spoIID Operon of Bacillus subtilis: Cloning and Sequence

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The locus spoIID, involved in the sporulation of Bacillus subtilis, was cloned into derivatives of the temperate phage φ105. Two recombinant phages were obtained which contain chromosomal DNA covering 1.6 kbp. They are both able to complement mutations spo-68 and spo-298. These mutations, which were believed to be in different loci, spoIID and spoIIC respectively, were shown to be closely linked, and both map at the position assigned to spoIID on the genetic map of B. subtilis. The sequence of 1656 bp carrying the spoIID locus was determined. Only one open reading frame was found; this codes for a protein of 343 amino acids. It is preceded by a ribosome binding site and possible recognition sequences for σ32- and σ29-RNA polymerases. Studies of the locus by means of integrational plasmid vectors defined the outer limits of the transcriptional unit. These results are completely compatible with the sequence data. The combination of sequence and mapping and the information obtained by the use of integrational plasmids confirm that the spoIID locus functions as a monocistronic operon.

INTRODUCTION

For the last two decades Bacillus subtilis sporulation has been studied as a primitive model of cellular differentiation. Much of the research has concentrated on attempts to understand the genetic complexity of the process. At least 50 sporulation-specific operons need to be expressed before viable spores are formed (Young & Mandelstam, 1979; Piggot et al., 1981). The number of sporulation genes that has been cloned is growing rapidly and several of them have been cloned in phage φ105 (Birdsell et al., 1969) or its derivatives. For example, the derivative p105J9 has been used to make DNA clone banks from which several genes have been isolated (Errington, 1984). Among the loci that have been cloned in φ105 are spoOF (Kawamura et al., 1981), spoIIIB (Jenkinson & Mandelstam, 1983), spoIIC (Anaguchi et al., 1984), spoIIA and spoVA (Savva & Mandelstam, 1984), spoIIG (Ayaki & Kobayashi, 1984) and gerE (James & Mandelstam, 1985). In this paper the cloning of the spoIID locus in φ105 is described.

When the sequence of a cloned fragment is known it is then fairly easy to determine the number of genes it contains and whether they are expressed as an operon (Fort & Errington, 1985). Knowledge of the sequence may also help in elucidating the function of a gene. spoIIG and the C protein of spoIIA, according to their sequences, code for sigma factor-like proteins and therefore have regulatory functions involving transcription of sporulation loci (Stragier et al., 1984; Errington et al., 1985). Other sequences have been less informative and the functions of the genes remain unknown.

Loci involved in sporulation at stage II are particularly interesting since during the last two to three years it has become apparent that most, perhaps all, of the known genes concerned with sporulation at stage 0–1 are expressed during vegetative growth (Losick, 1982). Those that control stage II – septation and the ensuing engulfment of the pre-spore – can consequently be

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considered as the first genes that are specifically concerned with sporulation. Mutations in spoII loci not only prevent the expression of biochemical markers concerned with later stages but also produce gross physical abnormalities such as multiple septation, formation of abortively disporic forms, etc. (see, e.g., Waites et al., 1970). It is of particular interest to determine the nature of these genes and, if possible, the functions of the proteins that they encode. We describe here the sequence and analysis of the spoIID locus.

**METHODS**

**Bacterial strains, phages and plasmids.** These are listed in Table 1.

**Induction of sporulation and measurement of marker events.** Cells were grown and induced to sporulate at 37 °C by the method of Sterlini & Mandelstam (1969). Serine protease was measured as described by Dancier & Mandelstam (1975) and alkaline phosphatase as described by Glenn & Mandelstam (1971). Methods for electron microscopy were those of Kay & Warren (1968).

**Preparation of DNA.** *B. subtilis* chromosomal DNA was prepared as described by Errington (1984) and phage ϕ105 DNA as described by Jenkinson & Mandelstam (1983). Plasmid DNA was extracted from *Escherichia coli* by the alkaline lysis method (Maniatis et al., 1982). Chromosomal DNA for transformations was prepared according to Ward & Zahler (1973).

**Buffers.** The ligation buffer normally used was 66 mM-Tris/HCl (pH 7.2), 5 mM-MgCl₂, 5 mM-DTT and 1 mM-ATP. For blunt-end ligations, 10 mM-Tris/HCl (pH 7.5), 50 mM-NaCl, 10 mM-MgCl₂, 5 mM-DTT and 1 mM-ATP was used. TBE buffer contained 90 mM-Tris/HCl (pH 8.3), 90 mM-boric acid and 2.5 mM-EDTA. Tris/acetate was 40 mM-Tris, 20 mM-acetic acid and 2 mM-EDTA (pH 8.1). Gel loading buffer was 30% (w/v) Ficoll, 25 mM-EDTA, 0.25% (w/v) bromphenol blue and 0.25% (w/v) xylene cyanol FF.

**Restriction endonuclease digestions.** Restriction enzymes were obtained from Amersham or BRL and used as recommended by the suppliers.

**DNA electrophoresis and removal of DNA from agarose gels.** Loading buffer was added to DNA, fragments were separated on horizontal agarose gels (0.8%, Sigma type II) in TBE buffer and viewed by ethidium bromide fluorescence. If DNA was to be recovered, low melting point agarose (BRL) was used and the DNA was extracted as described by Savva & Mandelstam (1984).

**DNA hybridization.** Agarose gels containing phages ϕ105D1:1t, ϕ105LD1 and ϕ105LD2 were transferred to nitrocellulose (Anderman), by the Southern transfer method (Southern, 1975). The 2 kbp PstI fragment of ϕ105LD2 (Fig. 2) recovered from a low melting point agarose gel was nick translated and labelled with 32P using a kit from Amersham. Filter preparation, hybridization and autoradiography were as described by Maniatis et al. (1982) and Savva & Mandelstam (1984).

**Isolation of phage.** Preparation of ϕ105 lysates, phage purification and measurement of titre were as previously described (Jenkinson & Mandelstam, 1983). Transducing lysates of PBS1 were made from donor strains essentially by the method of Karamata & Gross (1970), as described by Coote (1972).

**Transductions.** The procedure used for PBSI transductions has been described elsewhere (Coote, 1972). In transductions with phage ϕ105, the recipient strains were grown to mid-exponential phase in Penassay broth (Oxoid). Cells (0.2 ml) were mixed with 0.1–0.2 ml phage lysate and immediately plated on Schaeffer agar (Schaeffer et al., 1965) in glass plates.

**Construction of recombinant phages ϕ105LD1 and ϕ105LD2.** *B. subtilis* 168 DNA (20 μg) completely digested with EcoRI was ligated to 10 μg plasmid pDSMU7 (Savva & Mandelstam, 1984) cut with EcoRI and HindIII. The ligation mixture was used to transform competent MY2000.68 (lysogenic for phage ϕ105D1:1t) to Spo⁺ by prophage transformation (Kawamura et al., 1979). DNA from the resulting unstable phage ϕ105LD1 was partially digested with MboI to give pieces of a size not less than about 2 kbp. This DNA (300 ng) was ligated to ϕ105J9 (600 ng cleaved with BamHI) (Errington, 1984) and used to transfect protoplasts of strain CU267 using the method described by Errington (1984). A bank of recombinant phages was prepared from which ϕ105LD2 was isolated by selection for transduction of strain MY2000.68 to Spo⁺.

**Construction of recombinant plasmids. (a) Plasmids pSGLD1 and pSGLD2.** For pSGLD1, 5 μg phage ϕ105LD1 DNA was digested with EcoRI; 60 ng of the 4 kbp fragment, recovered from a low melting point agarose gel, was ligated to 5 ng EcoRI-cleaved pUC13 in a volume of 50 μl at 15 °C for 18 h and used to transform *E. coli* JM103. Plasmid pSGLD2 was constructed using the 1.6 kbp EcoRI–SalI fragment from ϕ105J11 (Errington, 1984) in a similar fashion.

(b) **Integralational plasmids.** Fragments of pSGLD1, as listed in Fig. 6, were recovered from low melting point agarose gels and ligated to pSGMU2 (Fort & Errington, 1985) in 50 μl volumes with 2–5 μM excess of insert fragments.

**Integralational analysis.** The Spo⁺ strain MB75 was transformed with about 1 μg DNA from the integralational plasmids (Fig. 6). Chloramphenicol-resistant transformants were selected on Schaeffer agar containing chloramphenicol (5 μg ml⁻¹). Mature colonies were easily distinguishable as Spo⁺ or Spo⁻ since Spo⁻ colonies tend
**spoIID sequence in Bacillus subtilis**

**Table 1. Bacterial strains, phages and plasmids**

<table>
<thead>
<tr>
<th>Designation</th>
<th>Genotype or description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacillus subtilis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MY2000.68</td>
<td>spoIID68 lys-1 pyrD1</td>
<td>M. Yudkin, Microbiology Unit, Department of Biochemistry, Oxford</td>
</tr>
<tr>
<td>SG43</td>
<td>spoIID68 trpC2</td>
<td>Experiment of MY2000.68</td>
</tr>
<tr>
<td>298.2</td>
<td>spoIID298 phe-12</td>
<td>Laboratory stock</td>
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<td>CU267</td>
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<td>S. A. Zahler, Cornell University, USA</td>
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<td>thr-5 trpC2 cysB3</td>
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</tr>
<tr>
<td>BD163</td>
<td>hisA1 argC4</td>
<td>D. Dubnau, Laboratory stock</td>
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<td>MB75</td>
<td>metC3 lys-1</td>
<td>This paper</td>
</tr>
<tr>
<td>SG42</td>
<td>metC3 lys-1 (pSGLD6 CmR)</td>
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<td><strong>Escherichia coli</strong></td>
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<td></td>
</tr>
<tr>
<td>JM103</td>
<td>N(lac-pro) thi strA supE endA sbcB1S F' traD36 proAB lacI ZAM15</td>
<td>Messing et al. (1981)</td>
</tr>
<tr>
<td><strong>Bacteriophages</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>φ10531:1t</td>
<td>φ105 carrying a 4.0 kb deletion of non-essential DNA</td>
<td>Flock (1977)</td>
</tr>
<tr>
<td>φ105311</td>
<td>spoIID+, 2.6 kbp BclI insert in φ10539</td>
<td>This paper</td>
</tr>
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</tr>
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<td>φ10531D2</td>
<td>spoIID+, 2 kbp MboI insert in φ10539</td>
<td>Takahashi (1961)</td>
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<td>PBS1</td>
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<td>Sequencing vectors</td>
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<td>pSGMU2</td>
<td>Ap⁸ Cm⁸ integrational vector</td>
<td>Fort &amp; Errington (1985)</td>
</tr>
<tr>
<td>pSGLD1</td>
<td>pUC13 + 4 kbp EcoRI of φ10531D1</td>
<td>This paper</td>
</tr>
<tr>
<td>pSGLD2</td>
<td>pUC13 + 1.6 kbp EcoRI–SalI of φ105311</td>
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<td>pSGLD3</td>
<td>pSGMU2 + 0.85 kbp HindIII of pSGLD1</td>
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<td>pSGLD4</td>
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<td>pSGLD6</td>
<td>pSGMU2 + 0.5 kbp HindIII–SalI of pSGLD1</td>
<td></td>
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<td>pSGLD8</td>
<td>pSGMU2 + 0.60 kbp AvA1–HindIII of pSGLD1</td>
<td>Savva &amp; Mandelstam (1984)</td>
</tr>
<tr>
<td>pDSMU7</td>
<td>pUC9 + EcoRI fragment E of φ105</td>
<td></td>
</tr>
</tbody>
</table>

to lyse. Colonies were also examined microscopically. The same procedure was used to map spo-68 and spo-298 with the integrational plasmids.

**Transformations.** B. subtilis recipient cells were made competent and transformed as described by Jenkinson (1983). The method used to transform E. coli JM103 was that described by Fort & Piggot (1984).

**Screening for recombinants and transductants.** (a) E. coli JM103. Inserts in plasmids pUC13 and pSGMU2 or phage M13 were selected after transformation by picking white colonies or clear plaques. If the selection plates contain 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside as substrate (Messing, 1983) the normal blue colour is lost if DNA is inserted into the polylinker. Initial selection was followed by one or more of the following: (1) transformation of appropriate B. subtilis strains to test for the presence of a particular region of DNA, (2) gel electrophoresis before and after restriction endonuclease digestion to check for size changes; (3) sequencing. The media used were as described by Fort & Piggot (1984) and Fort & Errington (1985).

(b) B. subtilis strains. Cotransduction or cotransformation of an unselected spo gene with a selected auxotrophic or drug resistance marker was determined by scoring for development of brown pigment on lactate/glutamate minimal medium or Schaeffer agar as described by Coote (1972). Linkage is expressed as percentage cotransduction or cotransformation. Colonies were also checked microscopically for the presence of spores. Spo⁺ transformants or transductants were selected on Schaeffer agar in glass plates by exposing the plates to chloroform vapour for 2 h at 55 °C (Hoch, 1971). Chloramphenicol resistant (Cm⁸) cells were selected on Schaeffer agar containing chloramphenicol (5 μg ml⁻¹).

**DNA sequencing.** DNA fragments from plasmid pSGLD2 were sequenced by the dideoxy chain termination method of Sanger et al. (1977) using subclones in M13 phage sequencing vectors (Messing, 1983). For exonuclease III–nuclease SI shortening, plasmid pSGLD2 was opened with EcoRI and progressively shortened as described by
Guo et al. (1983). The shortened fragments were cloned into M13 phage for sequencing. The cloning in M13 phage, isolation of recombinants in *E. coli* JM103, labelling of the DNA, running of buffer gradient gels etc. were all essentially as described by Amersham in the *M13 Cloning and Sequencing Handbook*. DNA was labelled with $^{35}$SdATP or $^{32}$PdATP.

**RESULTS**

*Isolation of the recombinant phage φ105LD1*

Using the prophage transformation method (Kawamura et al., 1979) an EcoRI fragment of the *B. subtilis* chromosome, able to complement spoIID68, was cloned as follows in φ105DI:1t, which is a derivative of φ105 with a deletion of 4 kbp (Flock, 1977). Plasmid pDSMU7 (Savva & Mandelstam, 1984), containing the EcoRI fragment E of phage φ105 into which DNA can be cloned, was used as a source of phage DNA which, when ligated to *B. subtilis* chromosomal DNA, could transform the prophage to give a recombinant phage. A spoIID mutant, MY2000.68, which had been made lysogenic for phage φ105DI:1t, was transformed with plasmid pDSMU7 previously cut with EcoRI and HindIII and ligated to fragments of EcoRI-digested *B. subtilis* DNA.

A total of 178 Spo+ transformants was obtained and pooled. A lysate, made by induction with mitomycin C (for details see Jenkinson & Mandelstam, 1983), was tested for ability to transduce

![Fig. 1. Restriction endonuclease analysis of phage φ105DI:1t DNA (lane 1) and its derivative φ105LD1 (lane 2), after digestion with EcoRI. Fragment sizes (kbp) are those reported by Bugaichuk et al. (1984). The faint 4 kbp band Z of the unstable phage φ105LD1 (lane 2) contains the spoIID locus.](image-url)
MY2000.68 to Spo+. A few Spo+ colonies were produced and lysates prepared from them showed transducing activity. However, again, the frequency of transduction to Spo+ was low (only about 1 per 10^3 phage plaques). One of the phages was purified and named φ105LD1. DNA from this phage was able to transform MY2000.68 to Spo+, indicating that it carried the relevant chromosomal fragment.

DNA from φ105LD1 and the parental phage φ105DI:1t was digested with EcoRI (Fig. 1). φ105LD1 produced three bands (X, Y and Z) not present in φ105DI:1t. In addition, bands C and F were very faint in the recombinant phage and band H was not detectable. Restriction patterns obtained with other enzymes also showed differences (results not shown). The variation in staining intensities between bands suggested that there was more than one type of molecule in the preparation. Attempts to separate these types were unsuccessful.

Isolation of recombinant phage φ105LD2

A new recombinant phage, able to complement spoIID more effectively, was constructed as follows. A partial MboI digest of φ105LD1 was ligated into the unique BamHI site in the cloning vector φ105J9 (Errington, 1984). By transfecting protoplasts of B. subtilis CU267 with this DNA (Errington, 1984), a bank of phages was obtained. The recombinant phage φ105LD2 was isolated from this bank by infecting a culture of MY2000.68 and selecting for transduction to spoIID+. A colony showing complementation was induced with mitomycin C. The resultant phage, φ105LD2, was able to complement spoIID68 and it transduced the Spo+ character with much higher efficiency than φ105LD1. DNA from φ105LD2 also transformed MY2000.68 to Spo+.

The fragments obtained after restriction endonuclease digestion of DNA from φ105J9 and its derivative φ105LD2, cut with EcoRI and PstI, are shown in Fig. 2. Since the BamHI cloning site of φ105J9 is flanked by two PstI sites (J. Errington, unpublished), any insert without a PstI site can be seen as a single extra band in a PstI digest. As shown in Fig. 2, the PstI-digested DNA showed one extra band of 2 kbp in φ105LD2 which was not present in φ105J9.

The EcoRI digest showed that the 7-6 kbp fragment B of φ105J9, which contains the unique BamHI cloning site, had been replaced by two fragments of 6 kbp and 3-6 kbp, indicating a site for EcoRI within the 2 kbp insert. However, since the insert came from a partial MboI digest of φ105LD1, it was possible that the insert contained some phage DNA in addition to the spoIID locus.

Hybridization analysis

To find out if there was any phage DNA in the 2 kbp insert of φ105LD2, hybridization experiments were done. The 2 kbp PstI fragment was recovered from an agarose gel and the DNA was used as a hybridization probe against both phage and chromosomal DNA.

There was a weak hybridization signal to the EcoRI fragment E of φ105DI:1t, indicating that the insert of φ105LD2 contained some DNA from fragment E. As described above, φ105LD1 DNA, which was used to make φ105LD2, has fragment E adjacent to the EcoRI chromosomal insert. There was a strong hybridization signal to the 4 kbp EcoRI band Z in φ105LD1 (Fig. 1) and to a band corresponding to 4 kbp in EcoRI-digested chromosomal DNA.

These results indicated that the mutation in spoIID68 is complemented by a region of chromosomal DNA less than 2 kbp in length bounded by EcoRI and MboI sites and that this fragment is contained within a 4 kbp EcoRI fragment.

Comparison of spoIID and spoIIC recombinant phages

We found that phages φ105LD1 and φ105LD2 were able to complement not only mutation spoIID68 but also spoIIC298. We then showed that φ105J11, a recombinant phage containing a 2.6 kbp BclI fragment of B. subtilis able to complement spoIIC298 (Errington, 1984), also complemented spoIID68. In addition, DNA from each of the three phages was able to transform strains containing either spoIID68 or spoIIC298 to spo+.

To get a more precise restriction map of the chromosomal regions cloned in the three phages, the 4 kbp EcoRI fragment of φ105LD1 and the 1-6 kbp EcoRI–SalI fragment of φ105J11 were
Fig. 2. Restriction endonuclease patterns of phage $\phi$105J9 and its derivative $\phi$105LD2. Fragment sizes (kbp) are from Bugaichuk et al. (1984) and Errington (1984). Lanes: 1, $\phi$105J9 cut with PstI; 2, $\phi$105LD2 cut with PstI; 3, $\phi$105J9 cut with EcoRI; 4, $\phi$105LD2 cut with EcoRI. Lane 2 shows an extra PstI fragment of about 2 kbp which contains the spoIID locus. In lane 4, the 7.6 kbp EcoRI fragment B of phage $\phi$105J9, which contains the spoIID insert, has been split into two fragments of 6 kbp and 3.6 kbp.

The relevant restriction maps are shown in Fig. 3. The cloned fragments in $\phi$105LD2 and $\phi$105J11 overlap as shown, giving a total of 5 kbp of chromosomal DNA cloned in the phages. The MboI fragment cloned in $\phi$105LD2 would be as shown, with a region of $\phi$105 DNA of about 0.4 kbp to the left of the EcoRI site. Mutations spoIID68 and spoIIC298 are contained within the EcoRI–MboI fragment of about 1.6 kbp which is common to the three phages, as shown by transformation and complementation.

Comparison of these restriction maps with that given by Anaguchi et al. (1984) suggested that phage $\phi$105IIC described by them contains the same EcoRI chromosomal fragment that is shown in Fig. 3.
spoIID sequence in Bacillus subtilis

(a) \( \phi 105J11 \)

\[
\begin{array}{cccccccc}
B & Sm & E & H & A & P & H & N & H & MS & B \\
\end{array}
\]

(b) \( \phi 105LD2 \)

\[
\begin{array}{cccccccc}
M & E & H & A & P & H & N & H & M \\
\end{array}
\]

(c) \( \phi 105LD1 \)

\[
\begin{array}{cccccccc}
E & H & A & P & H & N & H & MS & B & H & S & E \\
\end{array}
\]

Fig. 3. Restriction maps of the DNA fragments cloned in \( \phi 105 \) phages and which contain the spoIID locus. (a) Phage \( \phi 105J11 \), the 2.6 kbp \( BclI \) fragment cloned in phage \( \phi 105J9 \) by Errington (1984). (b) Phage \( \phi 105LD2 \), the 2 kbp \( MboI \) fragment from phage \( \phi 105LD2 \) cloned in phage \( \phi 105J9 \). The broken line represents the part of fragment E of phage \( \phi 105D1:1t \) which was also cloned when phage \( \phi 105LD2 \) was made. (c) Phage \( \phi 105LD1 \), the 4 kbp \( EcoRI \) fragment cloned in phage \( \phi 105D1:1t \). B, \( BclI \); Sm, \( SmaI \); E, \( EcoRI \); H, \( HindIII \); A, \( AraI \); P, \( PvuII \); N, \( NcoI \); S, \( SalI \); M, \( MboI \). Only the \( MboI \) site determined by the sequence is shown.

Table 2. Percentage cotransduction of spo-298 and spo-68 with cysB3 and hisA1

Recipient cells (1 ml) were transduced with phage PBS1 transducing lysate (0.1 ml) prepared from infected donor strains (see Methods). Spo- transductants were counted after microscopic examination of Aux+ colonies selected on appropriately supplemented lactate/glutamate minimal agar.

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>Selected phenotype</th>
<th>No. of Aux+ transductants examined</th>
<th>No. of Spo-</th>
<th>Percentage cotransduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD111 trpC2 cysB3 lys-1 pyrD1</td>
<td>MY2000.68 spoIID68</td>
<td>Cys*</td>
<td>260</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>298.2 spoIIC298 phe-12</td>
<td>Cys*</td>
<td>208</td>
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<td>His*</td>
<td>270</td>
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<td>298.2 spoIIC298 phe-12</td>
<td>His*</td>
<td>219</td>
<td>38</td>
<td>17</td>
</tr>
</tbody>
</table>

Transduction mapping of spoIID68 and spoIIC298 and examination of their associated phenotypes

According to the \( B. subtilis \) genetic map (Henner & Hoch, 1980), mutations spoIID68 and spoIIC298, which were originally mapped by PBS1 transduction (Piggot & Coote, 1976), are separated by at least a dozen non-sporulation loci, and therefore could not be located within a 1.6 kbp chromosomal fragment.

The spoIIC298 mutation was reported to be 57\% linked by PBS1 transduction to cysB3 and 42\% linked to hisA1 (Coote, 1972), whereas spoIID68 was 12–19\% linked to hisA1 but not linked to cysB3 (Piggot, 1973). These mapping results clearly contradicted our finding that a small insert of 1.6 kbp could complement mutations in both sporulation loci. We therefore decided to repeat the mapping by transduction with phage PBS1, using strains MY2000.68 spoIID68 and 298.2 spoIIC298 as donors. Results of crosses involving the markers cysB3 and hisA1 are shown in Table 2. According to these results, both spoIIC298 and spoIID68 are located at the position assigned to spoIID on the genetic map.

Since the mutants were originally isolated in this laboratory, we were able to compare cultures of them preserved at different times with the mutations in different backgrounds. We also
obtained strains from other laboratories. φ105LD2 and φ105J11 were able to complement all strains tested. Strains carrying the two mutations are also phenotypically indistinguishable.

Both produce serine protease and alkaline phosphatase, and electron microscopy shows that 5–10% of the cells in both strains have a spore septum which bulges, sac-like, into the mother cells – an unusual and very characteristic phenotype. A strain harbouring the original mutation, spo-298, was described as oligosporogenous (Coote, 1972) while the spoIIC strains we examined were all Spo-. No strain corresponding to previous descriptions of strains carrying the spoIIC298 mutation could be found in our collection of mutants.

Complementation tests were done to see if mutations spo-298 and spo-68 affected the same gene. Mutation spoIID68 was transferred to 105LD2 by prophage transformation, in the following way. Strain MY2000.68 (spoIID68 lys-I pyrDl), lysogenic for 105LD2 and so phenotypically Spo+, was transformed with chromosomal DNA from strain SG43 (spoIID68 trpC2). Ura+ transformants were selected and about 4% of these were Spo−. These Spo− colonies could have resulted either from transformation of the prophage (by congression with pyrD+) or from loss of the prophage. To distinguish between these possibilities, Spo− colonies were isolated and tested for immunity to infection by 105LD2. An immune Spo− colony was presumed to carry mutation spoIID68 on the prophage. Lysates were prepared from several of these and tested for loss of spoIID+ transducing activity. The presence of the prophage carrying the spoIID insert was confirmed by restriction analysis of the phage DNA. When strain 298.2 (spoIIC298) was lysogenized with this φ105LD2 carrying the spoIID68 lesion, the lysogens were Spo−. This lack of complementation confirms that both mutations are in the same gene.

Nucleotide sequence

The above results indicated that the spoIID gene was in the 1.6 kbp EcoRI–SalI fragment subcloned into pUC13 (pSGLD2 above). Subclones of this plasmid in M13 phage vectors (Messing, 1983) were then used for sequencing. The fragments sequenced are shown in Fig. 4. Only one large open reading frame was found. This, and short stretches on either side, were sequenced in both directions and all restriction sites were read through. The regions flanking the gene, although read in only one direction, were read from at least two independent clones.

The broken lines indicate sequences obtained after exonuclease III–nuclease S1 shortening (see Methods). This was necessary to order the small fragments in the centre of the open reading frame.

The sequence shown in Fig. 5 is from the HindIII site, at the extreme left in Fig. 4, to the SalI site. Results described below indicate that this fragment contains the complete spoIID transcriptional unit.
Fig. 5. Nucleotide sequence of the *spolID* locus. The sequence shown is from the *HindIII* site to the left of the open reading frame in Fig. 4 to the *SalI* site. The possible ribosome binding site and the putative terminator are underlined. The regions of homology with known promoter sequences (Johnson et al., 1983) are indicated thus: , the −10 and −35 regions recognized by σ32-RNA polymerase; , the −10 region recognized by σ29-RNA polymerase.
Fig. 6. Integrational plasmids used to analyse the spoIID locus. At the top is the region of the DNA fragment cloned in plasmid pSGLD1 (Fig. 3) used for subcloning into the integrational plasmid vector pSGMU2 (Fort & Errington, 1985). The hatched box represents the extent of the open reading frame as indicated by the sequence data. Below are the fragments contained in the integrational plasmids (designation above the line and insert sizes below). Only plasmid pSGLD8 (dotted line) gave a Spo⁻ phenotype when integrated into the chromosome of a Spo⁺ strain. The restriction map also shows the approximate positions of spo-298 and spo-68 as indicated from results shown in Table 3. H, HindIII; A, AcaI; S, SalI.

Analysis of the spoIID locus by integrational plasmids

The sequence showed only one gene in the spoIID locus. Whether or not the transcription extends beyond this open reading frame was tested by using integrational plasmids (Piggot et al., 1984) to find out if the transcription unit was the length expected from the sequence data. This method has been used to delimit the transcriptional units for spoIIA (Piggot et al., 1984) and for spoVA (Fort & Errington, 1985).

Integrational plasmids cannot replicate in B. subtilis but can express a selectable marker, usually drug resistance, when integrated into the chromosome. The integrational plasmid vector pSGMU2 (Fort & Errington, 1985) carries a chloramphenicol resistance (cat) gene that can be selected in B. subtilis and has several unique restriction endonuclease sites. Fragments of cloned DNA from pSGLD1 were subcloned into pSGMU2 to produce the integrational plasmids shown in Fig. 6.

These plasmids integrate into the chromosome by a single crossover in the region of homology (Haldenwang et al., 1980; Ferrari et al., 1983). If the region of homology is completely within a gene, the integration of the plasmid will disrupt the transcriptional unit and produce a Spo⁻ phenotype. However, if the homologous region lies partly outside the gene, the duplication generated by the crossover will leave one intact transcriptional region and thus a Spo⁺ phenotype will be observed.

Strain MB75 was transformed to chloramphenicol resistance with each of the integrational plasmids illustrated in Fig. 6. Only pSGLD8, which contains the 0·6 kbp AcaI–HindIII fragment, gave transformants that were Spo⁻ as well as chloramphenicol resistant. This indicates that only this fragment is totally within the transcriptional unit. Thus, the spoIID functional unit appears to lie between the HindIII site, to the left of AcaI, and the SalI site to the right (Fig. 6).

Mapping mutations with integrational plasmids

Strains carrying the spo-298 mutation can be transformed to Spo⁺ by pSGLD3 and pSGLD8 (Fig. 6). This indicates that the lesion is between the AcaI site and the nearer of the HindIII sites within the gene. Strains carrying the spo-68 mutation can be transformed to Spo⁺ by pSGLD4 and pSGLD6 (Fig. 6). Therefore, this mutation is between SalI and the nearer of the HindIII sites within the gene.

An even better estimate of the position of these mutations can be contained by mapping them with the integrational plasmids. For such mapping the plasmids used must have two properties. The DNA fragment carried must contain one end of the transcriptional unit and the region of DNA affected in a particular mutant. When such a plasmid is integrated into the chromosome of the mutant two types of Cm⁸ colonies will be produced depending on where the crossover
Table 3. Mapping mutations spo-68 and spo-298 with integrational plasmids

Competent recipient cells were transformed with the integrational plasmids shown in Fig. 6. Chloramphenicol resistant (CmR) transformants were selected on Schaeffer agar containing chloramphenicol (5 µg ml⁻¹). The number of Spo⁺ and Spo⁻ transformants was counted. The percentage of Spo⁺ transformants indicates the approximate distance of the mutation from one end of the insert in the donor plasmid (see text). The positions of the mutations based on these data are shown in Fig. 6.

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor plasmid</th>
<th>No. of CmR transformants</th>
<th>No. of CmR Spo⁺ transformants</th>
<th>Percentage of Spo⁺ transformants</th>
</tr>
</thead>
<tbody>
<tr>
<td>298.2 spo-298 phe-12</td>
<td>pSGLD3</td>
<td>834</td>
<td>275</td>
<td>33</td>
</tr>
<tr>
<td>MY2000.68 spo-68 lys-1 pyrD1</td>
<td>pSGLD4</td>
<td>314</td>
<td>121</td>
<td>38.5</td>
</tr>
<tr>
<td>MY2000.68 spo-68 lys-1 pyrD1</td>
<td>pSGLD6</td>
<td>381</td>
<td>269</td>
<td>70.6</td>
</tr>
</tbody>
</table>

occurs. If the crossover occurs between the mutation and the end of the transcriptional unit, the phenotype is determined by the mutation, in this case Spo⁻. If the crossover occurs on the other side of the mutation, the duplication will produce an intact transcriptional unit and thus a Spo⁺ phenotype. Assuming random integrations, the proportion of one or the other phenotype will depend on the position of the mutation in the fragment homologous to that cloned into the integrational plasmids.

In this case, we know from the results above that pSGLD3 (Fig. 6) contains the start of the spoIID transcription unit and transforms spo-298 strains. Plasmids pSGLD6 and pSGLD4 contain the end of the spoIID transcriptional unit and transform strains carrying spo-68 to Spo⁺. When the integrational plasmids were used to transform strains containing these mutations the results obtained were as shown in Table 3. Of the CmR transformants produced when plasmid pSGLD3 was used to transform strain 298.2, 33% were Spo⁺ and we can thus conclude that this mutation is about one-third of the distance from the end of the HindIII fragment towards the AatI site as shown in Fig. 6. When the experiment was done with strain MY2000.68, 71% of the transformants were Spo⁺ when the strain was transformed with pSGLD6 and 39% after transformation with pSGLD4. Therefore, the approximate position of this mutation is towards the SalI end of the fragment as shown in Fig. 6.

DISCUSSION

The spoIID locus is involved in the regulation of the sporulation of B. subtilis. Mutations in this locus prevent the expression of biochemical markers (like glucose dehydrogenase) and block the morphological development of the spore at stage II (Young & Mandelstam, 1979). Our results demonstrate the identity of loci spoIID and spoIIC. The 1.6 kbp chromosomal fragment we have cloned in a φ105 derivative carries a single sporulation locus able to complement mutation spoIID68 as well as the previously classified spoIIC298. Both are located at the position of spoIID on the genetic map of B. subtilis.

The data presented in this paper show that spoIID apparently codes for a single protein. The open reading frame of the gene starts with ATG and codes for a 343 amino acid polypeptide. The two mutations, spo-68 and spo-298, map within the limits of this open reading frame and show no inter-allelic complementation. Integrational plasmids have proved to be an easy and useful tool for mapping mutations very closely linked, allowing recombination to be converted roughly into physical distances. Also, integrational plasmids were used to determine the limits of the transcriptional unit. The number of genes in other sporulation loci so far cloned is variable. Thus, spoIIC has three genes (Piggot et al., 1984) while spoVA has five genes (Fort & Errington, 1985). The sequence of spoIIG (Stragier et al., 1984) indicates that this locus probably contains only one gene.

Ribosome binding sites in B. subtilis require extensive homology to the 3' end of B. subtilis 16S RNA (McLaughlin et al., 1981; Moran et al., 1982; Band & Henner, 1984). The sequence AGGAGG, located 9 bp before the ATG start codon, could bind to the B. subtilis 'Shine-Dalgarno' region 3'-UUUCCUCACU-5' (Shine & Dalgarno, 1974). The calculated free
energy (Tinoco et al., 1973) of this interaction is 16.6 kcal mol\(^{-1}\) (69.45 J mol\(^{-1}\)), which is within the range reported for \(B.\) subtilis (Moran et al., 1982; Fort & Piggot, 1984; Fort & Errington, 1985).

The stop codon TAG is the start of an inverted repeat sequence that may be the terminator. The region certainly has the properties associated with known terminators (Rosenberg & Court, 1979), and is very similar to sequences located immediately after the stop codon in other genes of bacilli (Vasantha et al., 1984).

In \(B.\) subtilis there are multiple forms of RNA polymerases, and different classes of promoters are recognized by forms of RNA polymerases containing different sigma factors (Losick & Pero, 1981). Two regions upstream from the \(spoIID\) gene have significant homology to sequences recognized by known \(B.\) subtilis sigma factors (Fig. 5). The sequence AAATC, separated by 17 nucleotides from the sequence TACTGGTTTTT, is a possible \(-35\) and \(-10\) promoter region for the \(\sigma^{32}\)-RNA polymerase (Johnson et al., 1983). However, there are 152 nucleotides from the end of the \(-10\) region to the ATG start. A sequence, CATATT, exactly homologous to the \(-10\) consensus sequence recognized by \(\sigma^{54}\)-RNA polymerase (Johnson et al., 1983) is located 60 nucleotides before the open reading frame. A possible \(-35\) region is less clear. Both possible promoter regions lie within the functional boundaries of the \(spoIID\) gene, as determined by the studies with integrational plasmids described above.

The amino acid sequence inferred from the \(spoIID\) gene nucleotide sequence shows a protein with an excess of basic (47) over acidic (27) residues. In addition, 20 of the first 26 amino acids are hydrophobic and no polar amino acid is present between residues 3 to 27. Hydrophobic and hydrophilic regions appear to be evenly distributed through the rest of the protein. Hydrophobic N-terminal regions similar to this are typical of pre-secretory proteins and act as signal sequences. Signal sequences, varying in length and amino acid composition, are usually preceded by a positively charged residue (Lys or Arg) and are followed by a signal peptidase recognition sequence, Ala-X-Ala, which determines the cleavage of the signal peptide during the export of the protein (Perlman & Halvorson, 1983). The \(spoIID\) signal sequence exhibits a positively charged amino acid (Lys) in position 2, preceding the hydrophobic region and shows a typical cleavage recognition sequence, Ala-Gly-Ala, at positions 32–34. However, the location of the \(spoIID\) product and its function remain unknown.

We have constructed transcriptional and translational fusions of the \(\beta\)-galactosidase gene of \(E.\) coli and the \(spoIID\) gene of \(B.\) subtilis, and we are using these to test the expression of \(spoIID\) in different mutant backgrounds. We expect that these studies will help us to understand how this gene is regulated, and which other sporulation genes are dependent on it for expression.

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REFERENCES


spoIID sequence in Bacillus subtilis


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