Structure and function of the spoIIIJ gene of Bacillus subtilis: a vegetatively expressed gene that is essential for $\sigma^G$ activity at an intermediate stage of sporulation

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The spo-87 mutation is one of two sporulation mutations originally used to define the spo0J locus of Bacillus subtilis. We now show that it blocks sporulation after completion of prespore engulfment (stage III). Surprisingly, the operon is expressed vegetatively, probably from a $\sigma^+$-dependent promoter, and its expression is shut down at the transcriptional level at about the onset of sporulation. DNA sequencing reveals that the locus defined by spo-87, which we now designate spoIIIJ, consists of a bicistronic operon. However, only the first gene is essential for sporulation; the function of the second cistron is cryptic. The predicted SpoIIIJ product has $M_r$ of 29409. It probably forms a lipoprotein and is rich in basic and hydrophobic amino acids. Mutations in spoIIIJ abolish the transcription of prespore-specific genes transcribed by the $\sigma^G$ form of RNA polymerase but not transcription of the spoIIIG gene encoding $\sigma^C$. The SpoIIIJ product could be involved in a signal transduction pathway coupling gene expression in the prespore to events in the mother cell, or it could be necessary for essential metabolic interactions between the two cells.

Introduction

Sporulation in Bacillus subtilis is a simple developmental system involving the differentiation of two cells known as the prespore and the mother cell. About 50 genes involved in the regulation of sporulation have been defined by spo mutations, which block sporulation but have no observable effect on vegetative growth (Pigott & Coote, 1976; Losick et al., 1986). Most of the spo genes have now been cloned and characterized, and their patterns of interaction have been partially elucidated (reviewed by Errington, 1992). It is clear that different programmes of gene expression occur in the prespore and mother cell from the time of their separation onwards (Errington & Illing, 1992; Losick & Stragier, 1992).

The spo-87 mutation abolishes the expression of genes in both the prespore and the mother cell from about the second hour of sporulation (Kunkel et al., 1988; Sandman et al., 1988; see below). The prespore-specific genes that are blocked are dependent on the $\sigma^G$ form of RNA polymerase, which is synthesized specifically in the prespore from the spoIIIG gene (Sun et al., 1989; Karmazyn-Campelli et al., 1989). The mother-cell-specific genes that are blocked are dependent on the $\sigma^K$ form of RNA polymerase (Kroos et al., 1989). The effects of spo-87 on $E\sigma^K$-dependent gene expression are probably indirect, being mediated by a mechanism that couples the formation of active $\sigma^K$ to gene expression in the prespore (Cutting et al., 1990, 1991). In contrast, events associated with the first two hours of sporulation, up to about the stage of prespore engulfment (stage III), are unperturbed by the spo-87 mutation.

It was originally thought that spo-87 was a spo0 mutation, both because it appeared to affect the early stages of morphological development and because genetic mapping seemed to place it in the same locus, spo0J, as the spo-93 mutation (Hranueli et al., 1974), which, on the basis of its effects on gene expression, is clearly a spo0 mutation (e.g. Errington & Mandelstam,
that electron microscopical studies of spoIIIJ, cloning and complementation experiments have shown to occur, in accordance with the results of Ryter, and hence propose that the gene defined by spo-87.

Conflicting results have been obtained by Hranueli et al. (1974) and Clarke & Mandelstam (1987) that cut in the polylinker: from SstI to XbaI inclusive (D. Foulger, unpublished results). The ermC gene can therefore be released by digestion with several enzymes, including SmaI.

We have also physically characterized the spoIIIJ locus. Disruption of the gene blocks the expression of late sporulation genes but has no observable effect on vegetative growth. A downstream gene, which is probably coordinately regulated, is not essential for either vegetative growth or sporulation. Surprisingly, although spoIIIJ is required only after engulfment, apparently for the appearance of σ^Sp activity, the gene is expressed in vegetative cells and turned off at the onset of sporulation.

Table 1. Bacterial strains, plasmids and bacteriophages

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>B. subtilis</td>
<td>trpC2 spo+</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>CU267</td>
<td>trpC2 ilvB2-leuB16 spo+</td>
<td>Errington &amp; Mandelstam (1986a)</td>
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<tr>
<td>SG38</td>
<td>trpC2 spo+</td>
<td>Stragier &amp; Mandelstam (1986a)</td>
</tr>
<tr>
<td>1.5</td>
<td>trpC2 spoIIAC1</td>
<td>Errington &amp; Mandelstam (1986a)</td>
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<td>Errington &amp; Mandelstam (1986a)</td>
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<td>55.3</td>
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<td>87.4</td>
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<td>Hranueli et al. (1974)</td>
</tr>
<tr>
<td>713</td>
<td>trpC2 Δ(spoIVA-lacZ cat)713</td>
<td>Stevens et al. (1992)</td>
</tr>
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<td>727</td>
<td>trpC2 Δ(spoIIIJ::ermC)727</td>
<td>This paper</td>
</tr>
<tr>
<td>729</td>
<td>trpC2 Δ(jag::ermC)729</td>
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<td>Plasmids</td>
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<td>pE194</td>
<td>ermC</td>
<td>Horinouchi &amp; Weisblum (1982)</td>
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<tr>
<td>pSG2</td>
<td>bla cat</td>
<td>Fort &amp; Errington (1985)</td>
</tr>
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<td>pSG28</td>
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<td>pSG139</td>
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<td>Foulger &amp; Errington (1989)</td>
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<td>φ105320</td>
<td>spoVA-lacZ cat</td>
<td>Errington &amp; Mandelstam (1986b)</td>
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<td>φ105349</td>
<td>spoIAA-lacZ cat</td>
<td>S. R. P. &amp; J. E., unpublished</td>
</tr>
<tr>
<td>φ105393</td>
<td>spoIIIJ+</td>
<td>Errington &amp; Jones (1987)</td>
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† Strain made isogenic with SG38 by transformation of strain CU267 as described by Errington & Mandelstam (1986a).

1986a; Guzman et al., 1988). More recently, however, cloning and complementation experiments have shown that spo-87 and spo-93 lie in separate loci (Errington & Jones, 1987). Conflicting results have been obtained by electron microscopical studies of spo-87 mutants. Hranueli et al. (1974) and Clarke & Mandelstam (1987) concluded that the mutant was blocked before the formation of the spore septum (i.e. at stage 0), whereas Ryter (unpublished results cited by Stragier et al., 1988) stated that it was blocked at stage III. Here we confirm that the spoIIIJ mutation allows prespore engulfment to occur, in accordance with the results of Ryter, and hence propose that the gene defined by spo-87 be redesignated spoIIIJ, to distinguish it from the spo0J gene defined by spo-93.

We have also physically characterized the spoIIIJ locus. Disruption of the gene blocks the expression of late sporulation genes but has no observable effect on vegetative growth. A downstream gene, which is probably coordinately regulated, is not essential for either vegetative growth or sporulation. Surprisingly, although spoIIIJ is required only after engulfment, apparently for the appearance of σ^Sp activity, the gene is expressed in vegetative cells and turned off at the onset of sporulation.

Methods

Bacterial strains, plasmids and bacteriophages. These are listed in Table 1, except for newly constructed plasmids, which are illustrated in Fig. 3. Plasmids pP5, pH27, pH28 and pSX3 contain fragments of DNA from the insert in phage φ105393 subcloned in pH2. Plasmids pSG173 and pSG1020 were constructed as follows. The 543 bp BclI-SstI fragment from 4105593, containing the spoIIIJ DNA could be removed by digestion with SmaI. This resulted in a plasmid pUC18BS from which the same fragment of spoIIIJ DNA could be removed by digestion with Smal (there being a SmaI site adjacent to the left end of the insert in 4105593, and one in the polylinker of pUC18). This Smal fragment was then cloned into pSG28 to construct a transcriptional lacZ fusion. Plasmid pSG250 contains the insertion in the opposite orientation. Plasmid pSG250 is a derivative in which the Smal fragment is oriented so that the spoIIIJ promoter drives transcription of the lacZ gene. Plasmid pSG173 has the insertion in the opposite orientation. Plasmid pSG250 contains the Strl-ClaI fragment of pE194, containing the ermC gene cloned into Strl/AccI-digested pUC18. The insertion occurred in an unusual manner such that the ermC fragment was inverted and is flanked by almost completely duplicated polylinker sequences. The ermC gene can therefore be released by digestion with several enzymes that cut in the polylinker: from Strl to XbaI inclusive (D. Foulger, unpublished results).

Construction of erm insertions in the spoIIIJ and jog genes. Plasmids pUC18BS, pPS and pSG250 were digested with Strl to completion, then extracted with phenol and precipitated with ethanol. The redissolved DNA samples were mixed in the ratio 3:3:1, to give a total.
of 0.5 μg DNA and added to a 10 μl ligation reaction. The ligation products were transformed into *B. subtilis* strain 168 with selection for the *erm* determinant from pSG250, on Oxoid nutrient agar containing 1 μg erythromycin ml⁻¹ and 25 μg lincomycin ml⁻¹. Spo⁻ transformants that were stable during repeated subculture in the absence of selection, and complemented by phage φ105J93, were checked by Southern hybridization for the expected chromosomal insertion of the *erm* gene. One such strain was isolated and designated strain 727.

To make an *erm* insertion in the *jag* gene, the *Tagl* fragment containing the *erm* gene from pE194 (see Horinouchi & Weisblum, 1982) was cloned into the unique *PstI* fragment of DNA polymerase. The resultant plasmid was transformed into strain 168 with selection for erythromycin resistance as described above. A chloramphenicol sensitive transformant was checked for the expected chromosomal insertion by Southern hybridization, and designated strain 729.

In both cases the orientation of the *erm* gene is the same as that of *spoIIIJ* and *jag*.

**Media and induction of sporulation.** Cells were induced to sporulate by the resuspension method of Sterlini & Mandelstam (1969). Nutrient exhaustion experiments were done by growth in Difco sporulation medium (DSM; Schaeffer et al., 1965).

**Electron microscopy.** Cells were fixed, sectioned and visualized as described previously (Illing & Errington, 1991b).

**DNA sequencing.** Fragments of the DNA originally cloned in *φ105J93* were subcloned and sequenced (both strands) by the chain-termination method (Sanger et al., 1977).

**Isolation of RNA and primer extension analysis.** RNA was isolated by the rapid procedure described previously (Fougeron & Errington, 1989). An oligonucleotide, 5'-TCACACTCGAGCATCCAGCC-3', complementary to the *spoIIIJ* mRNA was used for the primer extension reaction, which was carried out with AMV reverse transcriptase (Pharmacia) essentially as described by Moran (1990). The sequencing ladder was generated by an oligonucleotide complementary to a segment of DNA from the *murE* gene of *B. subtilis* (R.D. & J.E., unpublished results).

**lacZ fusion analysis.** Plasmids pSG173 and pSG1020 were transformed into *B. subtilis* by selection for chloramphenicol resistance. Purified transformants were induced to sporulate and assayed for β-galactosidase activity as described previously (Errington & Mandelstam, 1986a), except that lysozyme was used to permeabilize the cells (Mason et al., 1988).

**Results and Discussion**

**Ultrastructure of the spo-87 mutant**

In view of the conflicting reports on the phenotype of the *spo-87* mutant we induced strain 87.4 to sporulate and harvested the cells at *t*₅ for examination by electron microscopy. The majority of the cells (＞60%) appeared to have completed engulfment (stage III) at this time. Other cells contained completed asymmetric septa or were in the process of engulfment. The prespores in cells that had reached stage III had a very indistinct appearance, similar to that seen transiently in wild-type cells that have just completed engulfment but not yet initiated cortex synthesis (J.E., unpublished observations). Examples of cells in these various states are shown in Fig. 1. The indistinct appearance of the prespores of the *spo-87* mutant probably accounts for its earlier classification as a *spo0* mutant (Hranueli et al., 1974).

We conclude that the *spo-87* mutation causes sporulation to be arrested at stage III, in accordance with the results of Ryter (cited by Stragier et al., 1988). In view of the fact that the mutation is now known to lie in a distinct locus from the *spo-93* mutation (Mysliwiec et al., 1991), we propose to redesignate the locus defined by *spo-87* as *spoIIIJ*, whilst retaining the *spoOJ* designation for *spo-93*. We have passed over the next available *spoIII* designation *spoIIIH* in order to retain the suffix 'J'.

**Effect of a null mutation in the spoIIIJ gene on expression of other sporulation genes**

To define the role of the *spoIIIJ* gene in the expression of other sporulation genes, we introduced several *spo- lacZ* fusions into a newly constructed *spo IIIJ:* :ermC insertion mutant (strain 727). We then induced the strains to sporulate and compared the levels of β-galactosidase activity in isogenic mutant and wild-type strains (Fig. 2). We also measured alkaline phosphatase activity, which is a marker for early mother-cell-specific gene expression (Akrigg & Mandelstam, 1978; Errington & Illing, 1992). As expected (Clarke & Mandelstam, 1987), alkaline phosphatase synthesis was not blocked by the *spoIIIJ:* :ermC mutation, so the enzyme activity provided a control showing that sporulation was induced normally in each of the strains. Inactivation of *spoIIIJ* had little effect on expression of *spoIIA-*lacZ (Fig. 2a) or *spoVA-*lacZ fusions (not shown), as expected for genes expressed before stage III. It reduced expression of the prespore-specific genes *spoIIG* and *spoVA* about twofold and 10-fold respectively (Fig. 2b and c). Similar results have been obtained with the *spoIIIJ87* mutation (S. Maguire & J. Errington, unpublished results). Thus, *spoIIIJ* mutations seem to affect the expression of prespore-specific genes but not early mother-cell-specific genes (see below).

**Structure of the spoIIIJ operon**

We have previously reported the isolation of phages φ105J92 and φ105J93 that complement the *spoIIIJ87* mutation (Errington & Jones, 1987). Restriction maps of the two recombinants indicated that they contained overlapping insertions. Fragments of the insertion from phage φ105J93 were subcloned into plasmid pSG2 (Fig. 3) and the derivative plasmids were tested for insertion
inactivation of the spoIIIJ operon or for their ability to rescue the spoIIIJ87 mutation. None of the plasmids obtained gave an observable phenotypic effect when integrated into the wild-type strain SG38, indicating that their insertions do not lie wholly within the spoIIIJ locus (see Piggot et al., 1984). However, plasmids pH28 and pP5 gave a mixture of Spo⁺ and Spo⁻ colonies when transformed into strain 87.4. This indicated that the insertions overlapped the site of the spoIIIJ87 mutation and that one end of each insertion probably lay within the spoIIIJ transcription unit.

A region of 1830 bp from the left-hand part of the insertion in φ105J93 as shown in Fig. 3 was sequenced (Fig. 4). Three major open reading frames (ORFs) were
found, all reading left to right as shown in Fig. 3. ORF1 began at the BclI site marking one end of the insert originally cloned in phage φ105J93. This partial ORF was homologous to the rnrA gene of Escherichia coli. ORF2 began after a space of about 150 bp, and was capable of encoding a protein of 260 amino acid residues (M, 29409). This ORF ended about 20 bp before the PstI site at one end of the insert in plasmid p5, suggesting strongly that this ORF corresponded to the spoIIIJ gene. It contains an unusually larger proportion of basic residues compared with acidic residues (K + R, 27; D + E, 13), and has several extensive regions containing mainly hydrophobic side chains. The N-terminal region contains three basic amino acids, followed by a sequence of 20 mainly hydrophobic residues containing the sequence LAGC, suggesting that this is likely to form a lipoprotein (Pugsley & Schwartz, 1985).

Immediately adjacent to the putative spoIIIJ ORF was another ORF (ORF3) capable of encoding a polypeptide of 208 residues, again containing an excess of basic amino acids (K + R, 33; D + E, 23) but no extensive clusters of hydrophobic residues. Neither predicted protein showed significant homologies to heterologous proteins in the SWISSPROT database or to the translation products of GenBank/EMBL databases.

ORF2 and ORF3 were both preceded by reasonable ribosome-binding site (RBS) sequences (ΔG = -16-6 and -14-4 kcal mol⁻¹ [-69-4 and -60-2 kJ mol⁻¹], respectively). ORF2 has adjacent ATG and TTG codons near the RBS sequence: the latter is probably the major initiation site, given its distance from the RBS (Mountain, 1989). ORF3 has a likely translation initiation site at a GTG codon that overlaps the end of ORF2 in the sequence GTGA. This sequence could allow translational coupling. Since plasmids p5 and pH28 could integrate into the wild-type without generating an obvious phenotypic effect on either vegetative growth or sporulation, it seemed probable that ORF3 does not encode a function required for either of these activities. However, it was also possible that ORF3 could be expressed from a promoter within ORF2 or from a plasmid promoter.

To distinguish between these possibilities we inserted an erythromycin resistance gene into each ORF, generating strains 727 and 729 (Fig. 3). Both strains were constructed without difficulty and showed no obvious phenotype during growth (results not shown). Strain 729, with the insertion in ORF3, sporulated normally, but strain 727 was asporogenous and had a similar phenotype to that of the spoIIIJ87 mutant, as described above. These results confirmed that only ORF2 was essential for sporulation. We propose to designate ORFs 2 and 3 spoIIIJ and jag (spoIIIJ associated gene) respectively.

During the preparation of this manuscript, the sequence of this region of the chromosome was described by another group (Ogasawara & Yoshikawa, 1992). Our sequence differs from theirs at one position: our G at position 541 is A in the sequence of Ogasawara & Yoshikawa (1992). We are confident that our assignment is correct, because this base lies in a SstI restriction site that we used for some of the plasmid constructions. The change would not affect the translation product, so it is possible that the discrepancy is due to natural strain variation. As pointed out by Ogasawara & Yoshikawa (1992) the spoIIIJ gene product exhibits strong sequence similarity to proteins in the replication origins of both Pseudomonas putida and E. coli. Unfortunately, the
functions of these genes in the non-spore-forming organisms are unknown; this raises the possibility that SpoIIIJ may not only function during sporulation.

Regulation of spoIIIJ expression

To examine the regulation of the spoIIIJ locus we constructed plasmid pSG1020, which, on integration into the B. subtilis chromosome, would generate a spoIIIJ'-lacZ fusion. Plasmid pSG173 was similar to pSG1020 but with the promoter fragment in the reverse orientation (Fig. 3). The two plasmids were transformed into B. subtilis SG38 with selection for chloramphenicol resistance. The resultant strains were induced to sporulate by the resuspension method (Fig. 5a). As expected, β-galactosidase was produced by the strain containing pSG1020 but not by that containing pSG173 (apart from the low level of endogenous activity; Errington & Vogt, 1990). Surprisingly, the peak β-galactosidase activity in SG38::pSG1020 was found at t₀, in the first sample taken. Thereafter, the activity fell steadily, until sampling was stopped at t₅.

These results suggested that spoIIIJ expression occurs predominantly in vegetative cells. We therefore measured β-galactosidase production during growth and sporulation in DSM, in which sporulation is initiated by exhaustion of a limiting amount of glucose. Again, substantial β-galactosidase synthesis occurred in the vegetative state and the activity fell from about t₀ onwards (Fig. 5b). The double peak of β-galactosidase activity in Fig. 5(b) was reproducible but its significance is unclear.

Genes expressed predominantly in vegetative cells would be expected to have promoters recognized by the major vegetative form of RNA polymerase, EσA, and thus should be unaffected by mutations affecting gene expression during sporulation. To test this, we introduced the spoIIIJ'-lacZ fusion plasmids into strains containing mutations in various genes encoding regulators of sporulation gene expression: spoOA, spoOH, spoIIA and spoIIIG. Each derivative strain was induced to sporulate by resuspension and then assayed for β-galactosidase. In every case, the pattern of β-galactosidase synthesis was indistinguishable from the isogenic derivative with no spo mutation (results not shown). Thus, the spoIIIJ-gene seems not only to be expressed predominantly in vegetative cells, but also to be unresponsive to sporulation-associated regulatory effectors.

To confirm that the spoIIIJ gene is transcribed by the σA form of RNA polymerase, we used primer extension
analysis to determine its transcription start site in vivo (Fig. 6). A single predominant extension product was observed with RNA from vegetative cells, corresponding to initiation of transcription at nucleotide 352 in the sequence shown in Fig. 4. A specific transcript was barely detectable in the RNA from sporulating cells. As expected, the transcription start site lies just downstream from sequences that are quite similar to many promoters recognized by EaA (Fig. 4), particularly in the ‘−10’ and ‘−16’ regions (Moran et al., 1982; Henkin & Sonenshein, 1987), though there is no obvious ‘−35’ sequence in the appropriate position. This could indicate that transcription is facilitated by a positive regulator acting in conjunction with EaA. The location of this promoter within the region cloned in phage 4105593 would be consistent with the ability of this phage to complement mutations in the spoIIIJ gene. However, there is no clear transcriptional termination signal.
Fig. 5. Expression of spoIIIJ-lacZ during growth and sporulation. Strains SG38::pSG173 (○) and SG38::pSG1020 (●) were induced to sporulate by resuspension (a) or nutrient exhaustion (b; the latter strain only) and assayed for β-galactosidase activity. The onset of sporulation was defined by the time of resuspension in sporulation medium (a) or by the time at which the growth rate (b) deviated from exponential (b).

between the rnpA and spoIIIJ genes, so the possibility that some transcription of spoIIIJ occurs from a promoter upstream of rnpA cannot be excluded. Indeed weak higher Mr bands were observed in some of the primer extension experiments (data not shown).

Conclusions

The spoIIIJ locus probably consists of a bicistronic operon, which is expressed in vegetative cells and then turned off early in sporulation. The first gene but not the second is needed for sporulation. In the absence of spoIIIJ, sporulation is arrested at stage III: prespore engulfment is completed but expression of prespore-specific genes, such as spoVA, is blocked, indicating that the prespore sigma factor σG (Sun et al., 1989) is not made or is inactive. Since substantial expression of a spoIIIG-lacZ translational fusion occurs in a spoIIIJ mutant (Fig. 2b), it seems likely that the mutation affects the activity rather than the synthesis of σG (Stragier, 1992). The subsequent absence of late mother cell gene expression (Kunkel et al., 1988; Sandman et al., 1988) is probably due to the requirement for expression of one or more prespore genes, such as spoIVB, which somehow control the proteolytic activation of the late mother cell sigma factor σK (Cutting et al., 1990, 1991).

The apparent role of SpoIIIJ in σG activation is interesting. Mutations in several other genes – spoIIB, spoIID, spoIM, spoIIA and spoIIIG – cause substantial reductions in prespore-specific gene expression but have relatively minor effects on a spoIIIG-lacZ fusion
(Foulger & Errington, 1989; Karmazyn-Campelli et al., 1989; Mason et al., 1988; C. Karmazyn-Campelli & P. Stragier, unpublished results, cited in Stragier, 1992; S.R.P. & J.E., unpublished results). This could mean that their gene products act at the level of $\sigma^F$ activity, as suggested previously (Stragier, 1992), or its stability. At least two of these genes are expressed in the mother cell, as a result of transcription by the $\sigma^F$ form of RNA polymerase (Driks & Losick, 1991; Illing & Errington, 1991a). Stragier (1992) has suggested that the SpoIID product is required for engulfment, and that the SpoIIIA proteins are required for a signal transduction pathway that couples prespore gene expression (via $\sigma^G$ activity) to mother cell development. An alternative possibility would be that loss of the later phase of prespore gene expression is due to the sensitivity of the engulfed prespore to the metabolic state of the mother cell, or to metabolic communication between the two cells (Illing & Errington, 1990). In either case, the probable membrane location of the putative SpoIIIJ protein would be compatible with a function in some form of communication between the prespore and mother cell. Elucidating the mechanisms whereby the developmental programmes of the prespore and mother cell are coupled is likely to be a challenging and rewarding problem.

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References


