZM-white and at least three TZM-red recombinant colonies from each of the crosses was tested and in each case found to be identical to the parental strain with the similar TZM reaction. This confirmed the association between TZM phenotype and possession of the germination defect. Since the map location of the *ger-50* mutation was different from those of other germination mutants (Moir *et al.*, 1979) it was assigned the location letter J thus becoming *gerJ50*.

Two other mutations (*gerC58* and *gerC88*) are located near the *lys-1* and *trpC2* markers (Trowsdale & Smith, 1975). These differ from *gerJ50* in that they are associated with another marker *wrd* (from which they could be separated in PBS1 crosses; Moir *et al.*, 1979), their germination in L-alanine is temperature-sensitive and they are TZM-white. The parental strain (1408) from which the two *gerC*-containing strains (1488 and 1558) were derived was also found to possess a TZM-white marker (Moir *et al.*, 1979) and, later, to be defective in germination (R. J. Warburg, unpublished results). Crosses 8 to 11 (Table 2) demonstrate that a TZM-white marker near *aroC7* is present in strains 9020 (7% cotransformed), 1558 (10% cotransformed) and 1408 (16% cotransformed) and thus may be near to the *gerJ50* mutation (14% cotransformed with *aroC7*, cross 7). None of the spore suspensions prepared from the recombinants were temperature-sensitive in L-alanine, but had similar germination (loss of absorbance and appearance under phase contrast) and resistance properties to those of strain 4750 (see below).

Thus, it would seem that we have not located the *gerC58* and *gerC88* mutations but rather the TZM marker of strain 1408, and that this presumably spontaneous mutation is both genetically and physiologically similar to *gerJ50*. This marker can thus be referred to as *gerJ51*.

Before they could be used in the above crosses the markers *aroC7* and *aroB2* had to be transduced by phage PBS1 into strains 1506 and 1604, respectively. This was necessary because the original strains carry TZM-white mutations which made it impossible to score for the presence of *gerJ50* TZM marker. We have found that many laboratory strains have such markers, and in the case of the Aro⁻ strains we have found that they form spores that are unable to germinate in L-alanine but will do so in a mixture of glucose, fructose, asparagine and KCl. These properties are like those of strains with mutations in the GerA region (Moir *et al.*, 1979), which are the most commonly isolated germination mutants in our laboratory. The locations of the markers on the chromosome are in at least two areas—those linked to *lys-1* (*gerJ51*) and those unlinked to *lys-1* (TZM markers from the Aro strains, as shown by

Table 2. Location of *ger-50* relative to established markers on the *Bacillus subtilis* chromosome

Cross no.	Recipient	Donor	Selection	Recombinant class	No. in class	Linkage of <i>ger</i> or <i>spo</i> to selected marker (%)	Type of cross
1	<i>lys-1</i> 1506	<i>trpC2 ger-50</i> 4750	Lys ⁺	Trp ⁺ Ger ⁺ Trp ⁺ Ger ⁻ Trp ⁻ Ger ⁺ Trp ⁻ Ger ⁻	20 22 0 53	79	PBS1 transduction
2	<i>trpC2</i> 1604	<i>ger-50</i> 4756	Trp ⁺	Ger ⁺ Ger ⁻	8 92	92	PBS1 transduction
3	<i>trpC2 ger-50</i> 4750	<i>mir</i> SB455	Trp ⁺	Mir ^S Ger ⁺ Mir ^S Ger ⁻ Mir ^R Ger ⁺ Mir ^R Ger ⁻	2 47 10 26	14	SPP1 transduction
4	<i>aroB2 hisB2</i> 4783	<i>trpC2 ger-50</i> 4750	Aro ⁺	Ger ⁺ Ger ⁻	21 3	13	SPP1 transduction
5	<i>aroB2 hisB2</i> 4783	<i>leuA8 spoIV A67 tal-1</i> SL646	Aro ⁺	Spo ⁺ Spo ⁻ (All His ⁺)	40 60	60	SPP1 transduction
6	<i>aroC7 trpC2</i> 9098	<i>ger-50</i> 4756	Aro ⁺	Trp ⁺ Ger ⁺ Trp ⁺ Ger ⁻ Trp ⁻ Ger ⁺ Trp ⁻ Ger ⁻	4 6 50 19	32	Transformation
7	<i>aroC7 trpC2</i> 9098	<i>trpC2 ger-50</i> 4750	Aro ⁺	Ger ⁺ Ger ⁻	69 11	14	Transformation
8	<i>aroC7 trpC2</i> 9098	<i>trpC2 gerC88</i> TzM-white 9020	Aro ⁺	TzM-red TzM-white	65 5	7	Transformation
9	<i>aroB2 hisB2</i> 4783	<i>gerC88 trpC2 wrd</i> TzM-white 1488	Aro ⁺	TzM-red TzM-white (All His ⁺ Trp ⁻ Wrd ⁺)	85 14	14	SPP1 transduction
10	<i>aroC7 trpC2</i> 9098	<i>trpC2 gerC58 wrd</i> TzM-white 1558	Aro ⁺	TzM-red TzM-white (All Wrd ⁺)	87 10	10	Transformation
11	<i>aroC7 trpC2</i> 9098	<i>trpC2 TzM-white</i> 1408	Aro ⁺	TzM-red TzM-white	84 16	16	Transformation

Fig. 1. Changes in spores during germination, as seen by phase contrast microscopy. Dormant spores of the wild-type, 1604 (a) and mutant, 4750 (c) and germinated spores (incubated in 1 mM-L-alanine for 30 min at 37 °C) of the wild-type (b) and mutant (d) were mounted in 0.5% agar and photographed through a Leitz phase contrast microscope. The bar marker represents 10 µm.

PBS1 crosses used to transduce the Aro markers into strain 1506 or 1604). The presence of such TZM markers in strains with differing origins may well reflect some selective pressures for germination deficiencies during the storage and recovery of laboratory stocks.

Germination properties of mutant 4750 (gerJ50)

For each property, experiments were carried out on at least three separately prepared suspensions of both mutant and wild-type spores.

Loss of absorbance. After 30 min in the presence of L-alanine, or a mixture of glucose, fructose, asparagine and KCl, or PAB, the wild-type spore suspension lost 60% of its absorbance and the spores became phase-dark, whereas spores of the mutant lost 35% of their absorbance and the majority reached only a phase-grey stage (Fig. 1, 2a). About 10% of the mutant spores did become phase-dark after 30 min and in PAB could outgrow and undergo vegetative cell division; those that reached only the phase-grey stage did not outgrow even after 6 h in PAB. The recovery from spores of colonies on NA, however, was normal.

Loss of dipicolinic acid and resistance. Spores of the wild-type and the mutant contained similar amounts of DPA [6.6 ± 0.6 (S.D.) and $6.9 \pm 0.5\%$ of dry weight, respectively] and they released their DPA during germination in 1 mM-L-alanine in an identical manner (Fig. 2a); the loss of resistance to heat, octanol, chloroform and toluene showed a similar pattern. Thus, although spores of the mutant only reached a phase-grey stage, their loss of DPA and changes in resistances were similar to those of wild-type spores.

Requirement of spores for germination in L-alanine. Spores of the wild-type are able to germinate in a buffered solution of L-alanine (Moir *et al.*, 1979). As the concentration of L-alanine is increased the rate of germination (defined as the rate of loss of A_{580}) also increases up to a maximum. The concentration of L-alanine at which the rate of germination is half that of the maximum value (C_{50}) can thus be used as an estimate of the affinity of the spores for the germinant (Sammons *et al.*, 1981). The C_{50} value for the mutant ($3.5 \pm 0.7 \times 10^{-5}$ M) was not significantly different from that of the wild-type ($4.3 \pm 0.6 \times 10^{-5}$ M).

Release of hexosamine-containing fragments. Wild-type spores release these fragments at a similar rate to their loss in absorbance during germination. This reaction was complete after 30 min with approximately 35% of the total spore hexosamine ($3.3 \pm 0.2\%$ of dry weight)

Fig. 2. Loss of absorbance (A_{580}) and release of dipicolinic acid (DPA) and hexosamine-containing fragments during germination of spores in L-alanine. (a) The fall in A_{580} of a suspension of heat-activated spores in L-alanine was followed with time. At intervals 3 ml samples were taken and, after filtering, the DPA content of the filtrate was measured. The loss of A_{580} and release of DPA for wild-type spores at 60 min was taken as 100% completion of that event (at this time 60% of the initial A_{580} had been lost and 100% of the initial DPA content had been released into the supernatant). Release of DPA: ○, mutant (4750); ●, wild-type (1604). Loss of A_{580} : □, mutant; ■, wild-type.

(b) Heat-activated spores were suspended to an A_{580} of 40; at intervals 1 ml samples were taken and centrifuged for 2 min at 12 000 g. The hexosamine contents of both the pellets and supernatants were determined, and the A_{580} of a 1 in 30 dilution of each sample was measured. Percentage of the total spore hexosamine released into the supernatant: ○, mutant (4750); ●, wild-type (1604). A_{580} : □, mutant; ■, wild-type.

having been released. Spores of the mutant also released such fragments but both the rate and the total amount released (15%) were lower (Fig. 2b) although the total hexosamine content ($2.6 \pm 0.9\%$ of dry weight) was similar to that of wild-type spores. The kinetics of release of hexosamine-containing material and of the fall in absorbance, as seen from the shapes of the curves in Fig. 2(b), were similar to those for wild-type spores. This would argue against a slower lytic response in the mutant since the loss of fragments from both the mutant and wild-type stops at the same time.

Resistance levels of spores

The viable counts of spore suspensions of the wild-type and mutant were both unaffected by octanol (recoveries were 100 ± 28 and $90 \pm 9\%$ of the untreated control, respectively) while both were somewhat lower after toluene treatment (64 ± 18 and $60 \pm 10\%$, respectively). The chloroform resistance of both wild-type and mutant spores was low (17 ± 4 and $38 \pm 6\%$, respectively); this may reflect one effect of the sporulation medium on spore properties (normal resistance levels of 60–80% were observed for both wild-type and mutant spores prepared by the resuspension method of Sterlini & Mandelstam, 1969). Mutant spores were not sensitive to heating at 70 °C for 30 min, but unlike wild-type spores ($98 \pm 31\%$ recovery) the number of colony-forming units increased after this heating procedure ($198 \pm 20\%$). This might reflect a requirement of some mutant spores for heat activation before germination can occur, which is not possessed by wild-type spores. However, there was no evidence of such an effect when spore suspensions that had been heated for 0–60 min at 70 °C were germinated in L-alanine and the fall in absorbance was followed. Heating at 90 °C (Fig. 3) revealed that the mutant spores were considerably more sensitive than wild-type spores, with a D_{90} of about 8 min compared with 40 min for the wild-type.

We found no evidence of any sensitivity to lysozyme ($200 \mu\text{g ml}^{-1}$) even after 1 h at 37 °C for both the wild-type and strain 4750. Spore suspensions of the lysozyme-sensitive strain 4600 treated in a similar manner showed a sharp drop in A_{580} and complete lysis of the spores.

Fig. 3. Heat sensitivity of spores at 90 °C. The number of colony-forming units at each sampling time was expressed as a percentage of the colony-forming units at time 0. D_{90} was calculated as the time taken for the number of colony-forming units to fall to 10% of the initial value. O, mutant (4750); ●, wild-type (1604).

Morphological studies

No difference was detected between wild-type and mutant spores either in thin sections of KMnO_4 - or glutaraldehyde/osmium tetroxide-fixed preparations or in the freeze-fracture profiles of spores (Fig. 4 *a-g*). The volumes of coat, cortex and core were similar; the average number of coat layers was the same and the distance between surface striations observed in freeze-fracture preparations was equal. However, in sections of spores germinated in 1 mM-L-alanine for 30 min the cores of wild-type spores filled the cortical space whilst those of mutant spores had not swollen to such an extent (Fig. 4 *c, d*), as has been observed for other phase-grey spores (Santo & Doi, 1974). A few (4–5%) spores in germinated preparations of the wild-type had a similar morphology to germinated spores of the mutant (Fig. 4*e*), showing that the wild-type spores passed through the morphological stage reached by the mutant spores. The observation that approximately 10% of the mutant spores do become phase-dark was paralleled by a similar number of the sectioned spores having fully swollen cores. It is interesting to note the difference between the freeze fractures of wild-type spores prepared on PGYEA (Fig. 4*f*) and those prepared in the resuspension medium of Sterlini & Mandelstam (1969), the latter having a knobbled appearance compared with the smooth lines of the former (Fig. 4*h*). This feature was also seen in freeze fractures of mutant spores and represents an effect that the medium can have on spore structure (Waites *et al.*, 1979).

DISCUSSION

We have isolated a spore germination mutant, 4750, containing the mutation *gerJ50* that is unlike those previously described (Moir *et al.*, 1979). Spores of this mutant and of the wild-type both respond to all the germinants that have been tested; however, those of the mutant only reach a phase-grey stage, whilst wild-type spores pass through this stage and become phase-dark. This suggests that the trigger reaction (O'Connor & Halvorson, 1959) in these spores is normal, and this is further supported by the similar L-alanine concentration required for germination. When germination was measured by following the loss of resistance properties, or the release of DPA, we found that the mutant spores had an identical phenotype to those of the wild-type, showing that they are not only triggered but also undergo the first few reactions of the germination process normally. These observations correspond well to those made by Hashimoto *et al.* (1969 *a, b*) in their work on inhibitors and single spore germination in *Bacillus cereus*. They found that one phase, including the partial hydration of the core, the partial loss of absorbance and the release of DPA, took place in the presence of inhibitors, whilst the remaining reactions did not. Our mutant seems to carry out the processes involved in the first phase, but not in the later one.

Fig. 4. Electron micrographs of dormant and germinated spores. Suspensions of dormant spores of the wild-type, 1604 (a) and mutant, 4750 (b) and of germinated spores (30 min at 37 °C in 1 mM-L-alanine) of the wild-type (c, e) and mutant (d) were fixed in 1% KMnO_4 before embedding and cutting sections. Freeze fractures were made at -110°C on dormant spore suspensions of the wild-type (f) and mutant (g), and then coated with Pt/C. A freeze fracture was also made of a spore suspension of the wild-type (h) prepared in the resuspension medium of Sterlini & Mandelstam (1969) at 26 °C. The bar markers represent 0.1 μm .

Mutants of *B. cereus* with similar germination characteristics to mutant 4750 have been isolated (Aronson & Fitz-James, 1975; Cheng & Aronson, 1977; Cheng *et al.*, 1978; Stelma *et al.*, 1980), but all of these have been shown to be lysozyme-sensitive and/or to possess a defective spore coat. From the electron microscopy and the resistance properties this does not seem to be true for *gerJ50* spores, but further studies, especially of the coat proteins, are

needed. Some of the mutants of *B. cereus* also lack an intracellular protease activity (Cheng *et al.*, 1978) and this is accompanied by both a coat and germination deficiency. Again, this is not so for strains containing *gerJ50* for they have normal intracellular protease activities (R. J. Warburg, unpublished results). The germination mutant 4600 (containing *gerE36*) of *B. subtilis* (Moir, 1981) forms coat-defective spores but has a similar germination phenotype to strain 4750 as it reaches only a phase-grey stage. However, the map location of *gerE36* is different (99% cotransduced with *citF* by phage PBS1). In addition, spores of strain 4600 do not lose all of their DPA and behave in a less synchronous manner; only 50% of the spores become phase-grey after 30 min in L-alanine compared with 95% for *gerJ50*. From this it may be concluded that strain 4600 is probably blocked at a different stage of germination than 4750. *GerE36* is clearly pleiotropic, and this could also be so for *gerJ50* since the sensitivity of its spores to heating at 90 °C may well be due to a structural defect unassociated with resistance to the chemicals tested or to heating at 70 °C and undetectable by electron microscopy.

The slow and incomplete release of hexosamine-containing fragments during germination of the mutant spores may point to some form of a lytic enzyme deficiency. It may, on the other hand, indicate that fewer targets or sites in the spore peptidoglycan are susceptible to attack by a particular lytic enzyme, resulting in less release during germination. However, the hexosamine content of both wild-type and mutant spores is similar, and therefore any deficiency would have to be not in the total hexosamine but in its composition, probably in the structure or proportion of the cortical peptidoglycan. This would explain the observed heat (90 °C) sensitivity of the spores, because the cortical peptidoglycan may well be disrupted and so its function of keeping the core under pressure (Dring & Gould, 1975) could be impaired.

Whatever the cause of the deficiency in spores of strain 4750 it is a very useful mutant for the study of germination since it permits division of the process into three stages, i.e. the trigger response, the subsequent events that take place in both the mutant and wild-type and those that occur only in the wild-type.

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