Use of Integrational Plasmid Vectors to Demonstrate the Polycistronic Nature of a Transcriptional Unit (*spoIIA*) Required for Sporulation of *Bacillus subtilis*

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Plasmids carrying different portions of the polycistronic *spoIIA* locus, and unable to replicate autonomously in *Bacillus subtilis*, were able to transform a Spo⁺ *B. subtilis* strain, BR151, for the plasmid-determined chloramphenicol resistance by Campbell-like insertion into the region of homology on the chromosome. Two such plasmids, pPP35 and pPP36, yielded Spo⁻ transformants, indicating that the cloned regions of these plasmids were entirely within the chromosomal *spoIIA* transcriptional unit. The cloned regions overlapped the end of a known *spoIIA* cistron, so that the transcriptional unit was larger than this cistron, and was polycistronic. This is the first demonstration of such a polycistronic sporulation transcriptional unit. The DNA sequence of the region has now been determined (given in an accompanying paper) and suggests a transcriptional unit with three open reading frames. Two other plasmids yielded Spo⁺ transformants of BR151, and these define the outer limits of the transcriptional unit. The adjacent sporulation locus identified by the *spoVA89* mutation was not part of the same transcriptional unit.

INTRODUCTION

Formation of endospores from vegetative *Bacillus subtilis* is a system of cellular differentiation that is particularly amenable to study. It requires the functioning of at least 50 genetic loci that are specific to sporulation (Hranueli *et al.*, 1974; Piggot & Coote, 1976; Piggot *et al.*, 1981). A study of sporulation genes that are co-ordinately regulated might be particularly useful in unravelling how expression of such a large number of genes is controlled. To this end we have been analysing the *spoIIA* region. We have previously characterized a plasmid clone carrying part of the *spoIIA* locus and shown that the cloned piece would function to complement chromosomal mutations (Liu *et al.*, 1982). The maximum size of the functioning unit was shown to be about 700 bp and the minimum size about 300 bp using transposon mutagenesis (Lencastre *et al.*, 1983); mutations mapping within this region formed a single complementation group (Chak *et al.*, 1982). Here we report the cloning of the rest of the *spoIIA* locus on a plasmid, and analyse how the locus is transcribed.

Several plasmid vectors have been constructed that replicate in *Escherichia coli* and carry a drug-resistance determinant that can be expressed in *B. subtilis* although they cannot replicate in *B. subtilis*. These plasmids cannot transform *B. subtilis* unless they have *B. subtilis* DNA cloned in them; when selection is then made for the plasmid-determined drug-resistance, the plasmid integrates into the *B. subtilis* chromosome at the region of homology by a Campbell-like mechanism (Haldenwang *et al.*, 1980; Niaudet *et al.*, 1982; Ferrari *et al.*, 1983; Fig. 1). When the

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Fig. 1. Schematic representation of the integration of plasmids into the chromosome by a Campbell-type mechanism. (a) Cloned region is entirely within a transcriptional unit. (b) Cloned region includes an end of a transcriptional unit. The chromosome is drawn in bold lines, and plasmid in thin lines. The complete transcriptional unit is shown as a rectangle, incomplete units are indicated by serrated ends to the rectangle. The location of the plasmid-determined chloramphenicol resistance is indicated by Cm^R .

plasmid integrates into a transcriptional unit, then it necessarily interrupts that transcriptional unit. Provided the plasmid contains homologous DNA extending outside one end of the transcriptional unit, then a complete new transcriptional unit is created consisting of cloned DNA recombined with chromosomal DNA (Fig. 1b). However, when the homologous region is entirely within the transcriptional unit, then a complete transcriptional unit is not created and the function is lost upon integration (Fig. 1a). Here we use such plasmids to show that the *spoIIA* locus is transcribed as a polycistronic unit.

METHODS

Strains. The *B. subtilis* 168 strains used are listed in Table 1. The *E. coli* strain used, SL2034, was a transformable Dam^- strain; its origin is obscure. Plasmids were maintained in SL2034.

Plasmids. Plasmids used are listed in Table 2. pHM2 contains a 3.3 kbp region of *B. subtilis* DNA inserted by homopolymer tailing into the unique *Bam*HI site of pHV33 (Liu *et al.*, 1982); pHV33 is a chimaera of the enterobacterial plasmid pBR322 and the staphylococcal plasmid pC194 joined together at their single *Hind*III sites (Primrose & Ehrlich, 1981). The *B. subtilis* region of pHM2 is shown in Fig. 3, and the whole of pHM2 in an accompanying paper (Savva & Mandelstam, 1984). The unique *Sal*I site referred to in the text is in the inactivated tetracycline resistance gene of the pBR322 portion of the plasmid. The integrational vector pJAB1 (Fig. 2) was made by M. G. Sargent and J. A. Brannigan and kindly provided by them. It is a chimaera of the positive cloning vector pTR262 (Roberts *et al.*, 1980) and pJH101 which were both cut by *Pst*I and *Ava*I and ligated to each other; cloning into the single *Bcl*I, or *Hind*III, site of pJAB1 inactivates the λ repressor and allows expression of tetracycline resistance. The construction of pPP33 is described in Results. Plasmid pPP31 was constructed using pJAB1, whilst pPP35 and pPP36 were constructed using pJH101. The plasmids pPP31, pPP35 and pPP36 were derived by subcloning from pHM2 the 1.7 kbp *Hind*III–*Bgl*II fragment, the 1.2 kbp *Eco*RI–*Sal*I fragment and the 0.67 kbp *Eco*RI–*Bgl*II fragment, respectively (Fig. 3); the first of these pieces contains the entire *spoIIA69*-complementing region, whereas the other two pieces are able to recombine with *spoIIA69* to give *spo*⁺, but do not complement *spoIIA69* (Lencastre *et al.*, 1983). The *Eco*RI–*Sal*I fragment contains a portion of pHV33 DNA. In the construction of pPP31, pHM2 was restricted with *Hind*III and *Bgl*II, and pJAB1 with *Hind*III and *Bcl*I; the two mixtures were ligated together, and SL2034 was transformed to tetracycline resistance. From a transformant clone, a plasmid, pPP31, was isolated that was able to transform SL1013 (*spoIIA69*) to *Spo*⁺. Detailed analysis of pPP31 indicated that the 1.7 kbp *Hind*III–*Bgl*II fragment from pHM2 had apparently ligated through its *Bgl*II site to the *Bcl*I site of the 0.1 kbp *Hind*III–*Bcl*I piece from pJAB1, and this piece had been inserted into the

Table 1. *Bacterial strains*

Strain	Genotype	Reference
BR151	<i>metB10 lys-3 trpC2</i>	
SL401	<i>spoIIA1 trpC2</i>	El, Piggot (1973)
SL437	<i>spoIIA37 trpC2</i>	NG 1.82, Piggot (1973)
SL979	<i>spoIIA5 trpC2 metB10</i>	Liu <i>et al.</i> (1982)
SL980	<i>spoIIA4 trpC2 metB10</i>	Liu <i>et al.</i> (1982)
SL981	<i>spoIIA12 trpC2 metB10</i>	Liu <i>et al.</i> (1982)
SL983	<i>spoIIA26 trpC2 metB10</i>	Liu <i>et al.</i> (1982)
SL984	<i>spoIIA16 trpC2 metB10</i>	Liu <i>et al.</i> (1982)
SL1013	<i>spoIIA69 lys-3 trpC2</i>	Liu <i>et al.</i> (1982)
SL1018	<i>spoVA89 trpC2 metB10</i>	DNA 89.1 (Hranueli <i>et al.</i> , 1974) × BR151 → Lys ⁺
SL1089	<i>spoIIA1 trpC2 lys-3</i>	DNA SL401 × BR151 → Met ⁺

Table 2. *Plasmids*

Name	Phenotype in <i>E. coli</i>	Origin/Reference
pHM2	Ap ^R Cm ^R	Liu <i>et al.</i> (1982)
pHV33	Ap ^R Cm ^R Tc ^R	Primrose & Ehrlich (1981)
pJAB1	Cm ^R	M. G. Sargent
pJH101	Cm ^R Ap ^R Tc ^R	Ferrari <i>et al.</i> (1983)
pPP31	Cm ^R Tc ^R	This paper
pPP33	Cm ^R Tc ^R	This paper
pPP35	Cm ^R Ap ^R	This paper
pPP36	Cm ^R Ap ^R	This paper

Fig. 2. Restriction maps of plasmids. The upper three plasmids are pJAB1 and plasmids with inserts in pJAB1; the lower set are pJH101 and plasmids derived from it. Regions of *B. subtilis* DNA are shown in bold. Distances around the circumference for plasmids and inserts are on the same scale, shown (in kbp) for pJAB1 and pJH101; the sizes of the plasmids (kbp) are: pJAB1, 5.7; pPP31, 7.25; pPP33, 8.9; pJH101, 5.4 (Ferrari *et al.*, 1983); pPP35, 5.9; pPP36, 5.7. Restriction sites are: A, *AvaI*; Ba, *BamHI*; Bc, *BclI*; Bg, *BglII*; E, *EcoRI*; H, *HindIII*; P, *PstI*; S, *SalI*; V, *EcoRV*. Presumed sites of *BglII*-*BamHI* or *BglII*-*BclI* fusions are indicated by dotted lines.

*Hind*III site of another pJAB1 molecule (Fig. 2); there is, therefore, a duplication of the 0.1 kbp *Hind*III–*Bcl*I piece. The orientation of the *B. subtilis* insert is opposite to that which would have been obtained by direct ligation of the 1.7 kbp *Hind*III–*Bgl*II fragment with the large *Hind*III–*Bcl*I fragment from pJAB1. In the construction of pPP35 and pPP36, the required restriction fragments from pHM2 and pJH101 were purified by electrophoresis before ligation. Their structures, which were confirmed by restriction analysis, are shown in Fig. 2.

Plasmid preparation. Large-scale preparations were by the method of Guerry *et al.* (1973).

Preparation of chromosomal DNA. Cultures of *B. subtilis* derived from isolated colonies were grown in L-broth, or L-broth containing 5 µg chloramphenicol ml⁻¹ where appropriate. They were harvested and resuspended in 1/20 volume 20 mM-MgSO₄, 20 mM-Tris/HCl pH 7.4, 20% (w/v) sucrose containing 200 µg lysozyme ml⁻¹ and incubated at 45 °C for 10 min (or until protoplasting was complete). EDTA was then added to 0.05 M, Sarkosyl to 1% (w/v), Pronase to 0.1%, and the mixture incubated at 50 °C for 2 h. Two volumes of alcohol (4 °C) were then added, and the DNA spooled around a glass rod. The DNA was dissolved in TE (10 mM-Tris/HCl pH 7.5, 1 mM-EDTA), two volumes of ethanol added, and the spooling repeated. The DNA was dissolved in as small a volume of TE as possible and treated with RNAase A (Sigma; pre-heated to 100 °C for 15 min to inactivate DNAase activity) for 1 h at 37 °C. This mixture was extracted with an equal volume of chloroform/isoamyl alcohol/phenol (24:1:25). The aqueous phase was recovered, and any phenol removed from it by extraction with ether. The DNA was precipitated with two volumes of alcohol, spooled, and redissolved in TE; this last set of operations being repeated several times.

Enzymes. Restriction enzymes were purchased from P & S Biochemicals, Liverpool, UK, or from BRL. T4 DNA ligase was purchased from Boehringer. Enzymes were used according to the methods of Davis *et al.* (1980).

Gel electrophoresis. DNA was generally electrophoresed in horizontal 0.8% agarose gels as described previously (Liu *et al.*, 1982). DNA was extracted from gels by electrophoresis onto DEAE-paper (Dretzen *et al.*, 1981).

Southern blotting. Transfer of DNA from gels onto nitrocellulose filters, and subsequent probing by labelled DNA was by the method of Southern (1979). Approximately 0.5 µCi (18.5 kBq) probe was used for each blot. In general, fragments of less than about 1.0 kbp would not have been detected. Blots used to test for Campbell-like insertions were not designed to detect weak hybridization such as that exploited in 'chromosome-walking'.

Nick translation. DNA was labelled to high specific activity with [α -³²P]dATP by nick translation as described by Jeffreys & Flavell (1977).

Media. In general, *B. subtilis* strains were maintained on nutrient agar and *E. coli* strains maintained on L-agar. Appropriate antibiotics were added for strains harbouring plasmids: chloramphenicol (Cm), 5 µg ml⁻¹ for *B. subtilis*, 15 µg ml⁻¹ for *E. coli*; tetracycline (Tc) 20 µg ml⁻¹; ampicillin (Ap) 25 µg ml⁻¹. L-broth contained, per litre: Difco tryptone, 10 g; Difco yeast extract, 5 g; NaCl, 10 g; adjusted to pH 7.0. L-agar was L-broth containing 1.6% (w/v) Difco agar. Nutrient agar consisted of Difco nutrient broth solidified with 1.4% (w/v) Davis (NZ) agar. For transformation, GM1 consisted of 100 ml Bott & Wilson (1968) salts, 1.0 ml 50% (w/v) glucose, 0.1 ml 1 M-MgSO₄, 0.5 ml 20% (w/v) yeast extract and 5.0 ml Bott & Wilson (1968) amino acids. GM2, which was made up immediately before use, consisted of 10 ml GM1, 0.25 ml 0.1 M-MgCl₂ and 0.05 ml 0.1 M-CaCl₂. These were supplemented with 10 µg tryptophan ml⁻¹, and other amino acids at 100 µg ml⁻¹ where appropriate. Bott & Wilson (1968) salts consisted of K₂HPO₄, 1.24% (w/v); KH₂PO₄, 0.76%; trisodium citrate, 0.1%; (NH₄)₂SO₄, 0.6% adjusted to pH 6.7. Bott & Wilson (1968) amino acids contained valine, lysine, threonine, glycine, aspartic acid, methionine, histidine, tryptophan, and arginine, each at 500 µg ml⁻¹.

Transformation. *Escherichia coli* was transformed by the method of Brown *et al.* (1979). *Bacillus subtilis* was transformed by a method derived from that of Young & Wilson (1974). A single-colony inoculum of the recipient was grown overnight at 30 °C in 10 ml GM1 without shaking in a 15.2 × 1.9 cm test-tube. This culture was then transferred to a 100 ml conical flask and incubated at 37 °C with shaking. Growth was monitored by measuring the OD₆₀₀ of the culture with a Unicam SP6-550 spectrophotometer, and converting the reading to mg bacterial dry wt ml⁻¹ by means of a standard calibration curve. Approximately 90 min after growth had ceased to be exponential: either 1 ml culture was added to 10 ml pre-warmed GM2 in a 100 ml conical flask, and the culture shaken for 90 min at 37 °C, at which time cultures were highly competent for transformation; or fresh DMSO was added to 5% (v/v), the culture was frozen rapidly in 1 ml lots, at -70 °C, and stored at -70 °C; when required a sample was thawed (taking care that the temperature did not rise above 37 °C), transferred to 10 ml pre-warmed GM2, and incubated as above. In general, 0.5 ml lots of competent recipient were then incubated, with aeration, with 1 µg donor DNA for 30 min at 37 °C before plating on selective agar.

RESULTS

Cloning the rest of the spoIIA locus

Plasmid pHM2 could complement some *spoIIA* mutations, but did not carry the whole *spoIIA* locus (Liu *et al.*, 1982). Nick-translated pHM2 hybridized strongly to a 2.35 kbp fragment in a *Bgl*II digest of chromosomal DNA, and hybridized very weakly to fragments of 3.2 and 1.3 kbp.

Table 3. Transformation of *spo* mutants with pPP33, selecting for *Spo*⁺ transformants

The values are numbers of colonies obtained from 0.2 ml transformed cultures.

Recipient		Donor	
Strain	<i>spo</i> mutation	None	pPP33
SL437	<i>spoIIA37</i>	0	> 10 ³ *
SL979	<i>spoIIA5</i>	2	8.9 × 10 ⁴
SL980	<i>spoIIA4</i>	0	0
SL981	<i>spoIIA12</i>	6	5.3 × 10 ⁵
SL983	<i>spoIIA26</i>	4	4.1 × 10 ⁵
SL984	<i>spoIIA16</i>	2	1.8 × 10 ⁵
SL1089	<i>spoIIA1</i>	0	> 10 ⁴ *
SL1018	<i>spoVA89</i>	3	0

* The plates were too crowded to count accurately at the dilutions used.

Table 4. Sizes of restriction fragments obtained from pPP33

Enzyme	Fragment sizes (kbp)			
<i>Pst</i> I	5.5	2.25	1.27	
<i>Hind</i> III	8.9			
<i>Eco</i> RV	6.0	1.82	1.0	
<i>Bcl</i> I	7.8-8.5*			0.43
<i>Pst</i> I + <i>Hind</i> III	5.5†	2.25	1.29	
<i>Pst</i> I + <i>Eco</i> RV	4.5†	1.81	1.25	0.92
<i>Pst</i> I + <i>Bcl</i> I	5.3	2.09		1.04 (bands < 0.5 lost)
<i>Eco</i> RV + <i>Hind</i> III	6.02	1.85	0.83†	
<i>Eco</i> RV + <i>Bcl</i> I	5.5	1.82	1.0	0.43
<i>Bcl</i> I + <i>Hind</i> III	6.2	2.25	0.43	

* Band smeared.

† Band expected to be identical to band from pJAB1, where the sizes computed from sequences are: *Pst*I-*Hind*III, 5.3; *Pst*I-*Eco*RV, 4.5; *Eco*RV-*Hind*III, 0.81.

The 2.35 kbp fragment corresponded exactly to a *Bgl*II fragment obtained from pHM2 (Lencastre *et al.*, 1983). The other fragments presumably came from the adjacent chromosomal DNA, only a small portion of which was cloned in pHM2 (Fig. 3). A large *Hind*III fragment of 5.9 kbp derived from pHM2 has only the left end (in the sense of Fig. 3) of the *B. subtilis* insert in pHM2 and includes the *spoIIA69*-complementing activity of pHM2 (Lencastre *et al.*, 1983). This fragment hybridized weakly to the 3.2 kbp *Bgl*II chromosomal fragment as well as strongly to the 2.35 kbp fragment, but not at all to the 1.3 kbp fragment. This indicated the order of the *spoIIA* region. Accordingly, *Bgl*II-restricted chromosomal DNA was fractionated by electrophoresis in agarose. The fraction of about 3.2 kbp was eluted, ligated to pJAB1 cut with *Bcl*I, and the ligated mixture used to transform SL2034 to tetracycline resistance. Plasmid preparations were made from 190 individual clones and screened for their ability to transform *B. subtilis* SL1089 (*spoIIA1*) to *Spo*⁺. Four positive clones were identified, and one of these, with pPP33, was isolated and characterized in detail. Plasmid pPP33 also transformed a series of other *spoIIA* mutations, 5, 12, 16, 26 and 37 to *Spo*⁺ (Table 3). These were all the *spoIIA* mutations tested that had not been complemented by pHM2 (Liu *et al.*, 1982) with the exception of *spoIIA4*. A highly competent culture (indicated by its transformability to *Spo*⁺ by *Spo*⁺ chromosomal DNA, and to Cm^R by the autonomously replicating plasmid pHM2) of SL980 (*spoIIA4*) was not transformed to chloramphenicol resistance by pPP33, whereas all other strains were. It seems likely that *spoIIA4* contains a large deletion that removes any chromosomal homology with pPP33 and so prevents transformation by pPP33. It is concluded that the cloned regions on pHM2 and pPP33 cover the entire *spoIIA* locus. pPP33 did not correct the *spo-89* mutation in the adjacent *spoVA* locus.

Plasmid pPP33 contained a single *Hind*III site that was also present in the parental pJAB1, and contained no *Eco*RI sites. Its restriction map was constructed by analysing the sizes of fragments produced by single and double digestions with a series of restriction enzymes (Table 4). The restriction map of pPP33 is shown in Fig. 2; the *Bcl*I site of pJAB1 and the *Bgl*II sites at the ends of the *B. subtilis* insert were lost on ligation.

A restriction map of the *spoIIA* region of the chromosome was constructed from the maps of pHM2 (Lencastre *et al.*, 1983) and pPP33 (Fig. 3). To ensure that the cloned DNA was not rearranged in any way, and that the cloned regions of pHM2 and pPP33 overlapped in the manner shown, various ³²P-labelled DNA probes were used to hybridize to Southern blots of restricted chromosomal DNA from strain BR151. pPP31 and other probes derived from pHM2 hybridized to a 2.35 kbp *Bgl*II chromosomal fragment that was identical in size to the fragment derived from pHM2 (Table 5); pPP31 also hybridized to a 1.5 kbp *Eco*RI fragment identical to that obtained from pHM2 and homologous to part of pPP31, but not the other probes (Fig. 3). Similarly, pPP33, and a probe derived from it, hybridized to a 1.8 kbp *Eco*RV chromosomal fragment identical to the fragment derived from pPP33. These fragments covered most of the cloned regions, and the results indicated little, if any, rearrangement. pPP33 and probes derived from both pHM2 and pPP33 hybridized to an *Eco*RV fragment of approximately 3.4 kbp. This indicated that the clones came from very near each other; in addition, the size of fragment is that expected if the cloned regions overlap as shown (Fig. 3 and Table 5). Similarly, pPP33 and probes from both pHM2 and pPP33 hybridized to an *Eco*RI fragment of about 5.9 kbp. pPP33 and probes derived from it hybridized to a *Bgl*II fragment of approximately 3.2 kbp, as would be expected from the method of construction of pPP33. The 1.2 kbp *Eco*RI–*Sal*I fragment from pHM2 hybridized very faintly to a 3.2 kbp *Bgl*II fragment as expected (Table 5), although the hybridizations with pPP31 as probe were apparently too faint to detect the 3.2 kbp *Bgl*II band. The hybridization results are completely consistent with, and strongly support, the restriction map determined from the maps of the cloned DNA. They also provide information about the chromosomal regions outside the cloned DNA (Fig. 3).

Transformation of BR151 by pPP33

Transformation of the Spo⁺ strain BR151 by pPP33 yielded chloramphenicol-resistant transformants that were Spo⁺ (Table 6). Chloramphenicol-resistant clones were then analysed to confirm that they were formed by a Campbell-like mechanism. DNA was prepared from a clone, restricted by *Bgl*II, *Eco*RI and *Eco*RV and then analysed by Southern blotting using a series of probes. Sizes of bands giving strong hybridizations are shown in Table 7. The pHV33 probe hybridized to the DNA whereas it did not hybridize to DNA of the parental strain BR151. This confirmed that vector DNA (which has extensive homology with pHV33) had integrated into the chromosome. The sizes of the bands observed for all combinations of enzyme and probe are, within experimental error, the sizes predicted for a Campbell-like insertion by pPP33 (compare Table 7 and Fig. 4). In addition, no larger bands, 12.1 + (*n* × 8.9) kbp for *Bgl*II and 14.8 + (*n* × 8.9) kbp for *Eco*RI, were observed that would have indicated tandem repeats (no. *n*) of pPP33; nor were there any bands corresponding to free covalently closed circular plasmid. Thus the results indicated insertion of pPP33 by a Campbell-like mechanism, and are difficult to explain in any other way. As the clones with pPP33 inserted in the chromosome are Spo⁺, this is consistent with pPP33 containing at least one end of a *spoIIA* transcriptional unit.

Analysis of the portion of the spoIIA locus contained on pHM2

To see if the chromosomal *spoIIA* transcriptional unit was the same as, or larger than, the size of the functional unit that had been delimited on pHM2 (Lencastre *et al.*, 1983), sub-clones of pHM2 were constructed in integrational vectors (see Methods). These were pPP31, pPP35 and pPP36. pPP31 consisted of the 1.7 kbp *Hind*III–*Bgl*II fragment of pHM2 inserted in pJAB1 (Figs 2 and 3); this included the entire *spoIIA*₆₉-complementing region on pHM2 as defined by Tn1000 mutagenesis (Lencastre *et al.*, 1983). pPP35 consisted of the 1.2 kbp *Eco*RI–*Sal*I fragment of pHM2 cloned in pJH101; the *B. subtilis* portion of the insert in pPP35 started at the *Eco*RI site within the *spo-69*-complementing region and extended outside that region as far as

Table 5. Sizes of restriction fragments of BR151 chromosomal DNA that hybridized to different probes

Probe	Enzyme ...	Sizes of hybridizing DNA fragments (kbp)						
		<i>Bgl</i> II		<i>Eco</i> RI		<i>Eco</i> RV		
pPP31		2.45		1.5	5.95	5.5*	3.4*	
pPP33			3.37		5.75		3.4	1.97
0.67 kbp <i>Eco</i> RI- <i>Bgl</i> II fragment		2.4		ND			3.32	
1.2 kbp <i>Eco</i> RI- <i>Sal</i> I fragment		2.35	3.25f		6.0		3.22	
1.8 kbp <i>Eco</i> RV fragment			3.32		6.0			1.82
1.27 kbp <i>Pst</i> I fragment			3.45	ND			3.30	

ND, Not determined; f, faint.

* Digestion was incomplete, and only the major bands are indicated; the 5.5 kbp band is thought to be an artefact caused by the partial digestion.

Fig. 3. Restriction map of the *spoIIA* region of the chromosome. The map also shows the regions cloned in different plasmids. The restriction map of the chromosome was deduced from the restriction map of the cloned regions, and confirmed by Southern blots. The locations of restriction sites outside the cloned regions were determined by Southern blots. The anticipated regions of homology with various nick-translated probes are also indicated below the map. See the legend to Fig. 2 for restriction site abbreviations. The cloned region contained no *Sal*I sites. The minimum (hatched area) and maximum size of the *spoIIA69* complementing activity (CU; Lencastre *et al.*, 1983), and of the *spoIIA* transcriptional unit (TU) are indicated above the restriction map, as are the open reading frames (ORFs) identified in the DNA sequence (Fort & Piggot, 1984). The scale (in kbp) is shown at the bottom of the Figure.

the homopolymer tail formed in the construction of pHM2 (Figs 2 and 3). pPP36 consisted of the 0.67 kbp *Eco*RI-*Bgl*II fragment from pHM2 cloned in pJH101; again the insert started within, and extended outside, the functional part of the *spoIIA* locus on pHM2 (Figs 2 and 3).

These three plasmids all transformed the Spo⁺ strain BR151 to chloramphenicol resistance. However, whereas the transformants were Spo⁺ with pPP31, more than 99% were Spo⁻ with pPP35 and pPP36 (Table 6). The Spo⁻ Cm^R transformants exhibited the stage II phenotype

Table 6. *Transformation of BR151 to chloramphenicol resistance by various plasmids*

The values are numbers of colonies obtained from 0.2 ml transformed cultures. No Cm^R colonies were obtained with pJH101, or pJAB1 as donor, or in the absence of DNA.

Plasmid	10 ⁻¹ × No. of chloramphenicol-resistant transformants	
	Spo ⁺	Spo ⁻
pPP31	460	0
pPP33	970	0
pPP35*	0	174
pPP36	2	308
pHV33	467	0
None	0	0

* Separate experiment.

Table 7. *Sizes of restriction fragments of BR151/pPP33 chromosomal DNA that hybridized to different probes*

Anticipated regions of homology with the probes are shown in Fig. 4.

Probe	Enzyme	Sizes of hybridizing DNA fragments (kbp)			
		Bgl/II		EcoRI	
		Bgl/II	EcoRI	EcoRV	
pHV33		11.7	14.0-18.0*	ND	
pPP31		2.45 13.0	1.5 14.2	ND	
pPP33		12.6	13.7	3.45 2.0 1.08 6.0	
0.67 kbp <i>EcoRI</i> - <i>Bgl/II</i> fragment		2.4	ND	3.35	
1.2 kbp <i>EcoRI</i> - <i>Sal/I</i> fragment		2.4 11.2	ND	ND	
1.8 kbp <i>EcoRV</i> fragment		12.2	ND	1.8	
1.27 kbp <i>Pst/I</i> fragment		ND	ND	3.25	5.9

ND, Not determined.

* Band smeared.

Fig. 4. Effects of insertion of pPP33 into the *spoIIA* region by a Campbell-like mechanism. The restriction map of the *spoIIA* region of BR151 is shown at the top, and that anticipated for BR151/pPP33 is shown in the middle of the Figure. The region that is cloned in pPP33 is stippled; it is duplicated in BR151/pPP33 by Campbell insertion, and the region between the duplicated DNA is vector DNA. Restriction fragments unique to BR151/pPP33 are denoted by an asterisk. Anticipated regions of homology of BR151/pPP33 DNA with the various probes are indicated by the lines at the bottom of the Figure.

Table 8. Sizes of restriction fragments of BR151/pPPP31 chromosomal DNA that hybridized to different probes

Anticipated regions of homology with the probes are shown in Fig. 5.

Probe	Enzyme	Sizes of hybridizing DNA fragments (kbp)		
		<i>Bgl</i> II	<i>Eco</i> RI	<i>Eco</i> RV
pHV33		9.5	ND	ND
pPP31		10.5	1.5 7.3 5.85f	3.0 8.1
pPPP33		10.2 3.3	7.0 5.7	3.1 7.6 2.0
0.67 kbp <i>Eco</i> RI- <i>Bgl</i> II fragment		9.7	ND	3.0 7.7
1.2 kbp <i>Eco</i> RI- <i>Sal</i> I fragment		9.1 3.3f	7.6 6.2	2.9 7.5
1.8 kbp <i>Eco</i> RV fragment		3.35	6.0	1.82
1.27 kbp <i>Pst</i> I fragment		ND	ND	7.3

ND, Not determined; f, faint.

Fig. 5. Insertion of pPPP31 into the BR151 chromosome by a Campbell-like mechanism. The restriction map anticipated for the *spoIIA* region of the BR151/pPPP31 chromosome is shown; the region that is cloned in pPPP31, and duplicated upon Campbell insertion of pPPP31 into the chromosome, is stippled. Restriction fragments unique to BR151/pPPP31 are denoted by an asterisk. Anticipated regions of homology of BR151/pPPP31 DNA with the various probes are indicated by the lines below the restriction map.

when examined with a phase-contrast microscope. When they were sub-cultured in the absence of chloramphenicol they slowly segregated out Spo^+ Cm^S colonies (about 2% of colonies after 20 generations). This indicates that the transformation was reversible, and is consistent with the transformants having arisen by a Campbell-like mechanism as shown in Fig. 1(a). To confirm that this was the case, DNA was purified from Cm^R clones resulting from the crosses of BR151 with pPPP31, pPPP35 and pPPP36. The DNA is referred to as BR151/pPPP31 etc. The DNA was restricted by *Bgl*II, *Eco*RI or *Eco*RV, separated by electrophoresis in 0.8% agarose, and blotted on to nitrocellulose filters. A series of these blots were hybridized to different ^{32}P -labelled probes. All the chromosomal preparations hybridized to pHV33 (DNA from BR151 did not), indicating the presence of plasmid vectors in the chromosome. In general all the results (Tables 8 to 10) support insertion by a Campbell-like mechanism. Clones derived from the three different plasmids are now considered separately.

The major novel hybridization band seen in *Bgl*II digests of BR151/pPPP31 and not of BR151 was a band of 9.1 to 10.5 kbp (Table 8). This agrees with the size predicted for a Campbell insertion (Fig. 5): there was no sign of a band of 16.85 kbp or larger which would have been generated by tandem repeats, nor was there any band corresponding to free covalently closed circular plasmid. The novel bands seen in *Eco*RI and *Eco*RV digests, 7.0 to 7.6 kbp and 7.5 to 8.1 kbp, respectively, agree well with the sizes predicted, 7.25 and 7.75 kbp, respectively. In all cases where free plasmid pPPP31 cut with *Eco*RI was included, this had exactly the mobility of the large *Eco*RI band from BR151/pPPP31. The size of this was estimated from the restriction mapping as 7.25 kbp, and the spread of values from 7.0 to 7.6 kbp (Table 8) presumably represents the inaccuracy of our size measurements from Southern blots. The bands that were

Table 9. Sizes of restriction fragments of BR151/pPPP35 chromosomal DNA that hybridized to different probes

Anticipated regions of homology with the probes are shown in Fig. 6.

Probe	Enzyme	Sizes of hybridizing DNA fragments (kbp)					
		<i>Bgl</i> II		<i>Eco</i> RI		<i>Eco</i> RV	
pHV33			5.8		6.3		9.3
pPPP31		2.45	6.0	1.5	6.0		10.0
pPPP33			6.05	3.35	5.75	9.0*	2.0 15.3
0.67 kbp <i>Eco</i> RI- <i>Bgl</i> II fragment		2.38	5.6		5.5		8.9
1.2 kbp <i>Eco</i> RI- <i>Sal</i> I fragment		2.4	6.0	3.25f	6.1		8.9*
1.8 kbp <i>Eco</i> RV fragment				3.35		6.0	1.8
1.27 kbp <i>Pst</i> I fragment			ND		ND		8.5

ND, Not determined; f, faint.

* A second transformant clone was used for these blots.

Fig. 6. Insertion of pPPP35 into the BR151 chromosome by a Campbell-like mechanism. The restriction map anticipated for the *spoIIA* region of the BR151/pPPP35 chromosome is shown; the region cloned in pPPP35, and duplicated upon Campbell-like insertion, is stippled. Restriction fragments unique to BR151/pPPP35 are denoted by an asterisk. Anticipated regions of homology with the various probes are indicated by the lines below the restriction map.

predicted to be the same for BR151 and for BR151/pPPP31 were found to be so, within experimental error (compare Fig. 5 and Table 8).

For BR151/pPPP35, digestion with *Bgl*II gave a novel hybridizing fragment, not seen with BR151, of 5.6 to 6.1 kbp (Table 9) which had identical mobility to that of free pPPP35 cut with *Bgl*II in all gels where the two were compared. Again, the spread of size estimates indicates their imprecision. Digestion with *Eco*RI was predicted to produce two superimposed bands of 5.9 kbp; one also found in BR151, and one the linearized plasmid (Fig. 6). A band of about this size was seen for all probes tested. The pHV33 probe would only be expected to hybridize with one of the bands and the 1.8 kbp probe would only be expected to hybridize with the other; both hybridized with a band of about 5.9 kbp. Moreover, pHV33 did not hybridize with BR151 DNA, so that a new band of this size had been created in BR151/pPPP35. The major novel *Eco*RV band found was 8.9 to 10 kbp, and this agrees well with insertion of a single copy of pPPP35 by a Campbell mechanism. However, DNA from one clone also had an *Eco*RV band of 15.0 to 15.3 kbp, and this is consistent with a tandem duplication (predicted size 15.2 kbp) of the plasmid in the chromosome of some of the bacteria derived from this clone. Again the bands that were predicted to be the same for BR151 and BR151/pPPP35 were found to be so, within experimental error.

Digestion of BR151/pPPP36 with *Bgl*II yielded a major band, variously estimated to be between 7.5 and 8.3 kbp, for DNA from the clone with which most work was done (Table 10). This agreed well with the predicted value of 8.05 kbp for a single insert by a Campbell-like mechanism (Fig. 7). However, a second clone did not show this band, but rather showed strongest hybridization for material of 13 to 13.8 kbp. This suggests that the majority of the

Table 10. Sizes of restriction fragments of BR151/pPP36 chromosomal DNA that hybridized to different probes

Anticipated regions of homology with the probes are shown in Fig. 7.

Probe	Enzyme ...	Sizes of hybridizing DNA fragments (kbp)		
		<i>Bgl</i> /II	<i>Eco</i> RI	<i>Eco</i> RV
pHV33		7.9 (13)*	6.2	9.7 (16.3)*
pPP31		8.3	1.5 5.75	9.7
pPP33		8.2	5.7	8.4
0.67 kbp <i>Eco</i> RI– <i>Bgl</i> /II fragment		7.5 (13.8)*	ND	(14.0)*
1.2 kbp <i>Eco</i> RI– <i>Sal</i> I fragment		7.55	3.25f	5.9
1.8 kbp <i>Eco</i> RV fragment			3.35	5.95
1.27 kbp <i>Pst</i> I fragment			3.45	ND

ND, Not determined; f, faint.

* A second clone gave the bands shown in parentheses.

Fig. 7. Insertion of pPP36 into the BR151 chromosome by a Campbell-like mechanism. The restriction map anticipated for the *spoIIA* region of the BR151/pPP36 chromosome is shown; the region cloned in pPP36, and duplicated upon a Campbell-like insertion, is stippled. Restriction fragments unique to BR151/pPP36 are denoted by an asterisk. Anticipated regions of homology with the various probes are indicated by the lines below the restriction map.

inserted plasmid was tandemly duplicated in this clone (predicted size 13.75 kbp), a conclusion supported by the results of *Eco*RV digestion where the clone gave bands of 14 to 16.3 kbp (predicted size for tandem duplication 14.8 kbp) and no band of about 9.1 kbp – the size expected for a single insertion, and found for the other clone studied (estimates 8.5 to 9.7 kbp). Digestion of BR151/pPP36 with *Eco*RI gave a major hybridizing band of 5.7 to 6.2 kbp. This was assumed to be made up of two bands predicted to be 5.7 and 5.9 kbp that presumably were not resolved in our Southern blots. pHV33, which would be expected to hybridize to one of these, and the 1.8 kbp *Eco*RV fragment, which would be expected to hybridize to the other, both showed a band of about the correct size. Again the bands that were predicted to be the same for BR151 and BR151/pPP36 were found to be so, within experimental error.

DISCUSSION

Plasmids pPP31, 33, 35, and 36 carry different portions of the *B. subtilis spoIIA* locus. All were able to transform a *Spo*⁺ *B. subtilis* strain to chloramphenicol resistance. Analysis of the DNA of the transformants by Southern blots, using a variety of probes, indicated that the plasmids had integrated into the chromosome at the region of homology by a Campbell-like mechanism. In six out of eight cases a single copy of the plasmid was integrated into the chromosome, although a tandem duplication had occurred in DNA from two clones; multiple tandem repeats, found by Young (1983) when studying the *phe* region of the *B. subtilis* chromosome, and free plasmids, found by Wilson *et al.* (1981), were not present. Integration, and excision, was apparently by an

exact mechanism as removal of selection for the plasmid-determined chloramphenicol resistance led to slow segregation of chloramphenicol-sensitive *Spo*⁺ bacteria even where the original integration had made the organism *Spo*⁻; the detailed mechanism of integration and excision was not established. From what is known about the mechanism of transformation of *B. subtilis* by autonomously replicating plasmids with regions of chromosomal homology (Canosi *et al.*, 1981; Iglesias *et al.*, 1981; Chak *et al.*, 1982), there may well be extensive gene-converting activity between chromosome and incoming plasmid in the system employed here.

Plasmids pPP35 and pPP36 gave *Spo*⁻ chloramphenicol-resistant transformants, and so the cloned regions in these plasmids are entirely within a transcriptional unit for sporulation (Fig. 1*a*). (Insertion of pJH101 does not, in itself, cause asporogeny.) However, the cloned regions were not entirely within the *spoIIA69*-complementing cistron that had been delimited on pHM2 (Lencastre *et al.*, 1983). Therefore the transcriptional unit is larger than the cistron, and is presumably polycistronic as the other part of the locus is known to code for a protein (Yudkin & Turley, 1981; Liu *et al.*, 1982) (Fig. 3). This conclusion is reinforced by analysis of the DNA sequence of the *spoIIA* region (Fort & Piggot, 1984) which shows a cluster of three adjacent open reading frames (ORFs); ORF1 corresponds to the *spoIIA69*-complementing cistron. The cloned region of pPP36 extends from within ORF1 to within ORF2, and that of pPP35 from within ORF1 to within ORF3. This indicates a transcriptional unit of three (or possibly more) cistrons.

Plasmid pPP31 gave *Spo*⁺ *Cm*^R transformants of BR151. It differs from pPP35 in that the cloned region extends upstream from the ORF1 initiation codon and from the *Eco*RI site defining one end of the insertion in pPP35 by about 1 kbp to a *Hind*III site. Presumably this region contains the promoter of the transcriptional unit, although the precise position of the promoter remains uncertain (Fort & Piggot, 1984). Plasmid pPP33 also gave *Spo*⁺ *Cm*^R transformants of the *Spo*⁺ strain BR151, so that the promoter-distal end of the transcriptional unit is within the cloned region of pPP33. A plasmid subclone of pPP33, made by self-ligating the largest *Eco*RV fragment from pPP33 (Fig. 2), also gave *Spo*⁺ *Cm*^R transformants of BR151 and transformed to *Spo*⁺ all the *spoIIA* mutations transformable by pPP33 (unpublished observations) so that the transcriptional unit did not extend beyond the leftward of the two *Eco*RV sites in the region cloned in pPP33 (Fig. 3). This gives an upper limit of about 1.9 kbp to the size of the transcriptional unit. As pPP33 is unable to correct the *spoVA89* mutation by recombination, it can be concluded that the *spoVA* locus is not in the same transcriptional unit. It is tentatively concluded that the transcriptional unit identified here corresponds to the *spoIIA* locus. Sporulation loci were defined by groups of asporogenous mutations that map very close together and give rise to a similar phenotype (Piggot & Coote, 1976). The results here highlight the original reservation that locus is not synonymous with cistron (or gene).

This work provides the first demonstration that a polycistronic transcriptional unit is involved in sporulation of *B. subtilis*. Such structures have been anticipated for theoretical reasons (Mandelstam, 1976; Piggot, 1979; Piggot *et al.*, 1981). The *spo* loci are part of dependent sequences of events in which correct expression of early loci is necessary for expression of late loci. Thus any locus has the function, direct or indirect of switching on other loci. It seems teleonomically unlikely that a locus would have the sole function of switching on one other locus, although it could have the sole function of switching on several loci. However, many loci must also have functions to do with the physical assembly of the spore and some such loci could be polygenic. It seems likely that the *spoIIA* transcriptional unit is a polycistronic operon, but the possibility of RNA processing has not been ruled out. It is not yet known how the expression of the transcriptional unit is regulated. Mutations within the *spoIIA* locus are pleiotropic, preventing the expression of other sporulation genes (Waites *et al.*, 1970; Piggot & Coote, 1976), but it is not known how *spoIIA* controls expression of these other genes. In this context, it is intriguing that different mutations within the same *spoIIA* cistron show differing degrees of pleiotropy (Errington & Mandelstam, 1983).

The restriction map of the cloned regions on pHM2 and pPP33 agrees completely with the map of the chromosome determined by Southern blotting. There was no sign of structural instability of the plasmids. The cloned region includes part of the *spoVA* locus (J. Errington, personal communication) as well as the complete *spoIIA* locus. The map also agrees well with

that constructed by Savva & Mandelstam (1984) for a $\phi 105$ clone that covers the entire *spoIIA* and *spoVA* region. The only slight difference with that map is in the order of *Hind*III and *Eco*RI sites at the extreme right hand end of Fig. 3 (a region that was not cloned in a plasmid). This could result from imprecise estimates of fragment sizes from Southern blots, or from slight rearrangements in the $\phi 105$ clone. In any case the discrepancy is outside the *spoIIA*-*spoVA* region and does not affect the conclusions presented here, or in the accompanying papers.

Integrational vectors, such as those used here, are proving to be powerful tools in genetic analysis. They have been used to map cloned genes (Haldenwang *et al.*, 1980), to 'chromosome-walk' (Niaudet *et al.*, 1982), and to introduce genetically engineered mutations into the chromosome (Niaudet *et al.*, 1982; Guttererson & Koshland, 1983; Struhl, 1983). Here we add to the list by using integrational vectors to identify a polycistronic transcriptional unit.

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