Analysis of the Inhibition of Sporulation of *Bacillus subtilis* Caused by Increasing the Number of Copies of the *spo0F* Gene

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The *Bacillus subtilis* *spo0F* gene was cloned on a 6.3 kbp *Bgl*III fragment. The effect on sporulation of amplification of the *spo0F* region was examined. Sporulation was inhibited to less than 5% of that of the parental strain when as few as four copies of the *spo0F* region were present. Subclones, constructed in autonomous or integrative vectors, were used to demonstrate that the region responsible for the copy-number-dependent asporogeny corresponded closely with the *spo0F* structural gene. A possible mechanism for this effect is discussed.

INTRODUCTION

The formation of endospores by *Bacillus subtilis* requires the regulation expression of at least 50 genetic loci (Piggot *et al.*, 1981; Piggot & Hoch, 1985). Gene cloning has provided evidence that some sporulation genes, when present on multicopy plasmids, can inhibit sporulation. Many cloned sporulation genes show little or no copy number effect, but *spo0F* (Kawamura *et al.*, 1981); *spoII*G (Kobayashi & Anaguchi, 1985), *spoV*G (Banner *et al.*, 1983) and the intact *spoII*A locus (authors' unpublished observations) severely reduce sporulation when present in excess.

The mechanism of this copy-number-dependent asporogeny is unknown but two theoretical models can be proposed to account for this effect. In the site model, amplification of specific DNA sequences may titrate out regulatory proteins required to act elsewhere. In this case positively controlled *spo* genes may remain unexpressed and those under negative control would be prematurely expressed. Amplification of the *spoV*G promoter region alone imposes asporogeny, presumably by titration of regulatory proteins (Banner *et al.*, 1983). Alternatively, the product of the 'copy number' gene may be responsible for inhibition of sporulation when present in excess. Clearly such copy number genes must be precisely controlled to ensure spore formation and so may be situated at pivotal regulatory points in the whole process. Even if this is not so, analysis of the effects of gene copy number could prove a powerful analytical tool. In this paper we explore the effectiveness of this approach by analysing the copy number effect of the early sporulation gene *spo0F*.

Experimentally, plasmids unable to replicate in *B. subtilis* can give rise to transformants of *B. subtilis* if they contain sequences derived from the host genome. When selection is for an antibiotic resistance determinant of the vector, such plasmids integrate into the host genome by a Campbell-like mechanism at the region of homology provided by the cloned DNA (Haldenwang *et al.*, 1980; Ferrari *et al.*, 1983; Piggot *et al.*, 1984). By raising the selective pressure for the plasmid-determined drug resistance, it is possible to increase the number of copies of integrated plasmids carried by the chromosome (Young, 1984; Piggot *et al.*, 1984; Sargent & Bennett, 1985; Albertini & Galizzi, 1985). Thus the use of integrative plasmids facilitates the experimental manipulation of copy number. Here we report the controlled

Table 1. *Plasmids carrying fragments from the spo0F region*

Plasmid	Vector	<i>B. subtilis</i> DNA from the <i>spo0F</i> region (see Fig. 8)	Ability to replicate in <i>B. subtilis</i>
pPP41	pJAB1	6.3 kbp <i>Bgl</i> II fragment	+
pPP42	pHV33	2.2 kbp <i>Eco</i> RI fragment	—
pPP99	pJH101	2.2 kbp <i>Eco</i> RI fragment	—
pPP121	pHV33	2.3 kbp <i>Hpa</i> I fragment	+
pPP107	pDB1	0.7 kbp <i>Hpa</i> I– <i>Hind</i> III fragment	—
pPP44	pHV33	2.2 kbp <i>Eco</i> RI fragment deleted for the 0.7 kbp <i>Sst</i> I fragment	+
pPP45	pHV33	2.2 kbp <i>Eco</i> RI fragment deleted for the 0.3 kbp <i>Bcl</i> I fragment	+
pPP48	pHV33	0.3 kbp <i>Bcl</i> I fragment	+
pPP87	pHV33	0.7 kbp <i>Sst</i> I fragment inverted within pPP42	+
pPP109	pDB1	0.4 kbp <i>Pst</i> I– <i>Hind</i> III fragment	—

amplification of the *B. subtilis* *spo0F* locus in the *B. subtilis* genome and show that the presence of as few as four copies of the *spo0F* region severely inhibits sporulation. Integrative and autonomous plasmids carrying subclones of the *spo0F* region were used to show that the area which determined the copy-number-dependent asporogeny corresponds closely to the *spo0F* structural gene.

METHODS

Strains. The *B. subtilis* 168 strains were BR151 (*metB10 lys-3 trpC2*), JH649 (*spo0F221 phe-1 trpC2*) and SL989 (*metB10 lys-3 recE4*). The *Escherichia coli* strains were DH1 (Hanahan, 1983) and SL2034 (Piggot *et al.*, 1984). Plasmids were maintained in DH1 or SL2034 unless otherwise stated.

Plasmids. The plasmid vectors used were pHV33, a chimera of the enterobacterial plasmid pBR322 and the staphylococcal plasmid pC194 (Primrose & Ehrlich, 1981); pJH101, described by Ferrari *et al.* (1983); pJAB1, described by Sargent & Bennett (1985); pDB1, obtained from A. L. Sonenshein and described by Piggot *et al.* (1985), and pSG18, which was a gift from J. Errington and consisted of pUC8 (Messing, 1983) to which a 3.7 kbp DNA fragment carrying the pC194 CAT gene was inserted at the *Bam*HI site in the polylinker sequence. Novel plasmids constructed in this study are listed in Table 1 and the *B. subtilis* DNA fragments carried by each one are shown in Fig. 6.

To construct pPP41, *B. subtilis* 168T⁺ DNA was digested with *Bgl*II and fractionated by electrophoresis in agarose. The peak of *spo0F221* transforming activity was in material of 6 to 6.6 kbp. Fragments of this size were purified from agarose and ligated into the unique *Bcl*I site of the plasmid pJAB1 (Sargent & Bennett, 1985). A plasmid was isolated that had transforming activity for *spo0F221*, and was designated pPP41 (Fig. 1). pPP41 contains 6.3 kbp of *B. subtilis* DNA and carries the wild-type alleles of *ctrA1* (Trach *et al.*, 1985) and *rev-4* (J. Hoch, personal communication), both of which map near the *spo0F* region (Piggot & Hoch, 1985): this is the largest fragment of the *spo0F* region to be cloned.

Plasmid pPP42 was formed by the ligation of the 2.2 kbp *Eco*RI fragment from pPP41 into the single *Eco*RI site of pHV33. To construct pPP121 the 2.3 kbp *Hpa*I fragment from the *spo0F* region (Fig. 1) was ligated into the *Eco*RV site of pHV33. Plasmid pPP44 was constructed by self-ligation of a *Sst*I digest of pPP42 under conditions which favoured intramolecular ligation. pPP45 was formed by self-ligation of a *Bcl*I digest of pPP42. pPP48 was constructed by ligation of the 0.3 kbp fragment from pPP41 (containing part of the *spo0F* gene) into the *Bam*HI site of pHV33. pPP87 was formed by inversion of the 0.7 kbp *Sst*I fragment within pPP42.

Plasmid pPP99 was constructed by ligation of the 2.2 kbp *Eco*RI fragment from pPP41 into pJH101. pPP107 carries the 0.7 kbp *Hpa*I–*Hind*III fragment from pPP41 cloned in pDB1. To facilitate cloning in pDB1, a *Hind*III site was generated adjacent to the *Hpa*I site by first inserting the 1.3 kbp *Hpa*I–*Eco*RI fragment from pPP41 into pSG18 linearized by digestion with *Sma*I and *Eco*RI. Removal of the 3.7 kbp *Bam*HI fragment placed the polylinker *Hind*III site adjacent to the *Hpa*I/*Sma*I junction. The 0.71 kbp *Hind*III fragment from the resulting plasmid thus contained the 0.7 kbp *Hpa*I–*Hind*III fragment from pPP41. This fragment was ligated into the single *Hind*III site of pDB1 to give pPP107. pPP109 was constructed by self-ligation of a *Pst*I digest of pPP107 under conditions that favoured circularization.

Media. In general, *B. subtilis* strains were maintained on nutrient agar and *E. coli* strains on L-agar. Antibiotics were added where appropriate: chloramphenicol (Cm) 5 µg ml⁻¹ for *B. subtilis*, 20 µg ml⁻¹ for *E. coli*; tetracycline

Fig. 1. Restriction map of the DNA cloned in plasmid pPP41. The upper line represents part of pPP41 in the region of the cloning site. The lower portion shows the restriction sites within the cloned DNA. Also shown are the location and direction of transcription of ORFS and *spo0F*.

Fig. 2. Effect of insertion of plasmid pPP41 into the *spo0F* region by a Campbell-type mechanism. The restriction map of the *spo0F* region of strain BR151 is shown at the top and that anticipated for BR151/pPP41 shown in the lower portion of the figure. The region that is cloned in pPP41 is stippled; it is duplicated by insertion of one copy of pPP41 into the genome. The vector DNA, inserted between these duplicated blocks, is shown as single line. Insertion of one copy of pPP41 increases the size of the *spo0F* BglII fragment from 6.3 kbp to 18.3 kbp. The size of this fragment will increase by 12 kbp for each further copy of pPP41 integrated. Restriction sites are Bgl, BglII; E, *EcoRI*; P, *PstI*. The size of the large *PstI* fragment is 14 kbp.

20 $\mu\text{g ml}^{-1}$; ampicillin 25 $\mu\text{g ml}^{-1}$. Medium MSSM (see Results) contained Difco nutrient broth 16 g l^{-1} , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g l^{-1} , KCl 2 g l^{-1} , $\text{Ca}(\text{NO}_3)_2$ 1 mM, MnCl_2 0.1 mM, FeSO_4 0.001 mM, glucose 1%; pH 7.0.

DNA preparation. *B. subtilis* chromosomal DNA was prepared by the method of Piggot *et al.* (1984). Large-scale preparation of plasmid DNA was done by the method of Guerry *et al.* (1973).

Enzymes. Restriction enzymes were purchased from P & S Biochemicals, BRL or Boehringer. T₄ DNA ligase and calf intestinal phosphatase were purchased from Boehringer.

Transformation. *E. coli* was transformed by the method of Brown *et al.* (1979), and *B. subtilis* as described by Piggot *et al.* (1984).

Southern blotting. Transfer of DNA from gels onto nitrocellulose filters (Schleicher & Schüll) and subsequent probing with ³²P-labelled DNA was done by the method of Southern (1975). Approximately 0.5 μCi (18.5 kBq) probe was used for each blot. The probe DNA was labelled to high specific activity with [³²P]dATP by nick-translation (Jeffreys & Flavell, 1977).

Copy number measurement. (i) *Measurement of the size of the reiterated region.* Integration of pPP41 into the genome of BR151 at the *spo0F* region will increase the size of the 6.3 kbp BglII fragment by 12 kbp for every copy of the plasmid integrated (Fig. 2). To measure the size of the reiterated region, chromosomal DNA was digested to completion with BglII and the fragments were separated by electrophoresis at low voltage (0.5 V cm^{-2}) in 0.3% agarose. The fragments were transferred to nitrocellulose and the *spo0F* BglII fragment was identified by hybridization with pPP42, which carries part of the *spo0F* region. The size of this fragment was measured with reference to the mobility of restricted and unrestricted λ DNA standards.

(ii) *Differential hybridization.* The copy number of pPP99 and pPP107 was estimated by comparison of the ratio between single-copy restriction fragments and fragments carried by the plasmid which become reiterated on

Fig. 3. Effect of insertion of plasmids pPP99 and pPP107 into the *spo0F* region by a Campbell-type mechanism. The stippled region represents the *spo0F* DNA cloned in the plasmid and the single line represents vector DNA. The upper portion of the figure represents integration of two copies of pPP99 into the *spo0F* region of strain BR151. The 14 kbp *Pst*I fragment remains in single copy irrespective of the number of plasmids integrated but the copy number of the 5.8 kbp *Pst*I fragment, an internal fragment of pPP99, increases in proportion to the number of copies integrated. The lower portion of the figure represents insertion of two copies of pPP107 into the BR151 genome. In this case a 1.56 kbp *Hind*III fragment remains in single copy whilst a 0.7 kbp *Hind*III fragment increases in number for each copy of the plasmid integrated. Restriction sites are as in Fig. 2.

amplification of plasmid sequences within the chromosome. On integration of multiple copies of pPP99 the 14 kbp *Pst*I fragment which spans the cloned DNA remains in single copy but the copy number of the 5.8 kbp *Pst*I fragment increases with each copy of the plasmid integrated (Fig. 3). For copy number estimation DNA was prepared from strain BR151 transformed with pPP99 and approximately 1 µg was digested to completion with *Pst*I. The fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose. The filters were probed with 32 P-labelled 0.7 kbp *Bcl*I *Eco*RI fragment from pPP41 and the 14 and 5.8 kbp fragments identified by autoradiography. These fragments were excised from the filter and the radioactivity bound to each was measured in a Beckman liquid scintillation spectrometer. The radioactivity bound to a background sample, excised from the same filter, was also measured. The number of copies of the plasmid present was calculated by comparison of the amount of radioactivity bound to the single-copy fragment with that bound by the reiterated fragment after correction for background radioactivity. For BR151/pPP107 transformants a similar comparison was made between the single-copy 1.56 kbp fragment and the reiterated 0.7 kbp *Hind*III fragments using the 0.4 kbp *Pst*I-*Hind*III fragment from pPP41 as a probe (Fig. 3).

RESULTS

Inhibition of sporulation by the spo0F region carried on an autonomous plasmid

The 2.2 kbp *Eco*RI fragment from plasmid pPP41 (Fig. 1) contains the *spo0F* and ORF5 genes (Trach *et al.*, 1985). This fragment was shown by Kawamura *et al.* (1981) to inhibit sporulation when cloned into plasmid pUB110. To confirm these results the 2.2 kbp *Eco*RI fragment was cloned into pHV33 to give plasmid pPP42. pPP42 was introduced by transformation into strain SL989. This strain carries the *recE4* mutation that prevents recombination between plasmid and chromosomal sequences and so prevents integration of the plasmid into the chromosome. The resulting transformants failed to develop the brown pigmentation associated with sporulation, and examination by phase-contrast microscopy showed that sporulation was impaired (< 1 spore in 10^4). Strain SL989 transformed with pHV33 sporulated normally.

Inhibition of sporulation imposed by plasmid pPP42 was demonstrated in liquid medium by resuspension of exponentially growing pPP42 transformants in the sporulation medium of Sterlini & Mandelstam (1969), containing 3 µg Cm ml $^{-1}$. Only 0.01 % of the pPP42-containing cells produced spores. This is a similar frequency to that observed by Kawamura *et al.* (1981) for

the same fragment of *B. subtilis* DNA cloned in pUB110. pUB110 is present in at least 50 copies per cell whereas pPP42 has a copy number of about 17 (Chapman, 1985). Thus relatively few copies of the *spo0F* region have a marked effect on sporulation.

Amplification of the spo0F region in the B. subtilis chromosome

Autonomous plasmids carrying the *spo0F* locus were subject to segregational and structural instability. Moreover, it was impossible to lower their copy number without increasing the frequency of plasmid loss. Therefore integrative plasmids were used to manipulate the copy number of the *spo0F* region.

The Spo⁺ *B. subtilis* strain BR151 was transformed with pPP41 and recombinants were selected on nutrient agar containing 5 µg Cm ml⁻¹: all transformants were Spo⁺. Single BR151/pPP41 transformants were grown on nutrient agar containing 30 µg Cm ml⁻¹ to select for amplification of the plasmid integrated in the genome. When grown at 30 µg Cm ml⁻¹ the BR151/pPP41 transformants formed unpigmented, translucent colonies; phase-contrast microscopy revealed that these colonies contained <0.1% spores. When such Spo⁻ transformants were returned to growth at 5 µg Cm ml⁻¹ they recovered the Spo⁺ phenotype. This showed that the asporogeny at 30 µg Cm ml⁻¹ was not caused by irreversible deletion or rearrangement of the *spo0F* region. In a control experiment, strain BR151 was transformed with plasmid pPP31, an integrative plasmid carrying part of the *spoIIA* region cloned in pJAB1 (Piggot *et al.*, 1984). All clones were Spo⁺ when grown on media containing 30 µg Cm ml⁻¹. Thus the asporogeny of the BR151/pPP41 transformants was not an artifact of the method. Rather it suggested that multiple copies of the *spo0F* region integrated into the chromosome suppressed sporulation.

As plasmid pPP41 does not contain any *Bg*/II sites, integration of pPP41 will increase the size of the chromosomal 6.3 kbp *Bg*/II fragment from the *spo0F* region by 12 kbp (the size of pPP41) for each copy of the plasmid integrated. Chromosomal DNA, prepared from pPP41 transformants of strain BR151 grown in MSSM containing Cm at a variety of concentrations, was digested with *Bg*/II and separated by electrophoresis in 0.3% agarose. The DNA was transferred to nitrocellulose and probed with ³²P-labelled pPP42 (Fig. 4). The relationship between copy number and sporulation is shown in Table 2. Increasing the copy number of pPP41 from one to four severely inhibited sporulation. As BR151 already contained one copy of the *spo0F* region this is equivalent to increasing the *spo0F* copy number from two to five.

Although 0.3% gels effectively resolved DNA up to 40 kbp in size it was difficult to accurately measure copy number from these gels when four or more copies were present. The copy number of integrated plasmids can be estimated by quantitative hybridization (Young, 1984; see Methods); this method was used to estimate the copy number of plasmids pPP99 and pPP107, which carry the 2.2 kbp *Eco*RI and 0.7 kbp *Hpa*I–*Hind*III fragments from the *spo0F* region, respectively. BR151 was transformed with pPP99 or pPP107 and the resulting transformants, selected at 5 µg Cm ml⁻¹, were grown on nutrient agar containing Cm at a variety of concentrations. These strains were then grown and sporulated in MSSM containing Cm at concentrations of up to 30 µg ml⁻¹. DNA was prepared from these cultures 30 min before the end of the exponential growth and the plasmid copy number was estimated from the ratio of the level of hybridization of specific probes to the single-copy and to amplified fragments as described in Methods (Table 3). Both the 2.2 kbp *Eco*RI fragment and the 0.7 kbp *Hpa*I–*Hind*III fragment inhibited sporulation when amplified in the chromosome. The titration of sporulation against copy number is shown in Fig. 5. It is clear that sporulation is extremely sensitive to *spo0F* copy number, as few as four copies of the region being severely inhibitory.

Localization of the region which determines copy number dependent asporogeny

To determine which part of the *spo0F* region was responsible for the copy number effect, subclones of this region were constructed in autonomous or integrational vectors. The effect on sporulation of these subclones in high copy number was determined (Fig. 6).

As discussed above, the 2.2 kbp *Eco*RI fragment from the *spo0F* region inhibited sporulation. Similarly, the 2.3 kbp *Hpa*I fragment carried by the autonomous plasmid pPP121 also inhibited

Fig. 4. Southern blot of DNA from strain BR151 transformed with plasmid pPP41. DNA, prepared from transformants grown in the presence of chloramphenicol at a variety of concentrations (see Table 2), was digested to completion with *Bgl*II; the fragments were separated by electrophoresis in 0.3% agarose and transferred to nitrocellulose. The blot was then probed with ³²P-labelled pPP42, which carries a portion of the *spo0F* region, to identify fragments from the *spo0F* region. The native *spo0F* *Bgl*II fragment is 6.3 kbp in size and increases by 12 kbp for each copy of pPP41 integrated at the *spo0F* region.

Table 2. *Inhibition of sporulation imposed by plasmid pPP41*

The copy number of pPP41 was estimated by analysis of *Bgl*II digests of BR151/pPP41 chromosomal DNA on a 0.3% agarose gel (Fig. 4). The figures shown in parentheses represent minor components of the population.

Strain and clone no.	Cm concn (µg ml ⁻¹)	Size of <i>spo0F</i> <i>Bgl</i> II fragment	pPP41 copy no.	Spores (%)
BR151/pPP41 1	5	18	1	28
BR151/pPP41 2	5	18	1	40
BR151/pPP41 3	10	18	1	48
BR151/pPP41 4	20	40	3	2
BR151/pPP41 5	20	50	4	0.4
BR151/pPP41 6	25	40 (50)	3 (4)	0.2

sporulation. Plasmid pPP107, which contains the 0.7 kbp *Hpa*I–*Hind*III fragment from the *spo0F* region, inhibited sporulation when amplified in the chromosome. Plasmids pPP121 and pPP107 carry the complete *spo0F* gene but code for only the first eight amino acids of ORFS product; thus the inhibition of sporulation by these plasmids conclusively demonstrated that the ORFS product was not responsible for the copy-number-dependent asporogeny. Plasmid pPP109, a derivative of pPP107 from which the 0.3 kbp *Hpa*I–*Pst*I fragment from the *spo0F*

Fig. 5. Inhibition of sporulation imposed by pPP41 (●), pPP99 (△) and pPP107 (▲) (from the data in Tables 2 and 3). The non-integral copy numbers were obtained from differential hybridization experiments. The values for sporulation of BR151 without plasmids were derived from phase-contrast microscopy of cultures grown overnight in MSMM.

Table 3. Effect of multiple copies of pPP99 and pPP107 on the sporulation of BR151

The copy number of each plasmid was estimated by comparing the radioactivity bound to the single-copy fragments which span the boundary of insertion with that bound to fragments contained entirely within the cloned DNA, after subtraction of background radioactivity determined from the same filter.

Strain and clone no.	Fragment	Radioactivity bound (c.p.m.)	Plasmid copy no.	Spores (%)
BR151/pPP99 1	14 kbp <i>Pst</i> I	35	5.1	0.013
	5.8 kbp <i>Pst</i> I	100		
BR151/pPP99 2	14 kbp <i>Pst</i> I	41	3.2	0.5
	5.8 kbp <i>Pst</i> I	130		
BR151/pPP99 3	14 kbp <i>Pst</i> I	66	3.5	0.6
	5.8 kbp <i>Pst</i> I	235		
BR151/pPP107 1	1.56 kbp <i>Hind</i> III	265	0.66	22
	0.7 kbp <i>Hind</i> III	173		
BR151/pPP107 2	1.56 kbp <i>Hind</i> III	440	0.63	35
	0.7 kbp <i>Hind</i> III	277		
BR151/pPP107 3	1.56 kbp <i>Hind</i> III	201	6.1	0.08
	0.7 kbp <i>Hind</i> III	1236		
BR151/pPP107 4	1.56 kbp <i>Hind</i> III	318	5.1	0.15
	0.7 kbp <i>Hind</i> III	1620		
BR151/pPP107 5	1.56 kbp <i>Hind</i> III	688	4.2	0.8
	0.7 kbp <i>Hind</i> III	2875		
BR151/pPP107 6	1.56 kbp <i>Hind</i> III	343	4.7	0.5
	0.7 kbp <i>Hind</i> III	1602

Fig. 6. Effect on sporulation of multiple copies of subclones from the *spo0F* region. The upper line shows a restriction map of part of the *spo0F* region. The boxes represent the extent of *B. subtilis* DNA carried by the various plasmids. pPP42, pPP121, pPP44, pPP45 and pPP87 are autonomous plasmids based on the pHV33 replicon. These plasmids were introduced into SL989 and their effect on sporulation was assessed. pPP99, pPP107 and pPP109 are integrative plasmids based on pJH101 or pDB1. These plasmids were amplified in the genome of BR151 by growth on nutrient agar containing at least $30 \mu\text{g Cm ml}^{-1}$. The minimum (hatched area) and maximum size of the inhibitory region is indicated above the restriction map, as are the locations of ORFS and *spo0F*. Restriction sites are Bc, *Bcl*I; E, *Eco*RI; H, *Hind*III; Hp, *Hpa*I; S, *Sst*I; P, *Pst*I.

region was deleted, had no effect on sporulation. These results show that the maximum region required for the copy number effect extends from the *Hpa*I site 5' to *spo0F* to the first *Hind*III site 3' to *spo0F* (Fig. 6).

The autonomous plasmid pPP45, which lacks the 0.3 kbp *Bcl*I fragment from pPP42, did not affect the sporulation of strain SL989. This, in conjunction with the results for pPP107, defines one end of the inhibitory region as lying between the *Hpa*I site and the *Bcl*I site within *spo0F*. The maximum rightward extent of the inhibitory region was defined by plasmid pPP44, in which the 0.7 kbp *Sst*I fragment was deleted from pPP42, and by pPP87, which carries this fragment alone. As neither plasmid inhibited sporulation, the copy number effect must require the integrity of at least one of the *Sst*I sites. Only the *Sst*I site within the *spo0F* gene lies in the maximum inhibitory region as defined by pPP107. Thus the rightward end of the inhibitory region must lie between this *Sst*I site and the first *Hind*III site 3' to *spo0F*.

The 0.3 kbp *Bcl*I fragment, cloned in pPP48, and the 0.7 kbp *Sst*I fragment in pPP87 carry the promoter regions for *spo0F* and ORFS (Trach *et al.*, 1985). As neither plasmid inhibited sporulation it is clear that it is not the titration of regulatory proteins by the upstream region of *spo0F* or ORFS that is responsible for the copy number effect.

DISCUSSION

Multiple copies of the *B. subtilis* *spo0F* region inhibit sporulation (Kawamura *et al.*, 1981; Piggot *et al.*, 1985). To determine the cause of the copy-number-dependent asporogeny a 6.3 kbp *Bgl*II fragment from the *spo0F* region was cloned. This fragment, carried by pPP41, contained the complete *spo0F* gene, an open reading frame of unknown function (ORFS) and the wild-type alleles of *ctrA1* (Trach *et al.*, 1985) and *rev-4* (J. Hoch, personal communication).

Subclones of pPP41 were used to locate the maximum and minimum extent of the region that exerted the inhibitory effect on sporulation. This region corresponded closely to the *spo0F* structural gene (Fig. 6). Yoshikawa *et al.* (1985) have also analysed the effects of deletion of this region on copy number inhibition and their results delimit one end of the inhibitory region more precisely. Although they interpreted their data in relation to an effect upon ORFS it can be deduced from their work that a deletion removing four residues from the C-terminus of the *spo0F* protein did not alter the copy number effect and that a deletion removing a further six residues from the C-terminus abolished the inhibition. Amplification of the *spo0F* promoter sequences lacking the intact gene did not inhibit sporulation. Thus the *spo0F* inhibition of sporulation differs from that imposed by *spoVG* (Banner *et al.*, 1983) and may be the first example where it is the *product* of a *spo* gene that inhibits sporulation when present in excess.

Sporulation is very sensitive to *spo0F* copy number (Fig. 5). The *spo0F* effect is a striking example of how gene dosage can affect sporulation and may be useful in determining the role of *spo0F* during sporulation. Mutations at the *spo0F* locus arrest cells at stage 0 of sporulation (Hoch & Mathews, 1973). Electron microscopy of *B. subtilis* strains carrying multiple copies of the *spo0F* locus showed that sporulation of these strains was arrested at stage 0, i.e. before any of the morphological changes associated with sporulation were apparent (results not shown). Thus the copy number effect appears to act at stage 0. However, although *spo0F* mutations, including deletions, are suppressed by the *sof-1* allele of *spo0A* (Hoch *et al.*, 1985) *sof-1* was unable to overcome the asporogeny imposed by pPP42 (J. Hoch, personal communication). Therefore the mechanisms by which *spo0F* mutation and overproduction of *spo0F* gene product affect sporulation are distinct.

The *spo0* products may constitute the system by which *B. subtilis* recognizes and responds to nutrient deprivation. It is possible that the overproduction of the *spo0F* product titrates an effector molecule also recognized by the *spo0A* product and thus deprives the *spo0A* product of the stimulus required for its activation. This mechanism may explain the copy-number-dependent asporogeny observed for *spo0F*. At this stage it is not possible to draw quantitative conclusions about the effect of the *spo0F* product as we do not know how gene copy number relates to the amount of product. Careful analysis of the copy number effect may shed light on the interaction between *spo0F* and *spo0A*. In more general terms the manipulation of the copy number of specific genes by the methods presented here will provide a powerful analytical tool in the study of sporulation.

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