Timing and genetic regulation of commitment to sporulation in *Bacillus subtilis*

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Sporulation in *Bacillus subtilis* is a simple developmental system involving the differentiation of two cell types called the prespore and the mother cell. The process is induced by nutrient deprivation and culminates with the formation of a mature spore, which is released by lysis of the mother cell. We have studied commitment to sporulation with several different assays. The results indicate that commitment occurs soon after the formation of the asymmetrically positioned division septum that separates the prespore and the mother cell. This is earlier than the previously postulated point of commitment, prespore engulfment by the mother cell. Commitment coincides approximately with activation of the early prespore- and mother-cell-specific sigma factors, σE and σF.

**Keywords**: sporulation, *Bacillus subtilis*, sigma factors, commitment, development

**INTRODUCTION**

Spore formation in *Bacillus subtilis* is a complex adaptive response to starvation (Errington, 1993; Losick & Stragier, 1992). About 1 h after the initiation of sporulation, a small prespore cell and a much larger mother cell are produced by asymmetric division. Later, the prespore is engulfed by the mother cell. Further development of the spore, including the formation of various protective layers, and the dehydration and mineralization of its core, then takes place within the cytoplasm of the mother cell. The mother cell devotes most of its resources to this process, and ultimately it lyses to release the mature spore.

The decision to initiate spore formation is important because it takes place at the expense of continued growth. Maturation of the single spore can take the equivalent of several vegetative generation times, so in the presence of adequate nutrients, cells that initiate sporulation may be considerably disadvantaged compared with cells that continue to proliferate. Several groups have therefore addressed the question of whether the initiation of spore formation represents an irreversible decision or whether the process can be aborted if conditions improve (reviewed by Freese & Heinze, 1984). The point at which spore formation becomes irreversible has been called 'commitment'. A variety of methods have been used to characterize commitment. Generally, samples from a culture of sporulating cells were enriched with a nutrient and the effects on the final spore yield, or on other markers of sporulation, were measured. Cells that were committed would ignore the enrichment and proceed to make a spore; uncommitted cells would revert to growth, resulting in a decrease in the ultimate spore titre. Freese and colleagues showed that the apparent point of commitment can be influenced by the method of enrichment. Thus, glucose enrichment gave a very early apparent time of commitment, due to the cells becoming unable to take up and utilize glucose (Freese et al., 1970), whereas other nutrients gave later times for commitment. For both *B. subtilis* and *Bacillus megaterium*, the latest time of commitment was obtained by enrichment with a hydrolysed casein (CH) medium (Cooney et al., 1977; Freese et al., 1975). This 'final' point of commitment was reported to coincide with stage III, complete engulfment of the prespore by the mother cell.

In other studies, electron microscopic examination of enriched sporulating cultures revealed the existence of outgrowing mother cells apparently associated with prespores that had developed to between stage II (asymmetric septum completed) and III (engulfment completed) (Fréhel & Ryter, 1969). Freese & Freese (1977) used a slightly different approach to measure the time of commitment. They showed that on certain media the plating efficiency of sporulating cultures decreased at about the
time that the first cells completed prespore engulfment. Their interpretation of the results was that the slow-growing (SG) colonies arose from cells in which the mother cell was committed to completion of sporulation and thus, ultimately, to cell lysis, and the prespore could only form a colony slowly, after completing sporulation and germinating. The time of mother cell commitment was again suggested to coincide with prespore engulfment. This conclusion was supported by studies of the programmes of gene expression and morphological development during sporulation (reviewed by Errington, 1993). The resuspension procedure for induction of sporulation (Sterlini & Mandelstam, 1969) has been particularly useful for such studies, because it provides a defined time of initiation, and the subsequent time course of sporulation is highly reproducible (Partridge & Errington, 1993). In this paper, we have therefore re-examined the question of commitment to sporulation in *B. subtilis*. Several lines of evidence show that commitment occurs soon after septation, but before engulfment is complete. The results are consistent with the early cell-specific sigma factors $\sigma^F$ and $\sigma^E$ being involved in the commitment of their respective cells.

**METHODS**

**Bacterial strains, plasmids and bacteriophage.** Bacterial strains used are listed in Table 1. Strain 637 was constructed by inserting the kanamycin-resistance determinant from plasmid pSG122 (Daniel & Errington, 1993) into the BamHI site of the *spoIIG* gene in the chromosome of strain SG38. Plasmid
pPS1280, carrying the *spoIV*-*2G-lacZ* fusion for insertion at the *amyE* locus of *B. subtilis*, was kindly provided by P. Setlow (Department of Biochemistry, University of Connecticut Health Center, Farmington, CT, USA) (this was the plasmid used to construct strain PS1287 of Sun et al., 1991). Phage #105J108, a *spoIVA* recombinant transducing phage, was described by Stevens et al. (1992).

**Conditions for growth and induction of sporulation.** Cultures growing in a CH medium were induced to sporulate by transfer to resuspension medium (SM), essentially as described by Sterlini & Mandelstam (1969) but incorporating the modifications of Partridge & Errington (1993).

**Measurement of lysozyme resistance.** The method of Jenkins et al. (1980) was used. Sporulating cultures, or enriched cultures derived from them, were incubated at 37 °C until 7 h after the time of initial resuspension in SM. Samples (0.1 ml) were removed and diluted with 4.9 ml of 'unsupplemented SM' (a partially formulated SM containing no glutamate, CaCl₂ or MgSO₄) containing 0.25 mg lysozyme ml⁻¹. This mixture was incubated for 10 min at 37 °C then further dilutions were made with unsupplemented SM. Dilutions were plated on nutrient agar (Oxoid) and counted after incubation overnight at 37 °C.

**Measurement of commitment by enrichment in liquid nutrient medium.** Samples (10 ml) were removed from sporulating cultures, centrifuged for 5 min at 5000 g. The supernatant was removed and the pellet was resuspended back to the original volume of the sample in warm CH medium. The resuspended culture was then placed in a prewarmed flask and incubated at 37 °C.

**Measurement of commitment by plating on minimal medium.** The minimal medium used, containing glucose, glutamate and alanine (GGA), was as described by Piggot (1973), except that the sodium lactate was replaced with 0.1% glucose. Samples were removed from sporulating cultures, sequentially diluted to 1 × 10⁻², 1 × 10⁻⁴ and 1 × 10⁻⁶. Usually, duplicate 100 μl samples of the 1 × 10⁻⁴ and 1 × 10⁻⁶ dilutions were spread on plates. These were dried for 10 min in a laminar flow cabinet (Intermed Pathfinder) and then placed at 37 °C. The number of visible colonies was counted several times during the incubation of the plates.

**Measurement of lysozyme resistance.** As shown in Fig. 1, lysozyme resistance began to escape from the effects of enrichment about 70–80 min after the initiation of sporulation. Samples were also taken from the unenriched culture for measurement of APase. Fig. 1 shows that escape from inhibition of lysozyme resistance coincided with the appearance of APase in the control culture. APase synthesis is controlled by σE (Errington & Illing, 1992). Thus, the commitment to form a resistant spore occurs early in sporulation, at about the time when the first cell-specific transcription factors, σF and σE, become active (Partridge & Errington, 1993). At this time, the first cells would have completed asymmetric septation (stage II) but not engulfment (stage III), which is only completed 25 min after septation (Partridge & Errington, 1993). Thus, in contrast to the conclusions of most earlier workers (reviewed by Freese & Heinze, 1984), our results suggested that commitment occurs before engulfment.

**RESULTS**

**Effect of nutrient enrichment on formation of lysozyme-resistant spores**

We began by measuring commitment to a very late event in sporulation, the formation of lysozyme-resistant spores. Wild-type strain SG38 was induced to sporulate and at intervals samples were enriched by resuspension in CH medium [Cooney et al. (1977) showed that enrichment with CH gave the latest time of commitment in *B. subtilis*]. Seven hours after induction of sporulation, the enriched cultures and the unenriched control were all assayed for lysozyme resistance. As shown in Fig. 1, lysozyme resistance began to escape from the effects of enrichment about 70–80 min after the initiation of sporulation. Samples were also taken from the unenriched culture for measurement of APase. Fig. 1 shows that escape from inhibition of lysozyme resistance coincided with the appearance of APase in the control culture. APase synthesis is controlled by σE (Errington & Illing, 1992). Thus, the commitment to form a resistant spore occurs early in sporulation, at about the time when the first cell-specific transcription factors, σF and σE, become active (Partridge & Errington, 1993). At this time, the first cells would have completed asymmetric septation (stage II) but not engulfment (stage III), which is only completed 25 min after septation (Partridge & Errington, 1993). Thus, in contrast to the conclusions of most earlier workers (reviewed by Freese & Heinze, 1984), our results suggested that commitment occurs before engulfment.

**Use of a minimal medium to study the timing of mother cell commitment**

As an alternative means of measuring the timing of commitment to sporulation, we followed the approach of Freese & Freese (1977). Briefly, their method relies on the idea that when the mother cell becomes committed, the only route to regrowth is via the prespore, which is relatively slow. Committed and non-committed cells thus differ in the time taken to form visible colonies when plated. Freese & Freese (1977) found that SG colonies, indicative of commitment, began to arise from sporulating cultures when the first cells reached stage III. Since this was at variance from the results described above, we wished to repeat the plating experiments on cells induced
to sporulate by the resuspension method. In preliminary experiments, we found that a minimal medium supplemented with glucose, glutamate and alanine (GGA) supported the rapid growth of vegetative cells, whereas colony formation by purified spores was retarded (data not shown). To test whether this medium could distinguish between committed and uncommitted sporulating cells, the wild-type *B. subtilis* strain SG38 was induced to sporulate, and at intervals samples were diluted and plated on GGA. Colonies were counted at several time-points during the subsequent incubation at 37 °C (Fig. 2a). Samples taken within the first hour after resuspension showed relatively uniform and rapid colonial growth; the colony count did not change appreciably between 22 and 44 h. (The overall colony count increased in this period because cells continue to divide for a while after resuspension.) However, from about the 80 min time-point onwards, a class of colonies that developed more slowly became evident. As sporulation progressed, the fraction of the population forming SG colonies increased, apparently at the expense of the more rapidly growing (RG) colonies. According to Freese & Freese (1977), the SG colonies result from cells that had become committed to sporulation and the RG colonies from cells that were not committed, at the time of plating. Typically, the fall in numbers of RG colonies between 90 and 300 min was about threefold. This would imply that about three-quarters of the population of cells commit to sporulation over this period. This interpretation would be consistent with the kinetics of morphological development in sporulating cultures of this type as observed by fluorescence or electron microscopy (Hauser & Errington, 1995; Partridge & Errington, 1993).

In Fig. 2(b), the accumulation of SG colonies over time is compared with the onset of APase synthesis. SG colonies began to be detected arising from samples taken about 90 min after resuspension, just after the onset of APase synthesis. In six similar experiments, the onset of APase synthesis and the first appearance of SG colonies always occurred within 10 min of each other (data not shown). Thus, two distinct assay methods suggested that commitment to sporulation occurs before prespore engulfment, the previously postulated point of commitment. 

### Effects of spo mutations on formation of RG and SG colonies

Having established by two methods the time of commitment to sporulation, it was interesting to use mutants to determine at what point in the well-charted genetic programme of sporulation commitment occurred. Lysosome resistance could not be used for this purpose, since the mutants would be sensitive, but it seemed possible that the plating method, which was readily applicable to mutant cultures, might shed further light on commitment. Unfortunately, it became apparent that colonial growth rate is strongly influenced by cell size and morphology, so most early blocked sporulation mutants gave responses that were very different from the wild-type and thus difficult to interpret (including strains with mutations in the *spo0A*, *spoIIA* and *spoIIG* genes). Nevertheless, these experiments did provide some new information. The *spoIIAC* and *spoIIGB* mutants both have a 'disporic' phenotype, in which prespore-like cells are formed at both poles of the sporulating cell, leaving an anucleate central compartment (Piggot & Coote, 1976; Setlow et al., 1991). Although the mutants are indistinguishable morphologically, they differ at the level of gene expression in that *spoIIGB* mutant prespores contain active σF, whereas those of the *spoIIAC* mutant do not (Lewis et al., 1994). We found that there was a reproducible difference in the behaviour of the mutants in plating experiments. In the typical experiments illustrated in Fig. 3(b, c), the viable count of the *spoIIGB* mutant fell after about 90 min, whereas that of the *spoIIAC* mutant continued to rise. This would be consistent with σF playing a role in prespore commitment (see Discussion).

The behaviour of the late blocked mutants (stage II onwards) was also informative. In all cases, the results were consistent with the mutants becoming committed normally, in that the fall in RG colonies after about 90 min was similar to that of the wild-type (see examples in Fig. 3a, d-f; summarized in Table 2). Surprisingly, however, most such mutants showed an interesting and reproducible departure from the wild-type behaviour in that the SG colonies were reduced or absent. If our supposition that in the wild-type SG colonies arise by prespore regrowth is correct, their absence in most of the
Commitment to sporulation in Bacillus subtilis

Fig. 3. Behaviour of cultures of sporulating strains after plating on GGA. Each strain was induced to sporulate and at intervals a sample was plated on GGA. Colony counts are shown 22 (□) and 44 (○) h after plating. The strains used were as follows: (a) SG38; (b) 1.5; (c) 55.3; (d) 792; (e) 298.4; (f) 23.4.

late blocked mutants suggests that prespore viability is seriously compromised. In accordance with this idea, the two mutant loci tested for which intermediate or normal numbers of SG colonies were obtained, spoVCB and spoVAE (Table 2), are both known to allow formation of relatively stable immature spores (Piggot & Coote, 1976; Errington & Mandelstam, 1984). The continued viability of some stage II mutants, but not of mutants blocked at stages III or IV, accords with the previous observations of Freese & Freese (1977).

Effects of nutrient enrichment on activation of σF and σE

The above experiments suggested that commitment occurs soon after the formation of the spore septum, which is about the time that the first cell-specific transcription factor activities appear. It was thus possible that commitment reflects the irreversible activation of the genetic programmes controlled by σF or σE. To investigate this, we measured the effects of nutrient enrichment on the initiation and maintenance of expression of lacZ reporter genes. A spoIVA-lacZ fusion (strain 1712) was used to measure σE activity, and a lacZ fusion to the spoE-2G promoter (Sun et al., 1991) to measure σF activity (strain 1713). In preliminary experiments, interpretation of the results obtained with the latter fusion was complicated by the increase in expression that occurs after t2 when the late prespore-specific sigma factor σG, which also recognizes this promoter (Sun et al., 1991), becomes active. To avoid this, both strains used for the experiments described below carried a mutation inactivating the gene encoding σG.

Cultures of strains 1712 and 1713 were induced to sporulate by resuspension. At intervals, samples were removed and enriched by centrifugation and resuspension in fresh CH medium. The samples were enriched at times close to and after the point (or points) of commitment deduced from the experiments described above. β-Galactosidase was then followed in both the enriched cultures and in the unenriched controls. The results of typical experiments are shown in Fig. 4. For both fusions, enrichment 60 min after induction of sporulation resulted in an almost complete elimination of β-galactosidase accumulation, indicating that few cells were committed to activation of σE or σF at that time. However, by 80 min a significant proportion of cells of both cultures were evidently committed, because β-galactosidase levels between 50 and 80% of the unenriched control were obtained. A higher level of commitment was consistently
**Table 2:** Effects of spo mutations on commitment and on production of SG colonies

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation</th>
<th>Sporulation phenotype*</th>
<th>Commitment phenotype†</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG38</td>
<td>spo*</td>
<td>Wild-type</td>
<td>++</td>
</tr>
<tr>
<td>43.9</td>
<td>spoA</td>
<td>0</td>
<td>NC</td>
</tr>
<tr>
<td>1.5</td>
<td>spoIIACI</td>
<td>II (Disporic)</td>
<td>NC</td>
</tr>
<tr>
<td>792</td>
<td>spoIIIB</td>
<td>IIi</td>
<td>+−</td>
</tr>
<tr>
<td>298.4</td>
<td>spoIIID</td>
<td>IIIi</td>
<td>+−</td>
</tr>
<tr>
<td>55.3</td>
<td>spoIIIG</td>
<td>II (Disporic)</td>
<td>NC</td>
</tr>
<tr>
<td>59.8</td>
<td>spoIIIA</td>
<td>III</td>
<td>+−</td>
</tr>
<tr>
<td>497.1</td>
<td>spoIIID</td>
<td>III</td>
<td>+−</td>
</tr>
<tr>
<td>36.3</td>
<td>spoIIIE</td>
<td>III†</td>
<td>+−</td>
</tr>
<tr>
<td>646</td>
<td>spoIIIG</td>
<td>III</td>
<td>+−</td>
</tr>
<tr>
<td>23.4</td>
<td>spoIVCBI</td>
<td>IV</td>
<td>+ (+)</td>
</tr>
<tr>
<td>89.10</td>
<td>spoVAE</td>
<td>V</td>
<td>+−</td>
</tr>
<tr>
<td>91</td>
<td>spoVB</td>
<td>V</td>
<td>+−</td>
</tr>
<tr>
<td>786</td>
<td>spoVD</td>
<td>V</td>
<td>+−</td>
</tr>
<tr>
<td>85.3</td>
<td>spoVE</td>
<td>V</td>
<td>+−</td>
</tr>
</tbody>
</table>

* Morphological stage reached by mutant cells. See Piggot & Coote (1977); Errington (1993).† Samples from a sporulating culture were plated on GGA and colonies were counted after 22 h. + +, Wild-type plating behaviour; NC, not classified; + −, a normal fall in the RG colony count occurred but SG colonies did not appear; + (+), as for + −, except that a few SG colonies appeared (about 20% of wild-type numbers). † Deficient in prespore chromosome partitioning (Wu & Errington, 1994).

seen for the σE-dependent fusion at this time (data not shown). Following enrichment at 100 min, there was little difference between the β-galactosidase activities of the enriched and control cultures, suggesting that most cells were committed to activation of σF and σE by this time. The slight overexpression seen in the samples enriched at late times suggests that cells sporulating in the enriched medium may synthesize more β-galactosidase than those remaining in sporulation medium. However, the magnitude of this increase did not appear to be sufficient to change the conclusion that a significant proportion of cells must be committed to activation of σF and σE after 80 min in SM.

**DISCUSSION**

**The timing of commitment**

We measured the timing of commitment to several different events of sporulation. Lysozyme resistance provided the most straightforward and easily quantifiable assay and gave a time of commitment of 70–80 min (Fig. 1). The plating assay gave a slightly later time of commitment of 80–90 min (Fig. 2). Although less precisely measured, commitment to expression of specific genes dependent on either σF or σE probably occurred in the same general period (Fig. 4). This period stretches roughly from the time that the first cells begin to complete asymmetric septation, about 60–65 min after resuspension (Partridge & Errington, 1993), to the onset of engulfment, beginning about 15–20 min later. All of the measured times precede by at least 10 min the completion of engulfment, which was previously reported to be the time of "final commitment" for B. subtilis (Cooney et al., 1977; Freese & Freese, 1977). There are several possible reasons for the discrepancy between our results and those of some earlier workers. As reviewed by Freese & Heinzle (1984), the composition of the medium, both before and after enrichment, is important. Alternatively, the resuspension method we have used, with a defined time of induction of sporulation, may allow the relative timing of commitment and morphological events to be more precisely related.

**Prespore and mother cell commitment and the possible involvement of σF and σE**

Commitment apparently occurs at about the time that σF and σE, the first cell-specific sigma factors, begin to become active (80 min; Partridge & Errington, 1993). It is therefore possible that commitment is related to the establishment of these transcription factor activities. We tried to examine this by testing various defined spo mutants...
Commitment to sporulation in Bacillus subtilis

with the plating assay but the results were complicated by non-specific effects on colonial growth rates apparently caused by the morphological aberrations of the early blocked mutants. Nevertheless, two tentative conclusions could be drawn from the results. First, $\sigma^F$ may play an important role in prespore commitment. Although the spoIG and spoIIA mutants are morphologically abnormal (in producing two prespore-like cells, rather than a prespore and a mother cell: Lewis et al., 1994; Piggot & Coote, 1976), they seemed to differ in plating efficiency. The apparent loss of viability in the spoIG mutant would be consistent with commitment of the prespore being controlled by $\sigma^F$. Magill & Setlow (1992) previously reported that spoIIA mutants produce stable, viable 'sporelets', but that spoIG mutants do not. Second, the results appeared to support the notion that commitment occurs at about stage II rather than later; mutants that activate $\sigma^E$ and $\sigma^F$ but are blocked at stage II or stage II–III (e.g. spoIB and spoIID mutants; see Errington, 1993) appeared to show normal commitment (Fig. 3; see also below).

The results with gene fusions suggested that mother cell commitment might occur slightly later than that of the prespore. In a series of experiments similar to that of Fig. 4, we reproducibly found slightly later commitment with the mother-cell-specific $\sigma^E$-dependent reporter than with the prespore-specific $\sigma^F$-dependent reporter (data not shown). The slightly later commitment of the mother cell would be consistent with the dependence of the mother cell sigma factor, $\sigma^E$, on the prespore sigma factor, $\sigma^F$ (Karow et al., 1995; Londoño-Vallejo & Stragier, 1995). It would also be consistent with the previous observations of Fréhel & Ryter (1969): ultrastructural examination of enriched sporulating cultures revealed apparently committed stage II prespores at the tips of filaments of vegetative cells, which were presumably derived by growth of uncommitted mother cells.

The establishment of $\sigma^E$ and $\sigma^F$ activities in their respective compartments represent key control points in the regulation of gene expression during sporulation. Our results suggest that they are important for commitment, but further work is needed to establish whether their involvement is direct, and whether prespore and mother cell commitment are completely separate or interdependent events.

Significance of the absence of SG colonies in mutants

In the plating assay, we used the disappearance of RG colonies as an indication of commitment. In the wild-type, such colonies were replaced after 80 or 90 min by a new class (SG colonies) exhibiting much slower growth. Following from Freese & Freese (1977), we suggest that the SG colonies represent cells in which the mother cell has become committed to sporulation and cannot resume growth. Their associated prespores can grow but they do so much more slowly than the vegetative cells from which they are derived. Perhaps there is an extended lag period before growth because prespores are much smaller and thus contain only a fraction of the cytoplasm of vegetative cells. Alternatively, or in addition, the proteins they contain, which begin adapting the cell for a prolonged period of dormancy, are iminical to rapid resumption of growth. Whatever the explanation for the slow growth, if SG colonies arise by the regrowth of prespores, their occurrence in mutant cultures should be dependent on prespore viability. Perhaps surprisingly, SG colonies were virtually absent in almost all of the mutants tested (Table 2). Several of the mutants (e.g. spoVD, spoVB and spoVE) are probably defective specifically in the synthesis of the spore cortex (Daniel et al., 1994; Henriques et al., 1992; Piggot & Coote, 1976; Popham & Stragier, 1991). A properly formed cortex may be needed for the prespore to withstand the osmotic shock of mother cell lysis. The significant numbers of SG colonies made by some mutants is probably due to them making more robust prespores. Thus, spoIVC mutants have been reported to produce relatively stable phase grey 'sporelets' (Piggot & Coote, 1976), and spaV-A mutants produce relatively stable phase 'white' spores, which are reported to be slow to regrow (Errington & Mandelstam, 1984). These considerations support the idea that in the wild-type an SG colony is formed when the mother cell has become committed and the only route to regrowth is via the prespore. It is possible that we may be able to exploit the loss of prespore viability in late blocked sporulation mutants to provide a means of selecting for mutations that overcome mother cell commitment, thus opening the way to a molecular dissection of commitment.

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