Cloning in *Bacillus subtilis* by Transfection with Bacteriophage Vector ϕ 105J27: Isolation and Preliminary Characterization of Transducing Phages for 23 Sporulation Loci

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Bacteriophage cloning vector ϕ 105J27, the construction of which is described in an accompanying paper, has been used for shotgun cloning of sporulation genes in *Bacillus subtilis*. Various genomic libraries have been constructed and screened for the presence of recombinant phages capable of transducing strains containing sporulation (*spo*) mutations to Spo⁺. Of a total of 30 *spo* loci tested, transducing phages have been isolated for 23, more than half of the known *spo* loci. Included are nine loci (*spo0D*, *spo0J*, *spoIIIA*, *spoIIIE*, *spoIIIF*, *spoIVF*, *spoVB*, *spoVH* and *spoVJ*) that do not appear to have been cloned previously. Preliminary genetic characterization of some of the new clones by a rapid screening procedure has enabled the status of various sporulation loci to be clarified.

INTRODUCTION

Endospore formation in *Bacillus subtilis* represents a simple model system for cellular development and differentiation. Since genetic methods for use in this organism are now quite sophisticated, this developmental system is amenable to study at the molecular level. The isolation of mutations that interfere with development (*spo*) has enabled many of the genes controlling the process to be identified. About 50 genetic loci have been identified (Piggot & Coote, 1976; Piggot & Hoch, 1985*b*). These 'loci' were defined mainly on the basis of genetic mapping of *spo* mutations by transduction or transformation, and by the phenotypic consequences of mutation. Gene cloning and sequencing have shown that some *spo* loci are polycistronic, while others are monocistronic (see for example: Lopez-Diaz *et al.*, 1986; Fort & Errington, 1985). However, the absence of a reliable and efficient means of cloning genes in *Bacillus* has, until recently, made similar analyses of the majority of *spo* loci difficult.

The accompanying paper (Jones & Errington, 1987) describes the construction of a new series of cloning vectors that improve the efficiency of a *B. subtilis* cloning system based on direct transfection of protoplasts with DNA from bacteriophage ϕ 105 (Errington, 1984). The general advantages of this cloning system have been fully discussed previously (Errington, 1984). This paper describes the use of one of the new vectors, ϕ 105J27, to construct *B. subtilis* genomic libraries, and the isolation and preliminary characterization of 31 new recombinant phages, capable of transducing to Spo⁺ strains harbouring mutations in a total of 23 *spo* loci.

METHODS

Bacterial strains. *B. subtilis* 168 *trpC2* is a sporogenic laboratory wild-type strain. The various *spo* mutants that were employed are listed in Table 1.

Preparation of phage. The methods of Jenkinson & Mandelstam (1983) were used, as modified by Jones & Errington (1987).

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Table 1. *Bacillus subtilis* strains

Strain	Genotype*	Origin†
2.3	<i>lys-1 spoIIIB2</i>	Piggot (1973) (A3)
8	<i>trpC2 spoIVC8</i>	Piggot (1973) (E13)
17.2	<i>metC3 tal-1 spo0H17</i>	Waites <i>et al.</i> (1970) (E22)
20	<i>trpC2 spoIIE20</i>	Waites <i>et al.</i> (1970) (N25)
21	<i>trpC2 spoIIE21</i>	Piggot (1973) (U27)
23.1	<i>trpC2 spoIVC23</i>	Waites <i>et al.</i> (1970) (E31)
26.2	<i>leuA8 tal-1 spoIIIA26</i>	Piggot (1973) (E34)
35	<i>trpC2 spoIIIA35</i>	Waites <i>et al.</i> (1970) (NG13)
36	<i>trpC2 spoIIIE36</i>	Piggot (1973) (NG1.67)
41.1	<i>leuA8 tal-1 spoIIG41</i>	Piggot (1973) (NG4.14)
43.6	<i>trpC2 spo0A43</i>	Piggot (1973) (NG6.21)
44.1	<i>metC3 tal-1 spoIIIA44</i>	Piggot (1973) (NG7.2)
46	<i>trpC2 spo0H46</i>	Piggot (1973) (NG7.17)
48.7	<i>trpC2 spoIIE48</i>	Piggot (1973) (NG9.3)
49.2	<i>metC3 tal-1 spoIIG49</i>	Piggot (1973) (NG10.2)
53.1	<i>trpC2 rpoB2 spoIIIA53</i>	Piggot (1973) (NG12.5)
55.2	<i>trpC2 rpoB2 spoIIG55</i>	Piggot (1973) (NG12.12)
59.2	<i>metC3 spoIIIA59</i>	Piggot (1973) (NG14.7)
60.1	<i>leuA8 rpoB2 spoIIE60</i>	Piggot (1973) (NG14.22)
61.1	<i>leuA8 rpoB2 spoIIE61</i>	Piggot (1973) (NG15.4)
64.1	<i>leuA8 rpoB2 spoIIE64</i>	Piggot (1973) (NG17.15)
65	<i>trpC2 spoIIIA65</i>	Piggot (1973) (NG17.17)
67	<i>trpC2 spoIVA67</i>	Piggot (1973) (NG17.23)
69.15	<i>trpC2 spoIIIA69</i>	Piggot (1973) (NG18.6)
71.2	<i>trpC2 spoIIE71</i>	Piggot (1973) (NG20.12)
79.2	<i>lys-1 spoIIIA79</i>	Hranueli <i>et al.</i> (1974)
81.1	<i>leuA8 rpoB2 spo0H81</i>	Hranueli <i>et al.</i> (1974)
83	<i>metC3 leuA8 tal-1 spo0H83</i>	Hranueli <i>et al.</i> (1974)
85	<i>trpC2 spoVE85</i>	Hranueli <i>et al.</i> (1974)
86.2	<i>metC3 tal-1 spoIIIA86</i>	Hranueli <i>et al.</i> (1974)
87.2	<i>leuA8 tal-1 spo0J87</i>	Hranueli <i>et al.</i> (1974)
88.7	<i>trpC2 spoIVF88</i>	Hranueli <i>et al.</i> (1974)
90.2	<i>metC3 tal-1 spoIIIA90</i>	Hranueli <i>et al.</i> (1974)
91	<i>trpC2 spoVB91</i>	Hranueli <i>et al.</i> (1974)
92	<i>trpC2 metC3 rpoB2 spoIVD92</i>	Hranueli <i>et al.</i> (1974)
93.2	<i>trpC2 spo0J93</i>	Hranueli <i>et al.</i> (1974)
133.1	<i>trpC2 spoIVC133</i>	Coote (1972 <i>b</i>) (Z7)
141	<i>trpC2 spo0K141</i>	Coote (1972 <i>a</i>) (Z31)
145	<i>trpC2 spoIIIA145</i>	Coote (1972 <i>a</i>) (Y9)
149.1	<i>pheA12 spoIVC149</i>	Coote (1972 <i>b</i>) (X2)
150.1	<i>pheA12 spoIIIA150</i>	Coote (1972 <i>a</i>) (X3)
153.2	<i>pheA12 spoVE153</i>	Coote (1972 <i>a</i>) (W5)
156	<i>trpC2 spoVD156</i>	Coote (1972 <i>a</i>) (W10)
158	<i>trpC2 spoIIIA158</i>	Coote (1972 <i>a</i>) (W12)
163	<i>trpC2 spoIIIA163</i>	Coote (1972 <i>a</i>) (P4)
207.2	<i>pheA12 spoIVF207</i>	Lamont (1983)
221.1	<i>trpC2 spo0F221</i>	Hoch & Mathews (1973) (<i>spoF221</i>)
298.2	<i>pheA12 spoIID298</i>	Coote (1972 <i>a</i>) (P9)
484.2	<i>trpC2 spo0E11</i>	Hoch & Mathews (1973) (<i>spoE11</i>)
485.1	<i>trpC2 spo0G14</i>	Ionesco <i>et al.</i> (1970) (14UL)
486	<i>trpC2 spo0D8</i>	Ionesco <i>et al.</i> (1970) (8H)
488.1	<i>lys-1 spo0B136</i>	Hoch & Mathews (1973) (<i>spoB136</i>)
496	<i>trpC2 spoIIIC94</i>	Ionesco <i>et al.</i> (1970) (94U)
497.1	<i>trpC2 spoIID83</i>	Ionesco <i>et al.</i> (1970) (83U)
498	<i>trpC2 spoIVE11</i>	Ionesco <i>et al.</i> (1970) (11T)
516	<i>trpC2 spoVH516</i>	Hill (1983)
517	<i>trpC2 spoVJ517</i>	Hill (1983)
518	<i>trpC2 spoVH518</i>	Hill (1983)
522.2	<i>trpC2 gerE36</i>	Moir (1981)
574.1	<i>lys-1 tal-1 spoVAA574</i>	Errington & Mandelstam (1984)
585	<i>lys-1 spoIVF585</i>	Lamont (1983)
586	<i>lys-1 spoIVF586</i>	Lamont (1983)
590.4	<i>trpC2 spoIIIF590</i>	Lamont & Mandelstam (1984)
IS233	<i>trpC2 pheA1 spo0HΔHind</i>	Weir <i>et al.</i> (1984)

* *tal-1*, resistance to β -thienylalanine.

† Strain isolation numbers or previous designations of mutant alleles are given in parentheses.

Preparation of DNA. Chromosomal DNA from strain 168, and phage DNA, were prepared as described previously (Errington, 1984).

Ligations. Chromosomal DNA (about 500 ng) and ϕ 105J27 DNA (*Bam*HI digested, about 200 ng) were ligated in 50 μ l reaction volumes for 16 h at 4 °C as described previously (Errington, 1984). The chromosomal DNA was prepared in three ways: partial digestion with *Bcl*I, complete digestion with *Bg*/II or partial digestion with *Mbo*I followed by size fractionation on BRL low-melting-point agarose (0.6%, v/v) to the range 3 to 4 kbp. This DNA was recovered using the method recommended by the suppliers.

Protoplast transfection. Protoplasts of strain CU267 were prepared using the method of Chang & Cohen (1979) as modified by Levi-Meyrueis *et al.* (1980). Methods for transfection with ligated DNA samples in the presence of polyethylene glycol, growth and recovery of recombinant phage pools were as described previously (Errington, 1984) except that the growth temperature was 30 °C.

Isolation of recombinant phages. Samples (50 μ l) of recombinant phage pools were mixed with phage-sensitive recipient cells (400 μ l of mid-exponential phase culture grown in Difco Penassay broth at 37 °C) and plated on Schaeffer's agar (Schaeffer *et al.*, 1965). After incubation for 40 h at 30 °C, *Spo*⁺ transductants were selected by treatment with CHCl₃ vapour (Hoch, 1971) for 4 h at 22 °C. *Spo*⁺ transductants, which appeared after 2 to 3 d reincubation at 30 °C, were pooled, and small-scale (5 ml) phage lysates were prepared by temperature induction (Jones & Errington, 1987). For highly oligosporogenous test strains containing mutations *spo0E11*, *spo0J87*, *spo0J93*, *spo0K141*, *spo0IIA26*, *spo0IVF88*, *spo0VH516* and *spo0VJ517*, it was not possible to identify *Spo*⁺ transductants among the large numbers of survivors. Instead, after several days of regrowth, the plates were replica plated to Oxoid nutrient agar and incubated for approximately 12 h at 30 °C to allow vegetative growth to be resumed. A lysate was then prepared from all of the pooled survivors. Lysates were tested for further transduction to *Spo*⁺ by one of two methods. Portions (20 μ l) of serial 100-fold dilutions were spotted on to lawns of mid-exponential phase cells (grown as already described) on Schaeffer's agar. After a further period of growth, selection for *Spo*⁺ cells was carried out (see above). Alternatively, and mainly for lysates made from purified strains, a 100 μ l sample of undiluted lysate (about 10⁹ p.f.u.) was spread over half of the surface of a Schaeffer's agar plate and allowed to dry. Strains to be tested for complementation (up to 12 per 9 cm plate) were then streaked across the plate from the unseeded area into the seeded area using a sterile toothpick. Growth and *Spo*⁺ selection were as above. (After incubation for only 12 h it was possible to check that the recipient strain was phage sensitive by the break in cell density apparent at the boundary between seeded and unseeded areas. This break did not occur in phage-resistant or immune strains and is a useful rapid test for phage immunity.) Complementation of the *spo* mutation was evident several days after treatment with CHCl₃ by confluent regrowth on only the phage-infected side of the plate. Where complementation did *not* occur, regrowth was either absent (for *Spo*⁻ strains), or partial and more or less uniform along the complete streak (for oligosporogenous strains). Moreover, when complementation occurred, the surviving lysogens were phenotypically *Spo*⁺ in terms of both pigmentation and the presence of phase-bright spores. Phages that did not complement but were able to transduce a *Spo*⁻ mutant to *Spo*⁺ by marker rescue recombination (see Results and Discussion and Fig. 1) gave rise to a stippled appearance following regrowth, as most of the infected cells remained *Spo*⁻. Again, the survivors were phenotypically *Spo*⁺ rather than mutant.

Induction of sporulation. Cells were grown in hydrolysed casein medium then induced to sporulate by resuspension in sporulation medium as described by Sterlini & Mandelstam (1969). Times (h) after resuspension are denoted *t*₁, *t*₂, *t*₃, etc. Growth and sporulation at 37 °C did not seem to cause significant loss of cells by phage induction.

Assay of heat resistant spores. A sample (1 ml) of sporulating culture at *t*₀ was heated at 85 °C for 15 min. Suitable dilutions were plated on Oxoid nutrient agar and incubated at 30 °C. Colonies were counted after 2 d at 30 °C.

RESULTS AND DISCUSSION

Construction and screening of B. subtilis genomic libraries

Chromosomal DNA from *B. subtilis* strain 168 was partially digested with *Mbo*I and the fragments were size fractionated to the range of 3 to 4 kbp. About 200 ng of this DNA was ligated to about 500 ng of the vector, ϕ 105J27, that had been cleaved at its unique *Bam*HI site. After transfection of strain CU267 protoplasts, about 10⁵ p.f.u. were obtained and the progeny phage were recovered as four independent pools of roughly equal size. A series of sporulation mutants were screened for transduction by the library of recombinant phages as described in Methods. *Spo*⁺ survivors were pooled, temperature induced and the sterilized lysates were tested for a second round of *Spo*⁺ transduction. Successful cloning was indicated by high frequency transduction of the *Spo*⁺ phenotype. Most strains were tested for transduction by a mixture of phage particles from all four pools. Several strains were tested against each individual pool and

Table 2. Screening *B. subtilis* genomic libraries constructed with phage $\phi 105J27$ for the presence of sporulation loci

Chromosomal DNA from *B. subtilis* strain 168 was prepared in various ways (see Methods) for cloning in $\phi 105J27$. After transfection of recipient strain CU267, the resultant pools of progeny phage were screened for the ability to transduce each of a series of strains containing *spo* mutations to *Spo*⁺ by selection for resistance to CHCl_3 vapour. A space indicates that screening was not carried out. A dash indicates that screening was performed but that no transduction was detected. Where selection was successful, the designation of the recombinant phage isolated is given.

spo mutation tested	Genomic library screened:			
	<i>Mbo</i> I-A	<i>Mbo</i> I-B	<i>Bcl</i> I	<i>Bgl</i> II
<i>spo0A43</i>	—			
<i>spo0B136</i>	$\phi 105J39$			
<i>spo0D8</i>		$\phi 105J96$	—	—
<i>spo0E11</i>	—	—	—	—
<i>spo0F221</i>	$\phi 105J40$			
<i>spo0G14</i>	—	—	$\phi 105J94$	—
<i>spo0H46</i>		$\phi 105J80$	—	—
<i>spo0J87*</i>	—	$\phi 105J92$	$\phi 105J93$	—
<i>spo0K141</i>	—	—	—	—
<i>spoIIA69</i>	$\phi 105J32$			$\phi 105J84$
<i>spoIID298</i>	$\phi 105J33, \phi 105J34$		$\phi 105J85$	
<i>spoIIE61</i>	—	—	—	$\phi 105J97$
<i>spoIIG55</i>	—	$\phi 105J95$	—	—
<i>spoIIIA26*</i>	—	—	$\phi 105J83$	—
<i>spoIIIB2</i>	$\phi 105J35$			
<i>spoIIIC94</i>	—			
<i>spoIIID83</i>	—	—	—	—
<i>spoIIIE36</i>	$\phi 105J30, \phi 105J31$			
<i>spoIIIF590</i>	—			
<i>spoIVA67</i>	—	—	—	—
<i>spoIVC23</i>	—	$\phi 105J79, \phi 105J91$	$\phi 105J78$	—
<i>spoIVE11</i>	—	—	—	—
<i>spoIVF88</i>	$\phi 105J36$			
<i>spoVAA574</i>	$\phi 105J41$			
<i>spoVB91</i>	—	—	—	$\phi 105J86$
<i>spoVD156</i>	—			
<i>spoVE85</i>	$\phi 105J37$			
<i>spoVH516</i>		$\phi 105J87^\dagger$	$\phi 105J88^\dagger$	$\phi 105J98^\dagger$
<i>spoVJ517</i>		$\phi 105J81$	$\phi 105J82$	—
<i>gerE36</i>	$\phi 105J44^\dagger$			

* *Mbo*I-A library was screened against mutations *spo0J93* and *spoIIIA59* rather than the mutations shown.

† These phages were isolated by S. M. Cutting.

in such cases more than one independent transducing phage was sometimes isolated. The results of the screening process, and the designations of the new recombinant phages, are shown in Table 2. In this original experiment ('*Mbo*I-A' library), ten transducing phages were obtained from the 26 loci tested, a success rate of about 40%.

All but two of the loci cloned (*spoIIIE* and *spoIVF*) have been cloned previously, whereas only 3 of the 16 loci not cloned (*spo0A*, *spoIIG* and *spoIVC*) have been cloned in $\phi 105$ in experiments reported elsewhere (although several have been cloned by other means; see below).

To determine whether other sporulation genes can be cloned in $\phi 105$ by transfection, three new genomic libraries were constructed and screened, using target DNA prepared in various ways. One ('*Mbo*I-B') was constructed by the same procedure except that the concentration of target DNA was increased by about twofold. The other libraries contained chromosomal DNA digested with *Bcl*I (partial digestion) or *Bgl*II (complete digestion). Table 2 also shows the results of the subsequent screening experiments, which were mainly concerned with *spo* loci that had not already been cloned. Seven of the loci not cloned in the first experiment, *spo0G*, *spo0J*,

spoIIG, *spoIIE*, *spoIIIA*, *spoIVC* and *spoVB*, were isolated from these new libraries. In addition, phages containing four loci that had not been screened for transduction in the first library, *spo0D*, *spo0H*, *spoVH* and *spoVJ*, were isolated. Mutations in two additional loci, *spo0A* and *spoIIIF*, were found to be complemented by phages transducing mutations in loci known to be closely linked (*spo0G* and *spoVB* respectively; see below). In summary, out of 30 loci tested in all, transducing phages were isolated for 23, a success rate of about 77%.

It appears, therefore, that transfection with $\phi 105J27$ is an effective means of cloning sporulation genes and, presumably, *B. subtilis* genes generally. Although each individual library gave a relatively poor success rate (between 23 and 47%), the overall rate was good. This suggests that cloning in this system is mainly dependent upon the distribution of restriction sites around each target gene. If more than one method for preparation of chromosomal DNA is used, the chances of success for any given gene appear to be enhanced. This may be a result of the relatively small size (about 4 kbp) of inserts that can be accommodated in the current $\phi 105$ vectors. It seems likely that further application of the methods described here and previously (Errington, 1984) (perhaps using other restriction enzymes to prepare the target DNA, and synthetic adaptor molecules), should enable us to clone most, if not all, of the remaining *spo* loci.

Further characterization of the transducing phages

An important advantage of the use of the $\phi 105$ transfection system for cloning *B. subtilis* genes is that non-defective phage vectors provide a rapid means of complementation analysis (Fort & Errington, 1985), and of mutant strain verification. The latter facility has become increasingly important in recent experiments in which the whole spectrum of *spo* mutations is being used to define the pathways of genetic regulation during sporulation (Errington & Mandelstam, 1986*a*, *b*; Clarke *et al.*, 1986; Turner *et al.*, 1986). The simple cross-streaking procedure described in Methods was used to determine the abilities of various phages to transduce other mutations thought to lie in or near the newly-cloned *spo* loci. An example of the results obtained with this test is shown in Fig. 1. The following loci were not considered further because they have already been characterized in some detail: *spo0B* (Ferrari *et al.*, 1985*a*); *spo0F* (Trach *et al.*, 1985); *spoIIA* (Fort & Piggot, 1984); *spoIID* (Lopez-Diaz *et al.*, 1986); *spoVA* (Fort & Errington, 1985); *spoVE* (Piggot *et al.*, 1986); *gerE* (Cutting & Mandelstam, 1986). The results of studies with the remaining clones are summarized below.

spo0D. Phage $\phi 105J96$ complemented the single known *spo0D* mutation, *spo0D8* (mutant 8H of Ionesco *et al.*, 1970). It did not complement mutations in *spoIIIC*, *spoIIID*, *spoIVC*, *spoIVD* or *spoIVE*; loci which are thought to lie nearby (Piggot & Coote, 1976). However, it did complement the *spo0B136* mutation, supporting the suggestion (J. A. Hoch, personal communication) that *spo0D8* is actually an allele of *spo0B*.

spo0G. The *spo0G* locus was defined (Piggot & Coote, 1976) by a single mutation, *spo-14* (mutant 14UL of Ionesco *et al.*, 1970), that appeared, on the basis of co-transduction frequencies, to be distinct from the *spo0A* locus. Indeed the latest *B. subtilis* genetic map (Piggot & Hoch, 1985*a*) shows *spo0A* and *spo0G* separated by seven other markers, including the *spoIIIA* and *spoIIIB* loci. However, it has been suggested (J. A. Hoch, personal communication) that mutation *spo0G14*, like *spo0D8* above, has been incorrectly mapped, and actually lies in the *spo0A* locus. Phage $\phi 105J94$ was therefore tested for complementation of mutation *spo0A43* and a positive result was obtained. Although it is possible that this phage contains non-contiguous fragments of chromosomal DNA it seems most likely that mutation *spo-14* lies within the *spo0A* locus, which consists of a single gene (Ferrari *et al.*, 1985*b*). Whether or not this is true, it appears that the *spo0A* locus has also been cloned in $\phi 105J27$.

spo0H. The homologous gene from *Bacillus licheniformis* has been cloned and sequenced (Ramakrishna *et al.*, 1984) but there remains some doubt about the integrity of the *B. subtilis* clone described by Weir *et al.* (1984). The plasmid isolated by these authors contained several non-contiguous fragments of *B. subtilis* chromosome and it did not appear to include the natural

Fig. 1. Example of the rapid plate test for complementation and marker rescue of *spo* mutations by phage $\phi 105$ derivatives. The right-hand side of the plate (a 9 cm glass dish containing Schaeffer's agar) was seeded with recombinant phage, $\phi 105J83$, which contains a part of the *spoIIIA* locus. A series of strains containing *spoIIIA* mutations, and a control *Spo*⁺ strain, 168, were streaked across the plate from left to right. After incubation at 30 °C for 36 h the plate was exposed to CHCl_3 vapour to kill vegetative cells and defective spores. The plate was then reincubated at 30 °C for 3 d to allow regrowth of the survivors. Strains (numbered on the left) showing extensive regrowth in the phage-seeded area of the plate, +, have a *spo* mutation that is complemented by the phage. The remaining strains are not complemented, -, but strain 86.2 shows relatively low-frequency survival in the phage seeded area (+), indicating that its *spo* mutation is not complemented but can be rescued by recombination with the DNA carried by the phage.

spo0H promoter. Phage $\phi 105J80$ complemented all of the *spo0H* mutations tested, including one, *spo0H* Δ *Hind*, derived by *in vitro* mutagenesis of the *spo0H* region (Weir *et al.*, 1984).

spo0J. Two mutations, *spo0J87* and *spo0J93*, define this locus but they are apparently quite widely separated in terms of transformation linkage (recombination index, RI, 0.39; Hranueli *et al.*, 1974). Phages $\phi 105J92$ and $\phi 105J93$ both gave clear positive results in complementation tests with strain 87.2 but results with the highly oligosporogenous strain 93.2 were less clear cut. Derivatives of this latter strain, lysogenic for each of the phages, were therefore induced to sporulate by the resuspension method and at *t*₀ the cultures were assayed for heat-resistant spores. Cultures of strain 93.2 lysogenic for $\phi 105J92$ or $\phi 105J93$ showed no more heat-resistant spores than did the non-lysogenic control strain (about 5×10^3 c.f.u. ml⁻¹). It also appeared that these phages did not rescue the *spo0J93* mutation. These results, together with the relatively large RI observed for these mutations, suggest that they probably lie in two separate loci.

spoIIE. Phage $\phi 105J97$ was isolated from a *Spo*⁺ transductant of strain 61.1 (*spoIIE61*). However, lysates made from this strain and from subsequent *Spo*⁺ transductants of the same strain gave only a relatively low frequency of transduction to *Spo*⁺. Most of the survivors of infection remained *Spo*⁻. We concluded that phage $\phi 105J97$ contains only a part of the *spoIIE* locus and does not complement *in trans* but can rescue the *spoIIE61* mutation at low frequency by homologous recombination. Similar results were reported previously for a phage, $\phi 105J15$, which can complement mutations in the first three genes of the *spoVA* locus but gives only low-frequency transduction, presumably by marker rescue recombination, of mutations in the fourth gene (Fort & Errington, 1985). Phage $\phi 105J97$ rescued four other *spoIIE* mutations, *spo-20*,

spo-60, *spo-64*, and *spo-71* but did not complement or rescue mutations *spo-21*, *spo-39* or *spo-48*. The *spoIIE* locus is thought to be large and possibly polycistronic (M. Young, personal communication) so it is not surprising that only part of the operon appears to have been cloned in this instance.

spoIIG. At least part of the *spoIIG* locus has been cloned and sequenced (Stragier *et al.*, 1984; Kobayashi & Anaguchi, 1985) but it has been suggested that the locus is complex and may contain other genes (Kobayashi & Anaguchi, 1985). Phage ϕ 105J95 complemented mutations *spo-41* and *spo-55*, those used to define the gene sequenced by the above authors, but a third mutation, *spo-49*, appeared only to be rescued by recombination (see above). This mutation must therefore define a second gene in the *spoIIG* locus. Mutations *spo-85* and *spo-153*, which lie in the nearby *spoVE* locus (Young, 1975), were not transduced by phage ϕ 105J95.

spoIIIA. A large number of *spoIIIA* mutations have been classified (Piggot & Coote, 1976). In view of the range of sporulation phenotypes associated with these mutations it was suggested that they might lie in several genes. Of 12 putative *spoIIIA* mutations tested, four, *spo-26*, *spo-44*, *spo-150* and *spo-158*, were complemented by phage ϕ 105J83. Mutation *spo-86* was rescued by recombination at low frequency, the remainder were not transduced at a detectable frequency (Fig. 1). There would appear, therefore, to be at least two genes in the *spoIIIA* locus, and the failure to rescue the majority of mutations suggests that a substantial part of the locus has not been cloned. Mutation *spo-2* in the nearby *spoIIIB* locus (Piggot & Coote, 1976) was not transduced by phage ϕ 105J83.

spoIIIB. This locus has been cloned previously in a defective derivative of phage ϕ 105 (Jenkinson & Mandelstam, 1983). Nucleotide sequence analysis and complementation analysis suggest that the locus contains a large gene and that the locus could be polycistronic (M. Deadman & J. Mandelstam, personal communication). Five newly-isolated *spoIIIB* mutations were all complemented by phage ϕ 105J35.

spoIIIE. phages ϕ 105J30 and ϕ 105J31 both complement all three known *spoIIIE* mutations, *spo-36*, *spo-47* and *spo-82*, and have been shown to contain a single complete transcription unit (P. Butler & J. Mandelstam, personal communication).

spoIVC. Dancer & Mandelstam (1981) showed that the *spoIVC* locus contains at least two different genes, separate complementation groups being defined by mutations *spo-23* and *spo-133*. This analysis has been extended to include 14 further *spoIVC* mutations, all of which fall into one or other of these cistrons (M. D. Yudkin, personal communication). A ϕ 105 derivative with *spoIVC*⁺ transducing activity has been reported previously but it was tested only for complementation of mutation *spoIVC133* (Fujita & Kobayashi, 1985). All three of the new *spoIVC*⁺ transducing phages, ϕ 105J78, ϕ 105J79 and ϕ 105J91, complemented all of the *spoIVC* mutations tested, which included representatives of both cistrons. None of the phages complemented mutations in the following loci, which it had been suggested were either in or closely linked to *spoIVC* (Piggot & Coote, 1976): *spoIIIC*, *spoIIID*, *spoIVD* and *spoIVE*.

It was noted that strain 23.1 lysogenic for any of the *spoIVC*⁺ recombinant phages had a rather translucent appearance on nutrient agar plates, unlike the normal appearance of Spo⁺ strains, including other *spoIVC* mutants transduced with these phages. Nevertheless, phase-bright spores were always abundant when the 23.1 derivatives were examined by phase-contrast microscopy. A similar phenomenon was observed in complementation of *spoVH* mutants (see below).

spoIVF. In addition to *spoIVF88*, strains containing 11 other *spoIVF* mutations have been isolated, all of which give an oligosporogenous phenotype (Lamont, 1983). These mutations are thought to lie in at least two distinct genes. Strains containing mutations *spo-88*, *spo-207*, *spo-585* and *spo-586* were tested, representative of both genes, and all of them appeared to be complemented by ϕ 105J36.

spoVB. A single mutation, *spo-91*, defines the *spoVB* locus (Piggot & Coote, 1976) but Lamont

& Mandelstam (1984) described a very closely linked (RI 0.05) mutation, *spo-590*, in a new locus designated *spoIIIF*. Phage $\phi 105J86$ transduced both of these mutations, thus confirming their proximity and supporting the suggestion of Lamont & Mandelstam (1984) that the mutations might lie in the same operon. Whatever the status of *spoIIIF* is, it has also now been cloned in $\phi 105J27$.

spoVH. This and the following locus, *spoVJ*, were defined by mutations with unusual spore resistance properties (Hill, 1983). Although the three mutations in these loci had rather similar properties and had a similar chromosomal location, two loci could be distinguished by measuring cotransformation frequencies. Mutations *spoVH516* and *spoVH518* were both complemented by phages $\phi 105J87$, $\phi 105J88$ and $\phi 105J98$. Like the complementation of *spoIVC23* described above, the complemented strains did not appear fully *Spo*⁺ when lysogenic for any of the three phages, although numerous spores were always evident when cultures of such lysogens were examined by phase-contrast microscopy. It seems unlikely that in all of the independently-isolated phages expression of the cloned gene is reduced by loss of important regulatory sequences. One possible explanation is partial dominance of the mutant allele; e.g. the polypeptide product of the mutant gene competes with the wild-type gene product expressed by the phage. None of the three phages transduced the *spoVJ* mutation or a mutation in the nearby *gerE* gene.

spoVJ. Phages $\phi 105J81$ and $\phi 105J82$, which complemented mutation *spoVJ517*, did not transduce mutations in the *spoVH* or *gerE* loci. We can conclude, therefore, that the *spoVH* and *spoVJ* loci are indeed distinct.

Preliminary characterization of these new clones has therefore enabled us to clarify the genetic organization of several *spo* loci. Further physical and genetic characterization should enable us to understand the general features of sporulation operon organization. Although some of the new phages do not contain intact operons they nevertheless provide us with DNA probes that could be used to clone adjacent regions of the chromosome.

Several of the cloned loci, *spoIIIA*, *spoIIIE*, *spoIIIF*, *spoIVF*, *spoVB*, *spoVH* and *spoVJ*, have not apparently been isolated previously. Characterization of their regulation by applying methods such as gene fusions (Errington, 1986) or mRNA hybridization (Savva & Mandelstam, 1986) should further our understanding of the patterns of regulation of sporulation genes. We have applied the former kind of analysis to four *spo* operons recently (Errington & Mandelstam, 1986*a, b*; Clarke *et al.*, 1986; Turner *et al.*, 1986). However, one of the practical problems with these experiments has been in maintaining and repeatedly handling 40 or so different strains of *B. subtilis* containing mutations representative of each of the known *spo* loci. The transducing phages described here provide a rapid means of verifying the identity of *spo* mutations, which can now be applied to more than half of the strain collection.

In conclusion, transfection of protoplasts with vectors such as $\phi 105J27$ provides a versatile and effective system for cloning in *B. subtilis*. It seems likely that a range of applications for this system may be developed in the future.

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