Protease Production during Sporulation of Germination Mutants of 
Bacillus subtilis and the Cloning of a Functional gerE Gene

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Early in sporulation, cells of wild-type Bacillus subtilis produce three proteases (b, c and d) with monomeric $M_r$ values of about 65000, 53000 and 43500, and a further protease, e ($M_r$ about 30000) at the time of coat assembly. An additional protease, f ($M_r$ about 15000) appears transiently in sporangia at about the time of spore release. Three strains with defective spore coats were examined for alterations in sporulation proteases. A strain carrying the $gerE^36$ mutation produces b, c and d normally, fails to produce e and accumulates f on or in its spores. A strain carrying the spoVIC610 mutation produces normal quantities of proteases b, c and d, but has a reduced amount of proteases e and f. A strain carrying both the $gerE^36$ and the spoVIC610 mutations accumulates neither protease e nor f. The wild-type allele of the gerE gene was cloned in the vector, phage $\phi$10539. Complementation tests with the cloned gene showed that the $gerE^36$ mutation is recessive to the wild-type allele.

INTRODUCTION

The involvement of proteases in sporulation has been recognized since Foster & Perry (1954) observed that three-quarters of the pre-formed (vegetative) proteins of Bacillus mycoides were degraded during the first 5 h of sporulation. Subsequent work has shown that a number of different proteases are required at different times during sporulation of Bacillus spp. Thus, Prestidge et al. (1971) described three early sporulation proteases of Bacillus subtilis, of which two are inhibited by phenylmethanesulphonyl fluoride (PMSF), and the third by EDTA. Mutant strains of B. subtilis which fail to produce the sporulation-associated extracellular serine protease are blocked at an early stage. Hageman & Carlton (1973) showed that a mutant which fails to make an EDTA-insensitive protease is asporogenous. The formation of heat-resistant spores is prevented if PMSF is added to cultures of B. subtilis during the first 3 h of sporulation (Dancer & Mandelstam, 1975). The addition of PMSF to sporulating cultures at any time between $t_4$ and $t_6$ prevents the deposition of the alkali-soluble spore coat protein and results in the formation of spores that are heat-resistant but lysozyme-sensitive (Jenkinson et al., 1980, 1981). A temperature-sensitive mutant of B. subtilis described by Sastry et al. (1983) lacks a trypsin-like enzyme and fails to process a protein which may form part of the spore undercoat. Moir (1981) described a mutant which lacks two major coat proteins, and this fails to produce a late sporulation protease (Jenkinson & Lord, 1983).

In Bacillus cereus T, PMSF inhibits germination, possibly by inhibiting a trypsin-like enzyme (Boschwitz et al., 1983) and a similar inhibition is found in B. subtilis (our unpublished results). A tetrameric protease of $M_r$ 160000 is responsible for the degradation of storage proteins during germination of Bacillus megaterium, but is not necessary for the loss of resistance and refractility during germination (Postemsky et al., 1978). This enzyme is synthesized as a precursor during sporulation (Hackett & Setlow, 1983).

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Abbreviation: PMSF, phenylmethanesulphonyl fluoride.
The aim of this work was to determine whether there was any relationship between the protease deficiency of gerE mutant strains and their structural and functional defects. Three approaches were used. Firstly, identification of sporulation proteases by a more sensitive assay than that used by Jenkinson & Lord (1983), whose analysis had detected only two of the proteases known to be involved in sporulation. Secondly, the proteases and spore properties were examined in another germination mutant, strain 610 pheA12 spoVIC610 (James & Mandelstam, 1985) and in a double mutant carrying both the gerE36 and the spoVIC610 mutations. Thirdly, the gerE gene was cloned in a temperate bacteriophage and the phenotype of a strain carrying both the wild-type and mutant alleles of the gene was examined.

METHODS

Bacteria. The strains used are listed in Table 1.

Growth and sporulation. Cells were grown in hydrolysed casein medium (Sterlini & Mandelstam, 1969) to a density of about 0.25 mg dry weight ml⁻¹ and were induced to sporulate by resuspension at the same density in the glutamate/inorganic salts medium of Sterlini & Mandelstam (1969). L-Amino acids (200 μg ml⁻¹) were added to the medium as appropriate. Incubation was at 37 °C. The time at which cells were transferred to sporulation medium is denoted t₀, and subsequent times (h) are denoted t₁, t₂, etc.

Measurement of resistance. Resistance of cells and spores to heat and chloroform was determined as described by Jenkinson et al. (1980). Resistance to lysozyme was determined by incubating a cell suspension (no more than 0.2 mg dry weight ml⁻¹) in sodium phosphate buffer (0.5 M), and NaCl (0.15 M) at 45 °C, and plating suitably diluted samples on nutrient agar (Oxoid).

Measurement of protease activities in sporulating cultures. Sporulating cells or purified spores were harvested and prepared for analysis of protease activity as described by Jenkinson & Lord (1983). After breakage of sporangia in a French pressure-cell at 83 MPa, the insoluble fraction was resuspended in extraction buffer (0.5 ml; Tris/HCl, 100 mM, pH 6-8) containing SDS (0.3%, w/v) and 2-mercaptoethanol (0.01%, v/v) and incubated for 30 min at 37 °C. The mixtures were then centrifuged (15000 g, 5 min) and the supernatants mixed with bromophenol blue (0.002%, w/v) and glycerol (10%, v/v). Proteases were analysed by the SDS-gelatin-PAGE method of Heussen & Dowdle (1980). A polyacrylamide gel [acylamide, 12.5% (w/v), bisacrylamide, 0.33% (w/v)] containing SDS (0.1% w/v) and gelatin (Difco; 0.1% w/v) was cast. Samples (40 μl) were electrophoresed at a constant current (40 mA per slab) at 4 °C. After electrophoresis, the gel was rinsed, first in Triton X-100 (Koch-Light; 2.5%, v/v) for 1 h at 30 °C, to remove SDS from the proteins and allow renaturation, and then in glycine/NaOH buffer (0.1 M, pH 8-3) containing NaCl (0.15 M) and MgSO₄ (40 mM) at 30 °C for 12 h. The gel was stained in Amido Black and destained in methanol (20%, v/v), acetic acid (10%, v/v), and NaOH (0.1%, v/v). M, standards (BDH) had approximate M, values of 13200, 17200, 25700, 45000, 66200 and 77000.

Germination studies. The methods used are described in the accompanying paper (James & Mandelstam, 1985).

Genetic transformation of B. subtilis using purified DNA. Chromosomal DNA was prepared by the method of Marmur (1961). Competent cells were produced by the method of Anagnostopoulos & Spizizen (1961) as modified by Jenkinson (1983). Samples of the transformation mixture were spread onto Schaeffer’s sporulation agar (Schaeffer et al., 1965) for the production of spores.

Selection of Ger+ recombinants. GerE+ spores were selected by resuspending cells which had been grown for 2 d on Schaeffer’s medium (Schaeffer et al., 1965) at 37 °C, in phosphate buffer (0.05 M, pH 5-8) containing NaCl.
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(0.15 M) and lysozyme (200 μg ml⁻¹). The suspension was incubated at 45 °C for 60 min and heated at 65 °C for 15 min to destroy vegetative cells and sensitive spores. Resistant colony-forming units (c.f.u.) were estimated by spreading appropriately diluted samples on nutrient agar.

Screening of GerE⁺ recombinants. The tetrazolium method of Irie et al. (1982) was used.

Preparation and manipulation of phage φ105 and its derivatives. These methods are described by Errington (1984).

RESULTS

Protease activities in sporulating cells of Spo⁺ strains

Extracts of sporulating cells were analysed for protease activities by SDS-gelatin-PAGE (Heussen & Dowdle, 1980; see Methods). Samples (10 ml) of sporulating cultures of strain 168 trpC2 were taken at intervals during sporulation. Proteins extracted from both the soluble and insoluble fractions were analysed. No proteases could be detected in either fraction at t₀, but, from t₁ until spore release (which began at about t₈), three proteases with different mobilities were found in both (Figs 1 and 2). For convenience, we call these proteases b, c, and d. After t₉, a fourth protease (e) of lower Mₑ was seen – predominantly in the insoluble fraction and in mature spores. A fifth protease, f, of still greater mobility was detected in sporangia sampled at t₈–t₁₀.

It was obviously desirable to have estimates of the Mₑ values of these sporulation proteases. Unfortunately, the use of proteolytic enzymes of known Mₑ as standards was unsatisfactory, and more conventional standards could not be distinguished against the Coomassie-stained gelatin background. However, the protein dye Amido Black, which is less sensitive than Coomassie Brilliant Blue R250, enabled protein molecular weight markers and protease bands to be seen in the same gel. The straight-line relationship between the mobility of protein standards and the logarithm of their Mₑ was used to estimate the Mₑ values of the sporulation proteases as follows: b, Mₑ = 65000; c, Mₑ = 53000; d, Mₑ = 43500; e, Mₑ = 30000; and f, Mₑ = 15000.

Several experiments were done in an attempt to characterize these proteases. SDS-gelatin gels were incubated in glycine (0.1 M) buffers of a variety of values of pH and electrolyte concentration and in the presence of EDTA (10 mM) or PMSF (saturating concentration). Neither EDTA nor PMSF had a noticeable inhibitory action. The proteases were relatively unaffected by a range of pH values from 5.5 to 9.1, but proteases b, c and d were more active in buffers containing monovalent and divalent cations. It was for this reason that NaCl (0.15 M) and MgSO₄ (40 mM) were therefor included in the glycine buffer (pH 8.3). All results shown in this paper were obtained with this modified buffer.

Protease activities in sporulating cells of Ger strains

We wished to find out whether the germination properties of strains were related to specific protease activities, since Jenkinson & Lord (1983) had demonstrated that GerE⁻ strains lacked a protease. We examined the sporulation proteases of strain 522 gerE36 trpC2 by SDS-gelatin-PAGE and compared them to those of the wild-type and of strain 610 pheA12 spoVIC610 (James & Mandelstam, 1985). A double mutant, strain DM2 gerE36 spoVIC610, was also examined, to see if the effect upon protease production of these two mutations was additive (see also James & Mandelstam, 1985). Like the wild-type, all these strains produced proteases b, c, and d. However, strain 522 failed to produce protease e but accumulated, very much later in sporulation, a low Mₑ protease activity (f) which was associated with the spore (Figs 1 and 3). Strain 610 produced a reduced amount of protease e, but accumulated no f. Strain DM2 produced neither protease e nor f (Fig. 3). The wild-type did produce a protease of the same Mₑ as f, but this was observed very late in sporulation, and was not detectable in the purified, mature spores.

The time course of accumulation of protease f in spores of strain 552 was then investigated. Samples of sporulating cultures were taken at intervals between t₁₀ and t₁₄ and analysed by SDS-gelatin-PAGE for protease activity. Protease f accumulated gradually in the insoluble fraction from about t₁₀ onwards, i.e. after sporulation was complete and all protein synthesis had finished. To determine whether mature mutant spores were abstracting proteases from the medium, an experiment was done in which the sporulated cells of strains 168 trpC2 and of 522
Fig. 1. Production of proteases in wild-type and GerE– strains during sporulation. Cells from sporulating cultures of strains 168 trpC2 and 522 gerE36 trpC2 were harvested at intervals, broken in a French pressure-cell, and the soluble and insoluble fractions were analysed for protease activity by SDS-gelatin-PAGE (see Methods). This schematic representation of the typical results of many experiments is drawn in the form of a hypothetical gel. The horizontal bands, designated by the letters in the centre, represent the mobilities and periods during which the protease activities were detectable. Solid bands represent proteases found principally in the soluble fraction of the cells, whereas cross-hatched bands represent proteases associated with the spore fraction. The positions of $M_r$ markers are indicated on the right.

Fig. 2. Separation of soluble sporulation proteases by SDS-gelatin-PAGE. Samples (10 ml) of sporulating cultures of strains 522 gerE36 trpC2 (lanes 5–8) and 168 trpC2 (lanes 1–4) were taken at the following times during sporulation: $t_0$, lanes 1 and 5; $t_1$, lanes 2 and 6; $t_2$, lanes 3 and 7; $t_3$, lanes 4 and 8. The cells were harvested and broken in a French pressure-cell. The soluble fractions were incubated in extraction buffer and the proteins were analysed by electrophoresis through an SDS-gelatin-gel.

gerE36 trpC2 were each transferred to the medium of the other strain at $t_{11}$, i.e. before the accumulation of protease $f$, and incubated until $t_{34}$. Spores of the mutant strain accumulated protease $f$ whether incubated in their own medium or in medium in which the wild-type had sporulated. Conversely, wild-type spores did not acquire protease $f$ activity after incubation in either medium (Fig. 4).
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Fig. 3. Spore-associated proteases of three Ger strains. Samples (10 ml) of sporulating cultures of strains 168 trpC2 (wild-type) (lanes 2 and 7), 610 pheA12 spoVIC610 (lanes 3 and 8), DM2 gerE36 spoVIC610 (lanes 4 and 9) and 522 gerE36 trpC2 (lanes 5 and 10) were taken at t8 (lanes 2–5) and t20 (lanes 7–10). The cells were harvested and broken in a French pressure-cell. The insoluble fractions of the t8 samples, and the purified spores of the t20 samples, were incubated in extraction buffer and the proteases were analysed by electrophoresis through an SDS-gelatin-gel as described in Methods. Lanes 1, 6 and 11 contain protein M markers as indicated in the legend to Fig. 1.

Fig. 4. Abstraction of protease f by spores of strain 522. Sporulating cultures of strains 168 trpC2 (lane 1) and 522 gerE36 trpC2 (lane 4) were sampled at t1. Also, spores collected at t1 were resuspended in their own medium and harvested at t24 after further incubation at 37 °C (168, lane 2; 522, lane 5). Further portions of spores from both strains collected at t1 were resuspended in the culture medium of the other strain, and again, harvested at t24 (168, lane 3; 522, lane 6). The spores in each sample were harvested by centrifugation and incubated in extraction buffer. The proteins were analysed by electrophoresis through an SDS-gelatin gel as described in Methods.
Preparations of spores of strain 522 often showed a large but variable number of higher \(M_r\) protease activities whose mobilities suggested a multimeric relationship to protease \(f\) (see e.g. Fig. 4). These bands were attenuated by incubation in extraction buffer at increasingly high temperatures, and the activity of the protease \(f\) band increased correspondingly. Protease \(f\) survived treatment in extraction buffer at temperatures up to 67°C. On the other hand, proteases \(b, c, d,\) and \(e\) survived treatment in extraction buffer at temperatures up to 55°C but did not appear to dissociate into protease activities of lower \(M_r\).

**Cloning of a fragment of wild-type DNA which complements the GerE defects**

We wished to clone the wild-type allele of the gerE gene as a preliminary to the study of the regulation, structure and function of the gene and gene product at the molecular level. Several approaches were tried using the temperate phage \(\phi 105\) as a vector, but these were not successful. A modified derivative, \(\phi 105\)J9, then became available (Errington, 1984). This phage has a single \(BamH\) site, can form plaques, and has a smaller genome, which should allow the cloning of fragments up to about 5 kb in size. Phage \(\phi 105\)J9, and derivatives carrying cloned DNA fragments, can integrate into the genome of *B. subtilis* at the attachment site for \(\phi 105\). If the lysogenic phage carries an intact gene, together with its promoter, for which the host is mutant, complementation can occur (Errington, 1984). Three pools of recombinant \(\phi 105\)J9 phage containing fragments of DNA from *B. subtilis* 168 cut with the restriction enzymes \(BclI, BglII\) or \(MboI\) (partial digest) were screened for the ability to complement the gerE36 mutation. The bank of recombinant phage was kindly supplied by J. Errington. The double mutant strain DM2 gerE36 spoV*IC610* was used as the recipient in these transduction experiments because its spores were even more sensitive to lysozyme than those of strain 522 gerE36 trpC2 (James & Mandelstam, 1985), thereby increasing the effectiveness of the Ger\(E^+\) selection. The culture of strain DM2 treated with phage from the '\(BclI\) pool' developed more than \(10^5\) lysozyme-resistant c.f.u. ml\(^{-1}\). The control culture, and those treated with phage from the other pools, developed about \(10^3\) lysozyme-resistant c.f.u. ml\(^{-1}\).

We wished to discover whether the ability of the '\(BclI\) pool' to confer lysozyme-resistance upon the double mutant was due to full complementation of the gerE36 mutation. Accordingly, phage were prepared by two rounds of lysogeny and introduction from single colonies of the lysozyme-resistant lysogens made with phage from the '\(BclI\) pool'. The purified phage was called \(\phi 105\)gerE. Strain 522 gerE36 trpC2, lysogenized with phage \(\phi 105\)gerE, was wild-type with respect to heat-, chloroform- and lysozyme-resistance (Table 2), germination properties (Fig. 5) and protease activity (Fig. 6). Strain 168 trpC2 when lysogenized with phage \(\phi 105\)gerE or \(\phi 105\)J9, and strain 522 gerE36 trpC2 when lysogenized with phage \(\phi 105\)J9, had the phenotype of the non-lysogenic, parent strain.

It was necessary to show that the apparently complemented strain still carried the gerE36 allele. Accordingly DNA was prepared from strains 522 gerE36 trpC2 (\(\phi 105\)gerE) and 522 gerE36 trpC2 and used to transform strains carrying the citF2 and leuB16 mutations to prototrophy; 200 transformants from each cross were scored for their Ger phenotype (see Methods). DNA from both strains was found to carry a ger mutation loosely linked to leuB16 (7\% and 5\% cotransformation, respectively) and closely linked to citF2 (62\% and 70\% cotransformation, respectively). This indicates that the strain carrying the lysogenic phage \(\phi 105\)gerE and whose phenotype had been shown to be Ger\(E^+\) still carried the gerE36 mutation.

There existed the formal possibility that the suppression of the Ger\(E^-\) phenotype in strains carrying the gerE36 mutation on the chromosome together with the \(\phi 105\)gerE prophage might not have been due to true complementation. For example, the recombinant phage could perhaps have carried the wild-type allele of a different gene which, when present in two copies per cell rather than one, could have suppressed the Ger\(E^-\) phenotype. To test this possibility, DNA prepared from phage \(\phi 105\)gerE was used to transform strain 522 gerE36 trpC2 and selection was made for Ger\(E^+\). A very high frequency of transformation was observed, the number of Ger\(E^+\) spores being \(3 \times 10^5\)-fold greater than that in an untransformed control, and the transformants (10 out of 10 tested) were not lysogenic for phage \(\phi 105\) or a derivative. We conclude that the recombinant phage carried the intact, wild-type allele of the gerE gene which is expressed *in vivo*, probably from its own promoter.
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**Fig. 5.** Complementation of the GerE− phenotype: germination deficiency. Spores of strains 168 trpC2 (φ105gerE) (○), 522 *gerE*36 trpC2 (φ105gerE) (■) and 522 *gerE*36 trpC2 (φ105J9) (□) were suspended in Tris/HCl (0·1 M, pH 7·4) and heat-activated (75 °C, 30 min). Germination at 37 °C was initiated by the addition of L-alanine (10 mM) and the OD580 of the suspensions was recorded. OD580 values were plotted as a percentage of the initial value against time. Each of the dots represents a data point; for clarity, only some of these points are shown as symbols.

**Fig. 6.** Complementation of the GerE− phenotype: protease deficiency. Sporulating cultures of strains 168 trpC2 (φ105gerE) (lanes 2 and 5), 522 *gerE*36 trpC2 (φ105J9) (lanes 3 and 6) and 522 *gerE*36 trpC2 (φ105gerE) (lanes 4 and 7) were sampled at t₁ (lanes 5–7) and t₂ (lanes 2–4), the spores harvested and their proteins extracted and analysed for proteases by SDS-gelatin-PAGE (see Methods). Lanes 1 and 8 contain *M*, markers as indicated in the legend to Fig. 1.

**Table 2.** Complementation of the *gerE*36 mutation by the recombinant phage φ105gerE: resistance properties

Sporulating cultures of the three strains indicated were sampled at t₂, and tested for resistance to heat, chloroform and lysozyme. The results are expressed as a percentage of the viable count.

<table>
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<tr>
<th>Strain</th>
<th>Viable count (c.f.u. ml⁻¹)</th>
<th>Resistance to</th>
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<tr>
<td>168 <em>trpC2</em> (φ105gerE)</td>
<td>4·1 × 10⁸</td>
<td>Heat</td>
<td>Chloroform</td>
</tr>
<tr>
<td>522 <em>gerE</em>36 <em>trpC2</em> (φ105J9)</td>
<td>3·1 × 10⁸</td>
<td>100</td>
<td>85·4</td>
</tr>
<tr>
<td>522 <em>gerE</em>36 <em>trpC2</em> (φ105gerE)</td>
<td>5·3 × 10⁸</td>
<td>23</td>
<td>2·9</td>
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<td></td>
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<td>90·6</td>
<td>88·7</td>
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An attempt was made to see whether the gerE gene coded for protease e, by infecting maxi-cells of GerE− strains with phage φ105gerE (Kobayashi et al., 1981), and analysing them by SDS-gelatin-PAGE. We were not able to see any change in intracellular proteases in maxi-cells prepared during sporulation after invasion with phage φ105gerE. This result does not support the idea that the gerE gene encodes protease e.

In order to begin to characterize the cloned gene, we purified DNA from the phages φ105J9 and φ105gerE and analysed it by restriction enzyme digestion and agarose gel electrophoresis. Additional sites for the enzymes HindIII and EcoRV were found in phage φ105gerE which were not present in φ105J9, and the cloned insert was about 1.5 kb in length (Fig. 7).

**DISCUSSION**

Of the protease activities described in this work, three were found in cells soon after the beginning of sporulation. Although these may correspond to the three sporulation proteases described by Prestidge et al. (1971), we were not able to show whether any of them were specifically inhibited by PMSF or EDTA. Protease e is probably the same as protease B described by Jenkinson & Lord (1983) because both enzymes are produced at the same stage of sporulation, are found in mature spores, are sensitive to heating and are absent from GerE− mutant strains. The difference in our estimates of its Mₙ (30000 rather than 25000) probably results from our ability to infer this value from the mobility of six markers, rather than one marker (chymotrypsinogen A, Mₙ 25000).

Even with the more sensitive method of Heussen & Dowdle (1980), we were not able to detect a serine protease, presumed to be synthesized at the time of spore coat deposition, which is necessary for deposition of the 12K coat protein (Mₙ 12000) (Jenkinson et al., 1981; Goldman & Tipper, 1978). This could be due to various factors, e.g. sensitivity of the enzyme to the denaturing conditions, multimerism, etc.

Protease f has not, as far as we are aware, been previously reported. It appears to be synthesized in sporulating cells of all the strains we tested, but it appears in small quantities and only just before spore release in the wild-type. Now, GerE− spores tend to aggregate, as do wild-type spores which have been treated with NaOH or disulphide-reducing agents which remove some of the spore coat protein. This suggests that coat proteins which are abnormally exposed, either because of faulty synthesis and assembly of the spore coat (in the case of GerE− strains; see Jenkinson & Lord, 1983) or because of chemical extraction of the coat, tend to adhere to proteins similarly exposed on other spores. Furthermore, since small amounts of protease f were
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sometimes observed in the sporangia of the wild-type at \( t_6 \) (shortly before spore release; see Fig. 3) it seems most likely that the coat defect in spores of strain 522 causes it to abstract a protease \( f \) from the medium which, in the wild-type, is normally lost during spore release.

We also observed that PMSF, added to sporulating wild-type cultures at \( t_6 \), prevented not only the deposition of the 12K coat protein \( (M_r = 12000) \) but also spore release (unpublished results), suggesting that a serine enzyme, which might be protease \( f \), is involved in spore release. It should be noted that although GerE- strains fail to produce the 12K coat protein \( (M_r = 36000) \) and protease \( e \), they do produce protease \( f \) and accomplish spore release, both of which are later events of sporulation.

The cloning of the gerE gene (briefly reported by Errington, 1984) has enabled us to carry out complementation tests and to show that gerE36 is recessive to the wild-type allele. It is now possible to sequence the gene, determine whether it is likely to code for any of the known sporulation proteases or spore coat proteins, determine the time of expression of the gene and generate new mutations in the region. This work is currently in progress (S. Cutting, personal communication).

REFERENCES


