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Germination Properties of a Spore Coat-Defective Mutant of *Bacillus subtilis*

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The presence of the *gerE36* mutation in strains of *Bacillus subtilis* 168 resulted in poor germination of their spores in a range of germinants, as measured by the fall in absorbance of spore suspensions. Although resistant to heat and organic solvents, spores were sensitive to lysozyme; electron microscopy revealed that their coat structure was incomplete. These spores responded to germinants by losing heat resistance and changing from phase bright to phase gray. The release of dipicolinic acid and the fall in absorbance of spore suspensions reached only 75 and 50% of wild-type levels, respectively, but followed the same time course as the loss of heat resistance. Although the germination response was incomplete, the concentration of L-alanine required to elicit it was the same for the mutant as for the wild type. The properties of mutant spores suggest that an intact spore coat is not required for the initial interaction between germinant and spore, but that the coat layers may contain molecules important in later stages of germination. In transduction with phage SPP1, the *gerE36* mutation mapped between *citF* and *ilvB* and was 90% cotransduced with *citF2*. The *gerE* mutation identifies the location of a gene important for the progress of late stages of spore formation.

The complex differentiation cycle between vegetative cell and endospore is characteristic of a variety of species of bacteria (25) but has been studied most extensively in bacilli and clostridia. The change of the spore from the dormant and resistant state to a metabolically active form which is sensitive to heat and to organic chemicals can be stimulated by a variety of small molecules; one of the most widely used and effective is L-alanine. The changes in the spore, collectively termed "germination," include degradation of the cortex, alteration of coat layers, excretion of small molecules, ion fluxes, and rehydration and swelling of the spore core. They are accompanied by the loss of refractility and phase brightness of individual spores and a fall in the absorbance of spore suspensions (10). The rapidity of germination and its insensitivity to inhibitors of RNA and protein synthesis suggest that no macromolecular synthesis is required (16). Little is understood of the nature or control of the underlying reactions.

The stages of the process are hard to distinguish, since the measurable changes occur very rapidly in individual spores, whereas the response of the population to the germinant is asynchronous. It is possible to block germination before any irreversible changes have occurred by the addition of inhibitors, such as D-alanine (13) or methyl anthranilate (21), or by mutation (19, 31). Spore germination has been blocked after this stage but before its completion in *Bacillus megaterium* by the action of mercuric chloride (18, 22) or in *Bacillus cereus* by heat damage, high Ca^{2+} concentration (12), or mutation (6). This paper describes the effects of a mutation in *Bacillus subtilis* which, in addition to altering sporulation properties and spore structure, prevents the completion of germination. The isolation of a strain carrying this *gerE36* mutation and its approximate map location have already been described (19).

MATERIALS AND METHODS

Bacterial strains. Strains of *B. subtilis* 168 used in this study are listed in Table 1.

Media. Penassay broth (antibiotic medium no. 3, Difco Laboratories) was used for routine growth of bacteria. The growth and resuspension media of Sterlini and Mandelstam (29) as modified by Dworkin et al. (8) were used to obtain synchronous sporulation. Solid media included potato-glucose-yeast extract agar (7), tryptose-blood agar base (Difco) solidified with 1.5% (wt/vol) Difco agar, and TY agar (3). MA lactate was minimal salts (1) supplemented with sodium DL-lactate (5 mg/ml) and required amino acids (50 μg/ml) and solidified with 1.5% (wt/vol) Difco agar.

Preparation of spores. Incubation, harvesting, and washing of spores from potato-glucose-yeast extract agar were as previously described (17). For spore preparation by the method of Sterlini and Mandelstam

TABLE 1. *Strains of B. subtilis 168*

Strain	Genotype	Origin or construction
4822	*citF83 leu-2*	B. Rutberg (20)
4823	*citF2 leu-2*	B. Rutberg (20)
JH402	*citF2 trpC2*	J. Hoch (20)
JH326	*citF78 leu-2*	J. Hoch (20)
CU229	*trpC2 ilvB2*	S. Zahler (32)
CU371	*trpC2 ΔilvB1*[a]	S. Zahler (32)
BD40	*pheA12 argA3*	D. Dubnau
1604	*trpC2*	This laboratory (19)
4600	*gerE36 trpC2 his*	UV mutagenesis of 1604 (19)
4670	*ger*$^+$ *pheA*$^+$ *argA*$^+$	4600 → BD40[b]
4651	*gerE36 pheA12*	4600 → BD40[b]
4673	*gerE36*	4600 → BD40[b]
4735	*gerE36 ΔilvB1*	CU371 → 4651[b]
4736	*gerE36 ilvB2*	CU229 → 4651[b]
4751	*gerE36 leu-2*	4736 → JH326[c]
4825	*gerE*$^+$ *leu-2*	4736 → JH326[c]

[a] *ΔilvB1* is a deletion which covers all known points in *ilvB* but does not enter *ilvC*. It probably also covers all point mutations in *azlA* (S. Zahler, personal communication).

[b] Transduction by PBS1. Where strains were constructed by transduction crosses, the arrows point to the recipient strain.

[c] Transduction by SPP1.

(29), a culture was grown by inoculating a fresh single colony into 200 ml of growth medium and incubating at 25°C for 15 to 18 h to an absorbance at 580 nm (A_{580}) of 0.6 to 0.8. Bacteria were harvested by centrifugation at room temperature, suspended to an A_{580} of 0.6 in 200 ml of prewarmed resuspension medium, and incubated at 25°C until spore formation and release occurred after 24 to 28 h. Spores were harvested by centrifugation at 3,000 × *g* for 20 min at 4°C and suspended in 100 ml of sterile distilled water. After 10 cycles of washing in 100 ml of sterile distilled water, spores were stored at 4°C in sterile distilled water at an A_{580} of 10. To monitor spore formation at 42°C, an overnight culture in growth medium at 25°C was diluted to an A_{580} of 0.15, grown to an A_{580} of 0.6 at 42°C, harvested at room temperature, and suspended to an A_{580} of 0.6 in resuspension medium at 42°C. Samples were removed at intervals for microscopy, to determine the proportion of heat- or toluene-resistant organisms, or to measure dipicolinic acid (DPA).

Measurement of germination. Spores were diluted to an A_{580} of approximately 1.0 in 10 mM Tris-hydrochloride (pH adjusted to 8.4 at 20°C) and equilibrated at 37°C. L-Alanine (to 10 mM) and KCl (to 1 mg/ml) were added to initiate germination, and the A_{580} was measured at intervals. Portions (0.05 ml) were removed for measurement of heat resistance, and 2.5-ml quantities were passed through a membrane filter (Millipore; 0.45-μm pore size) before assay of DPA in the supernatant. To measure the dependence of germination rate on L-alanine concentration, 3 ml of spore suspension in 10 mM Tris-hydrochloride, pH 8.4 (A_{580} = 0.4), was incubated at 37°C in a Pye Unicam SP1800 spectrophotometer, and the fall in absorbance after addition of L-alanine was measured on a Pye Unicam AR25 linear recorder.

Determination of heat resistance during sporulation and germination. Samples (0.1 ml) were removed from cultures, diluted 50-fold into sterile distilled water at 80°C, and incubated for 10 min. After cooling on ice, appropriate dilutions were made in sterile distilled water, and amounts were spread on tryptose-blood agar base agar.

Determination of toluene resistance. Samples (0.1 ml) were removed from cultures and diluted into 4.9 ml of sterile distilled water at room temperature, 0.5 ml of toluene was added, and the tubes were shaken for 30 s and then left to stand for 15 min. Portions were then removed, diluted in sterile distilled water, and spread on tryptose-blood agar base agar.

Determination of DPA. A 20-ml amount of a sporulating culture was centrifuged at 4°C for 15 min at 2,000 × *g*, and the pellet was suspended in 1.25 ml of distilled water and autoclaved for 15 min at 15 lb/in^2. A 1.0-ml amount was removed for measurement of DPA by the method of Jannsen et al. (14). This method was used for determining the DPA contents of sporulating cells and of washed spores. For measurement of DPA released from spores during germination, the assay procedure of Scott and Ellar (24) was used.

Measurement of the resistance of spores to heating at 90°C. A 0.1-ml amount of washed spores was diluted into 4.9 ml of sterile distilled water at 90°C to an A_{580} of 0.3, and 0.1-ml portions were removed at intervals and diluted into 4.9 ml of ice-cold sterile distilled water. Appropriate dilutions were carried out in sterile distilled water, and samples were spread on tryptose-blood agar base agar.

Light microscopy. Light microscopy under phase-contrast optics was performed as previously described (17).

Electron microscopy. Electron microscopy was carried out by Unilever Research, Colworth House, Sharnbrook, United Kingdom. Spores were fixed and stained in 2% $KMnO_4$ at 37°C for 90 min and then washed extensively in distilled water. After suspension in 1% agar, they were dehydrated in an alcohol series and embedded in Spurr resin (28). Sections were examined in a JEOL JEM 100C microscope at 80 kV.

Genetic studies. PBS1 transduction was as previously described (19). SPP1 phage (obtained from U. Canosi) was propagated on *B. subtilis* strains, and transduction was carried out as described by Ferrari et al. (9), except that vegetative bacteria rather than spores were used in the preparation of lysates. Cit$^+$ transductants were selected on MA lactate, and a tetrazolium overlay technique (17) was used to test the germination phenotype of transductants.

RESULTS

This paper describes the characterization of strains carrying the *gerE36* mutation. Since the original isolate, strain 4600, was derived from a mutagenized culture, detailed studies were carried out using derivatives in which the *gerE36* mutation had been transduced into an unmuta-

genized background, comparing these with derivatives from the same cross in which the *ger*$^+$ allele had been introduced. These strains included 4673 (*gerE36*) and 4670 (*ger*$^+$), constructed by PBS1 transduction, and 4751 (*gerE36 leu-2*) and 4825 (*ger*$^+$ *leu-2*), constructed by SPP1 transduction (Table 1). Initial characterization was performed with PBS1 transductants; in these, up to 5 to 8% of the chromosome may have been transduced. The recombinants from SPP1 crosses, in which a smaller segment (up to 1% of the chromosome) can be transduced, are likely to contain a smaller proportion of the original mutagenized chromosome and thus should define rather more precisely the effect of the *gerE36* mutation. The comparison between mutant and wild type was therefore repeated and extended by using strains 4751 and 4825.

Properties of spores of strains carrying *gerE36*. (i) Spore formation. The preliminary observation distinguishing strain 4600 and recombinants carrying the *gerE36* mutation was that their spores produced on potato-glucose-yeast extract agar appeared smaller than those of other germination mutants or of wild type (19). Spores of *ger*$^+$ and *gerE36* transductants, both PBS1 and SPP1, were produced on potato-glucose-yeast extract agar at 26, 37, and 42°C. Sporangia of *gerE36* strains formed at 42°C contained small, phase-gray spores which were not released from the mother cell. During sporulation at lower temperatures the spores became fully phase bright, but were still small in size, and many remained within their partially lysed sporangia. This behavior was noted in all *gerE* PBS1 and SPP1 transductants tested (12 of each type). Continued incubation did not lead to further development of the mutant spores.

Synchronous sporulation induced by the replacement method of Sterlini and Mandelstam (29) at 25°C resulted in the formation of spores at a high frequency for both PBS1 and SPP1 transductants. Phase-bright prespores of both wild-type and mutant strains appeared 12 to 14 h after suspension, and by 24 to 28 h the yield of phase-bright mature spores from both cultures was high. The spores of *gerE36* transductants were released from lysing sporangia at a slower rate than were those of *ger*$^+$ and still appeared slightly smaller. Sporulation of the *gerE36* PBS1 transductant 4673, but not of the *ger*$^+$ PBS1 transductant 4670, was temperature sensitive; at 42°C, the proportion of cells in the total population reaching the later stages in sporulation was progressively lower; although 35% of the cells developed forespores which were partially phase bright, 7% became toluene resistant, and only 1% became heat resistant. This was not the case for the *gerE36* SPP1 transductant, strain 4751, however; it completed sporulation at 42°C, producing phase-bright spores which were octanol, toluene, and heat resistant, but lysozyme sensitive, and which were smaller than wild type. It is clear that the presence of the *gerE36* mutation does not of necessity result in a temperature-sensitive block in sporulation by the Sterlini and Mandelstam method, since this behavior is not manifested by SPP1 recombinants carrying *gerE36*. Whether the difference in sporulation at 42°C between *gerE36* strains 4673 and 4751 is due to a second mutation in the original strain, close enough to be transduced by PBS1 but not by SPP1 phage, or whether it is the result of differences in the genetic background affecting the expression of the *gerE36* mutation, has not been explored.

Since sporulation by the resuspension method at 25°C yielded homogeneous preparations of spores of both wild type and mutant, these conditions were used to generate spores for germination studies.

(ii) Electron microscopy. When sections of spores were prepared and examined, a striking difference between wild-type and mutant coat structure was observed. Figure 1 demonstrates the morphology of spores of PBS1 transductants 4670 and 4673 prepared at 25°C by the resuspension method. The coat structure of wild type (strain 4670) comprised a close-packed multilamellar inner coat and a thick, electron-dense outer coat in which individual layers were hard to discern; a layer at the outer edge of the coat, which has been compared to an exosporium (27), was visible in some sections. The coat of the mutant, however, was incomplete, comprising at most three concentric layers of more diffusely organized material. The cortex of mutant spores sometimes appeared to have an extra outer, less electron-dense layer, but since this was most marked where the coat layers were damaged, it was probably an artifact of the preparation procedures, resulting from a less firm adhesion of coat to cortex in the mutant under the conditions used. Electron micrographs of spores of the SPP1 transductants (R. Warburg and A. Moir, unpublished data) showed that strain 4825 (*ger*$^+$) possessed a coat structure similar to strain 4670, whereas the *gerE36* strain 4751 contained only small amounts of coat material arranged diffusely, giving an appearance identical to that of spores of strain 4673. The most dramatic effect of the *gerE36* mutation on spore size was seen in spores prepared at 37 or 42°C on potato-glucose-yeast extract agar (Fig. 2). The overall dimensions of the spores decreased;

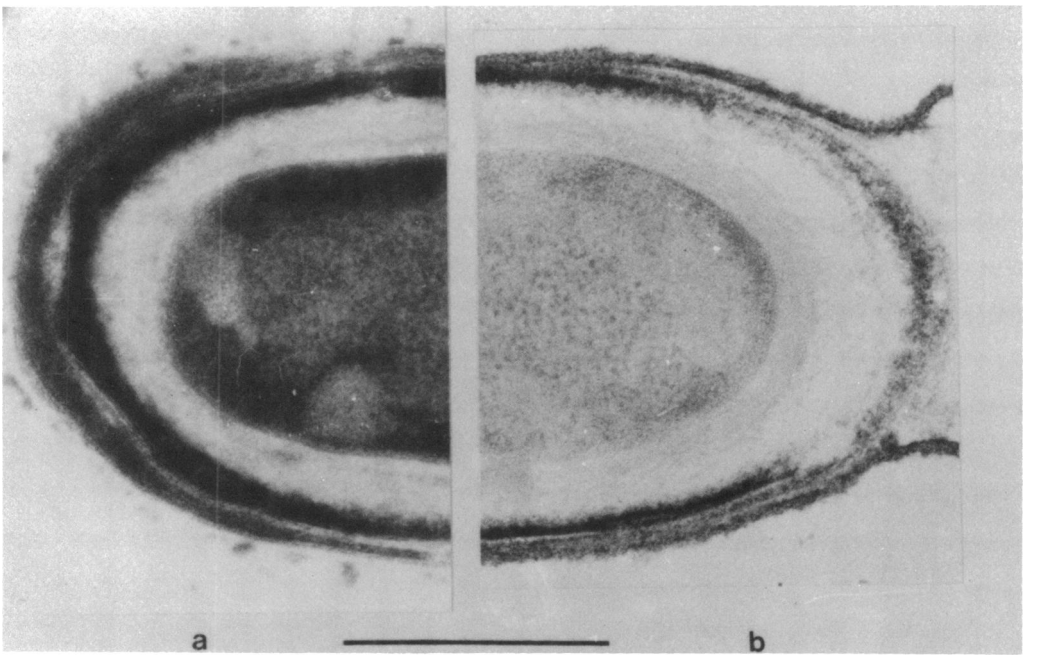

FIG. 1. *Sections through permanganate-stained spores of (a) strain 4670 (ger$^+$) and (b) strain 4673 (gerE36) produced by the resuspension method (29) at 25°C. Bar, 0.5 μm.*

spores possessed smaller cores and very little coat material. The reason for this pleiotropic effect on the size of the developing forespore is not obvious, but deserves consideration in the interpretation of the possible defect caused by the *gerE36* mutation.

(iii) Resistance properties. Spores of *ger*$^+$ and *gerE* transductants prepared by the resuspension method at 25°C showed similar resistance to heating at 90°C (Fig. 3; measured only on PBS1 transductants). All other resistance properties described in this section were measured using both PBS1 and SPP1 transductants. The results for the two "wild-type" strains corresponded, and where differences between wild-type and mutant strains were obtained, these were observed in both PBS1 and SPP1 transductants. *ger*$^+$ and *gerE36* spores were fully resistant to toluene and to octanol, and their DPA contents, expressed as a proportion of the total dry weight, were also similar (50 to 60 μg/ml). Unlike wild type, however, spores of strains containing the *gerE36* mutation were very sensitive to lysozyme. Even after several hours of incubation in the presence of lysozyme at 100 μg/ml, wild-type spores showed a negligible change either in the A_{580} of suspensions or in their appearance under phase-contrast microscopy. In contrast, *gerE36*-containing spore suspensions lost absorbance very quickly, even at a low concentration of lysozyme (Fig. 4). The fall in A_{580} reflected phase darkening and lysis of the spores; when the suspension was osmotically stabilized with 0.3 M sucrose, protoplasts were formed. The extreme sensitivity to lysozyme suggests that the spore integuments of strains carrying *gerE36* are much more permeable to enzymes, allowing lysozyme access to the peptidoglycan of the cortex and germ cell wall. This is consistent with the observation of altered coat layers in thin sections of the mutant spores (Fig. 1).

(iv) Germination of mutant spores. On incubation with L-alanine, with a mixture of glucose, fructose, L-asparagine, and KCl or with Penassay broth, spore suspensions of strains carrying the *gerE36* mutation showed a slow fall in A_{580}; the extent of the fall in 90 min varied from 15 to 30% of the original absorbance, depending on the age of the spores and the genetic background of the strain. The experiments described analyzing germination in more detail were carried out using SPP1 transductants 4751 (*gerE36*) and 4825 (*ger*$^+$). All except the measurement of DPA release have also been performed with PBS1 transductants 4673 (*gerE36*) and 4670 (*ger*$^+$). Analogous results showing the same defect in both strains carrying *gerE36* were obtained.

For spore suspensions of strain 4825, germi-

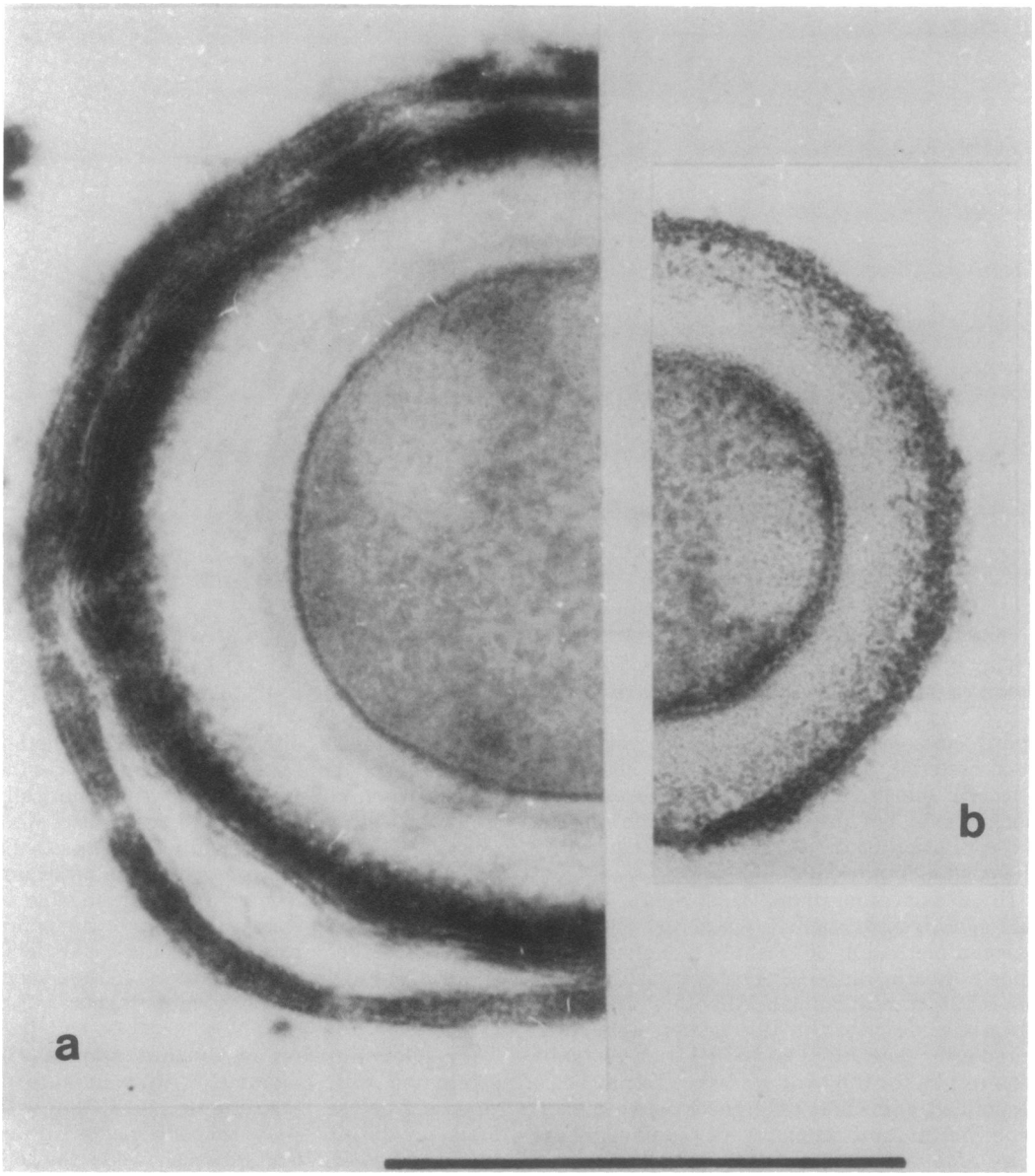

FIG. 2. *Sections through permanganate-stained spores of (a) strain 4670 (ger$^+$) and (b) strain 4673 (gerE36) produced on potato-glucose-yeast extract agar at 37°C. Bar, 0.5 μm.*

nation results in a loss of 60% of the original A_{580}, the loss of heat resistance in 95 to 100% of the population, and the release of all the DPA contained in the spores (Fig. 5a). However, the strain 4751 spores responded in a different manner. Although the absorbance loss was only about one-half that of the wild type, 90% of the spores lost heat resistance (Fig. 5b). The release of DPA from the spores was incomplete, reaching only 70% after 90 min. At this time the spores were gray under phase-contrast microscopy, and only about 30% were stainable with methylene blue. Thus the germination response of *gerE36* strains was blocked at an intermediate stage. Although germination-like changes commenced in response to the germinant, not all the germination-associated events were completed.

When germination was expressed as a proportion of the total change in any of the parameters in 90 min (Fig. 6), the sets of data for loss of absorbance, heat resistance, and DPA became virtually superimposable. Therefore it was pos-

sible to use any of them to describe the germination response. The asynchrony of germination was such that it would be difficult to establish from these data the temporal sequence of the three events. Since the loss of A_{580} was the easiest to monitor at frequent intervals, this parameter was used to characterize further the residual germination response of the mutant. The dependence of the germination rate of strain

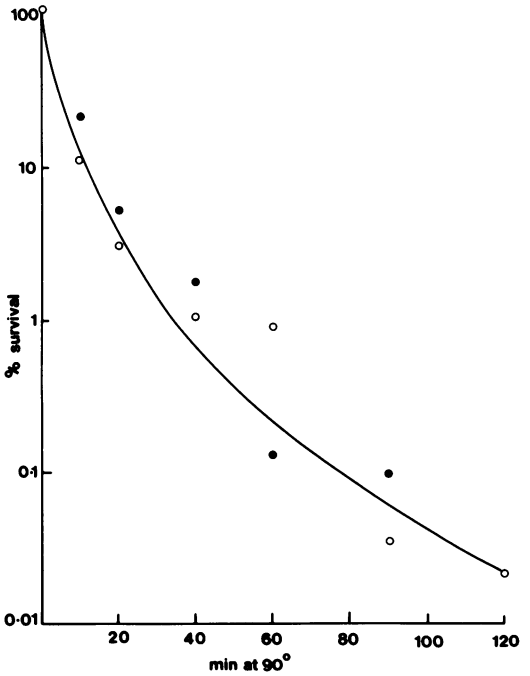

FIG. 3. *Heat resistance of spores at 90°C. The viable count after incubation at 90°C was expressed as a proportion of that of the unheated control.* ●, *Strain 4670 (ger⁺);* ○, *strain 4673 (gerE36).*

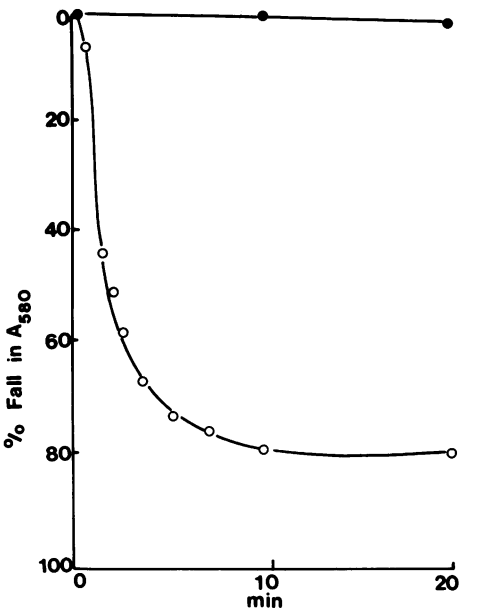

FIG. 4. *Effect of lysozyme on the absorbance of spore suspensions. Spores of* (●) *strain 4670 (ger⁺) and* (○) *strain 4673 (gerE36) were suspended at* A_{580} *= 0.3 in 0.85% saline. Hen egg white lysozyme was added to 6.7 μg/ml, and the* A_{580} *was measured at intervals up to 20 min.*

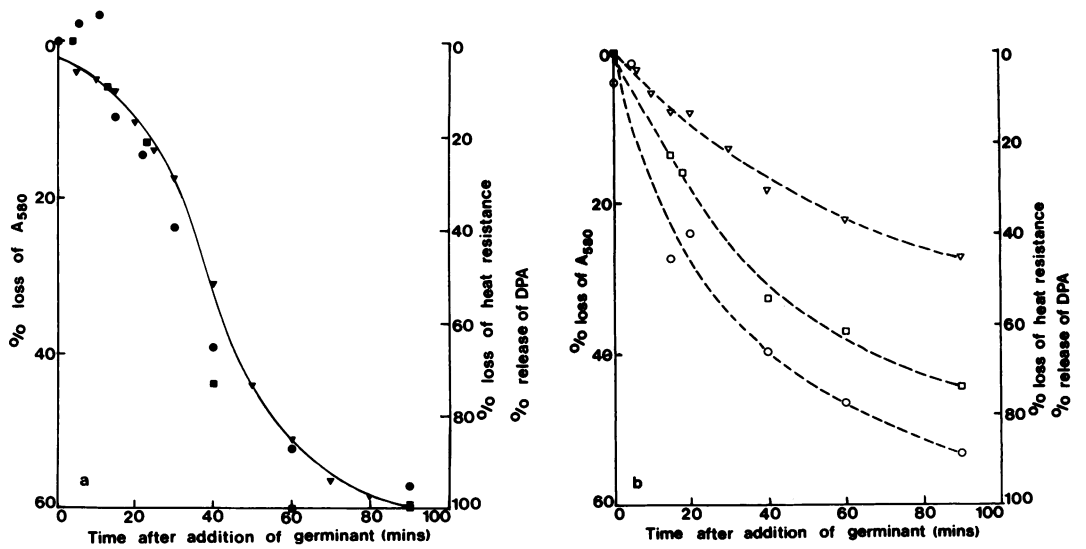

FIG. 5. *Changes in spore properties on germination. Spores of (a) strain 4825 (ger⁺) and (b) strain 4751 (gerE36) were germinated in 10 mM* L*-alanine with 1 mg of KCl per ml.* ▼ *and* ▽, *Loss of* A_{580}; ● *and* ○ *loss of heat resistance;* ■ *and* □, *release of DPA.*

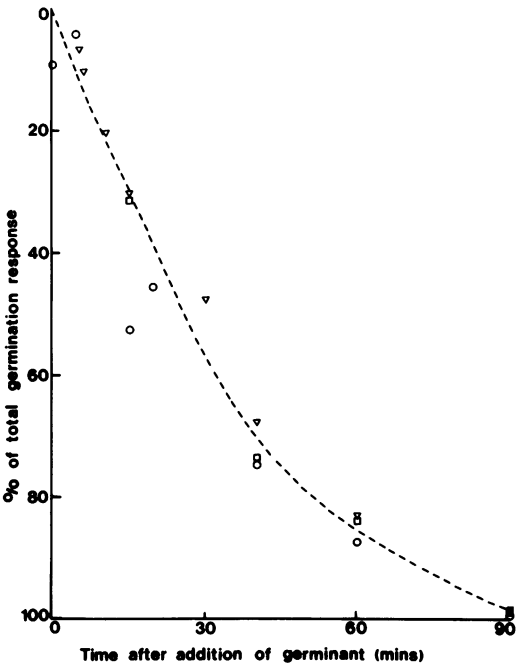

FIG. 6. *Changes in spore properties of strain 4751 (gerE36) on germination expressed as a proportion of the total change in each parameter in 90 min.* ▽, *Loss of* A_{580}; ○ *loss of heat resistance;* □, *release of DPA.*

4751 and 4825 spore suspensions on the concentration of L-alanine was measured (Fig. 7). The concentration required to achieve half of the maximum germination rate (23a, 28) was estimated as 37 μM and 35 μM for wild type and mutant, respectively. Thus the requirement for L-alanine was unchanged in the mutant. Although the subsequent stages of germination of the mutant spores are incomplete, there is no evidence of any alteration in the initial interaction between germinant and spore.

In fact, the response of mutant spores to germinant reveals a rather shorter average microlag, expressed as the length of the period between exposure to germinant and detection of the response, compared to wild type (compare, for example, the loss of absorbance in 30 min in Fig. 7a and b). This is particularly prominent at limiting concentrations of germinant. It is tempting to speculate that this may be directly correlated with the increased permeability of spore coat layers. However, more trivial explanations, such as that molecules leaching from the increased amount of cell debris even in well-washed spore preparations of *gerE36* may alter the microlag, are possible.

The residual germination response of mutant spores is still sensitive to inhibitors of early stages in germination. Inhibition by D-alanine is effective at the same concentrations of D- and L-isomers in the mutant as in wild type, and the partial losses of absorbance and phase brightness of mutant spores in L-alanine are inhibited by methyl anthranilate (data not shown).

Heat activation of *B. subtilis* 168 *ger*$^+$ strains increased the rate of response of the spores to germinant, but had no effect on the total extent of absorbance loss and did not markedly (two-fold or less; this change borders on statistical significance) change the concentration of germinant required to induce germination (R. Sammons, Ph.D. thesis, University of Birmingham, Birmingham, United Kingdom, 1979). The experiments described above were performed without heat activation; heat activation of the spores in distilled water at 80°C for 10 min before use slightly increased the rate of fall of absorbance for both wild type and mutant, but did not affect the extent of absorbance loss in either case.

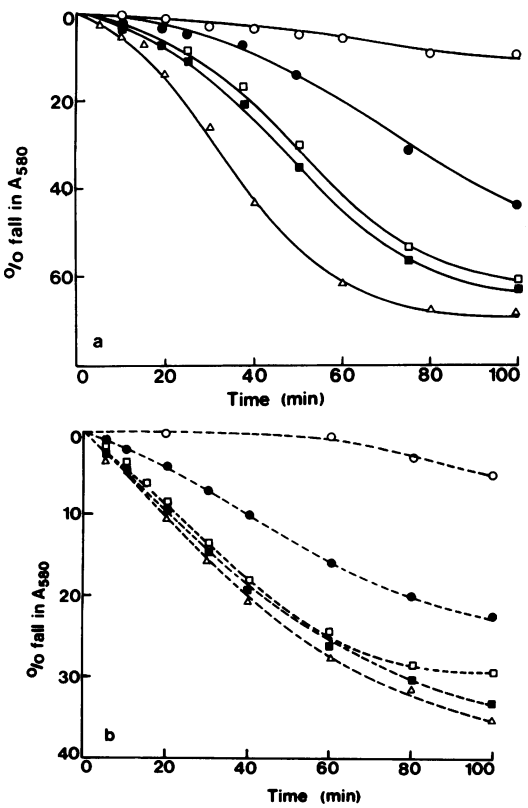

FIG. 7. *Effect of* L-*alanine concentration on germination of (a) strain 4825 (ger*$^+$*) and (b) strain 4751 (gerE36). Concentrations of* L-*alanine were* (○) *10,* (●) *25,* (○) *50, and* (■) *75,* μM *and* (△) *10 mM.*

Mapping the *gerE36* mutation. In phage PBS1 transduction, *gerE36* was closely linked to *citF* and *leuA* genes and was tentively located on the side of *citF* distal to the *leu* cluster (19), but the high frequency of cotransduction of *gerE36* with *citF* and *leuA* made accurate location difficult. Phage SPP1, which transduces fragments equivalent to approximately 1% of the *B. subtilis* chromosome (34), was used to improve the mapping of the region aroung *gerE36*.

The *citF* locus is a complex one, almost certainly comprising more than one gene (20), and *citF* mutants, although all deficient in succinate dehydrogenase activity, comprise several distinct phenotypic groups (23). The mutations have been ordered (20), the extremes being *citF78* farthest from and *citF2* and *citF83* closest to the *ilv-leu* gene cluster. SPP1 transduction between strains carrying *citF*, *gerE36*, *ilv*, and *leu* mutations (Table 1) were carried out. The results of three- and four-factor crosses (Table 2) show that the *gerE36* mutation mapped between *citF* and the *ilv* or *leu* mutations whether *citF2*, *citF83*, and *citF78* mutants were used as recipients. The *gerE36* mutation was approximately 90% linked to *citF2* and to *citF83*, but was considerably further away from *citF78*. All the data obtained in SPP1 transduction were consistent with the order *citF78*-(*citF2*, *83*)-*gerE36*-(Δ*ilvB1*, *ilvB2*)-*leu-2*. These data seem quite conclusive, but conflict with the previously published order from PBS1 transduction (19). The earlier PBS1 data were obtained using PBS1 phage, previously grown on strain 1604, propagated on the appropriate donor in a single cycle of lytic infection. Phage particles transducting strain 1604 (*gerE*$^+$ *trpC2*) DNA could have been carried over from a previous cycle of growth to provide a low but significant proportion of the recombinants and would have formed part or the whole of a critical recombinant class. This emphasizes the importance of growing phages for several cycles on the donor strain. In the SPP1 transduction, this was achieved by

TABLE 2. *Mapping of gerE36 by SPP1 transduction crosses*[a]

Recipient	Donor	Recombinant class	No.	Order suggested
citF2 leu-2	*gerE36*	Ger$^+$ Leu$^+$	1	*citF2-gerE36-leu-2*
		Ger$^+$ Leu$^-$	15	
		Ger$^-$ Leu$^+$	24	
		Ger$^-$ Leu$^-$	60	
citF2 trpC2	*gerE36 ilvB2*	Ger$^+$ Ilv$^+$	12[b]	*citF2-gerE36-ilvB2*
		Ger$^+$ Ilv$^-$	2	
		Ger$^-$ Ilv$^+$	81	
		Ger$^-$ Ilv$^-$	50	
citF83 leu-2	*gerE36 his trpC2*	Ger$^+$ Leu$^+$	0	*citF83-gerE36-leu-2*
		Ger$^+$ Leu$^-$	11	
		Ger$^-$ Leu$^+$	30	
		Ger$^-$ Leu$^-$	73	
citF83 leu-2	*gerE36* Δ*ilvB1*	Ger$^+$ Ilv$^+$ Leu$^+$	0	*citF83-gerE36-*Δ*ilvB1-leu-2*
		Ger$^+$ Ilv$^+$ Leu$^-$	11	
		Ger$^+$ Ilv$^-$ Leu$^+$	2	
		Ger$^+$ Ilv$^-$ Leu$^-$	0	
		Ger$^-$ Ilv$^+$ Leu$^+$	0	
		Ger$^-$ Ilv$^+$ Leu$^-$	58	
		Ger$^-$ Ilv$^-$ Leu$^+$	29	
		Ger$^-$ Ilv$^-$ Leu$^-$	17	
citF78 leu-2	*gerE36 ilvB2*	Ger$^+$ Ilv$^+$ Leu$^+$	0[c]	*citF78-gerE36-ilvB2-leu-2*
		Ger$^+$ Ilv$^+$ Leu$^-$	117	
		Ger$^+$ Ilv$^-$ Leu$^+$	2	
		Ger$^+$ Ilv$^-$ Leu$^-$	0	
		Ger$^-$ Ilv$^+$ Leu$^+$	0	
		Ger$^-$ Ilv$^+$ Leu$^-$	112	
		Ger$^-$ Ilv$^-$ Leu$^+$	53	
		Ger$^-$ Ilv$^-$ Leu$^-$	15	

[a] Cit$^+$ was selected in each cross, and unselected markers were scored.
[b] Data summed from two experiments.
[c] Data summed from three experiments.

preparing the phage by confluent lysis of a bacterial lawn, where plaque formation requires multiple cycles of infection.

DISCUSSION

The properties of strains carrying the *gerE36* mutation indicate that the mutation affects the structure of the spore, particularly of the coat layers, and the response of the spore to germinant. This is the first structurally deficient spore germination mutant to be recognized in *B. subtilis* 168, and, although originally identified as altered in germination, it might with equal validity be described as a mutant defective in sporulation, blocked either in the synthesis or assembly of coat layers. The mutation may have a more complex effect, however, since, under some conditions, the mutant spores also have a smaller core diameter. As already discussed, a temperature-sensitive block in sporulation occurs (under certain growth conditions) in a PBS1 transductant carrying *gerE36*. Since, however, this is not also the case for a SPP1 trandsuctant which contains a smaller proportion of the originally mutagenized genome, the temperature sensitivity may be the result of a second mutation fairly close to *gerE36*, or might be dependent on some other aspect of the genetic background of the recipient strain into which *gerE36* was transferred.

The mutation responsible for generating spores with defects in at least spore coat structure and germination mapped by SPP1 transduction between the *citF* and *ilvB* loci (Table 2). Since the defects in coat structure and in germination have not been separated in SPP1 transduction there is no indication they they are the result of more than a single mutation. Reversion studies are necessary, however, before it can be stated with confidence that *gerE36* is a point mutation in a single gene and does not represent a deletion or two very closely linked mutations. Nevertheless, the location of *gerE36* identifies one or more genes whose product is essential for successful spore development; whether the *gerE* gene is directly involved in coat protein synthesis or deposition as a regulatory or structural gene, or has some more indirect, pleiotropic effect on coat structure, is not yet clear.

Spores carrying the *gerE36* mutation are defective in both structure and function. The spore coat contains a less extensive arrangement of lamellae (Fig. 1 and 2) and is permeable to lysozyme (Fig. 4). Correlations between coat structure abnormalities and germination deficiencies have been observed in other spore-forming bacteria. Some, but not all, chemical treatments of spores to extract coat material can prevent subsequent germination (30). Coatless spores of *Clostridium perfringens* do not respond to germinant, but can be germinated by lysozyme (4), and lysozyme-dependent spore germination-deficient mutants of *B. cereus* (2, 5, 6) are also coat deficient. In these strains, the lysozyme-resistant inner layer of the cortex, the germ cell wall, remains intact to form the cell wall of the outgrowing spore. In contrast, both the cortex and germ cell wall peptidoglycan of *B. subtilis* are sensitive to lysozyme (33), and *gerE36* spores lyse completely on treatment with lysozyme, unless osmotically stabilized, when protoplasts are formed. In some of the coat-defective *B. cereus* mutants (5, 6) a deficiency in the major intracellular serine protease has been identified; it has not been established whether the impaired germination is due directly to the lack of active protease in the spore or indirectly to the effect of absence of protease on coat processing and structure. Since not all the coat-defective mutants of *B. cereus* are similarly protease deficient, the latter is perhaps more probable. That the activity of intracellular serine protease is essential for sporulation in *B. subtilis* has been shown by the study of mutants (11, 15), but these are blocked at early stages in sporulation; it would be interesting to learn whether the protease has a number of roles at different stages of the sporulation process. Intracellular serine protease activity should be tested in *gerE36* strains, and it would be interesting to identify and compare the map location of the protease-deficient mutant of Kerjan et al. (15) with that of *gerE* and other germination mutants.

Despite the coat deficiency, *gerE* spores still show the same dependence on germinant concentration as wild type, although only some of the normal stages of germination are completed. Clearly the primary interaction of spore with germinant is unaffected by the mutation. It is thus probable that the location of this interaction is not in the spore coat. However, since later changes associated with germination, such as the release of DPA, uptake of water soluble dye, and phase darkening, are not completed, the coat layers may contain molecules important for these later stages. The block in germination after the loss of heat resistance and much of the DPA is reminiscent of the behavior of coat mutants of *B. cereus* (6) and of *B. cereus* T spores at high temperatures or high concentrations of Ca^{2+} (12). It has already been reported that loss of heat resistance and loss of DPA are among the earlier germination-associated events (18). Whether other germination events proceed, such as release of mucopeptide, activation of meta-

bolic capacity, or release and hydrolysis of small acid-soluble proteins, should be tested.

The *gerE* mutant defines an important sporulation gene, with associated effects on spore structure and germination. It provides another type of sporulation defect which can be studied at several levels. The *gerE* gene product has a role in sporulation and is probably developmentally regulated, and this regulation may be examined. The coat abnormality may be useful in studying coat structure and assembly. The block in germination could be studied in more detail, to identify which process can occur without the completion of others. Because of their sensitivity to lysozyme, *gerE36* spores could prove useful in the preparation of spore protoplasts of *B. subtilis*, or to provide a gentle means of breaking open spores, e.g., to study their enzyme complement, without the need to resort to potentially denaturing physical or chemical extraction procedures. Although the interpretation of the development defect in *gerE36* strains is confused by the pleiotropic phenotype conferred by the mutation, such strains may thus have a place in the study both of the establishment and of the disruption of the dormant state in bacterial endospores.

Finally, the *gerE* mutation is closely linked in SPP1 transduction to mutations near one end of the *citF* cluster; the properties of strains carrying *gerE36* do not resemble those of *citF* mutants, however (19), and it seems probable that the *gerE* mutation represents a separate gene from *citF*. Another group of germination mutants, those mapping in *gerA*, are also fairly closely linked to a citric acid cycle gene, *citG*, responsible for fumarase activity (Sammons et al., in press). It is not known whether or not these genetic relationships reflect any functional relationship between the citric acid cycle and germination.

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