Cloning of the *Bacillus subtilis* *lys* and *spoIIIB* Genes in Phage ϕ 105

By H. F. JENKINSON† AND J. MANDELSTAM*

Microbiology Unit, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, U.K.

(Received 13 December 1982; Revised 31 January 1983)

The *lys* gene of *Bacillus subtilis* was inserted into prophage ϕ 105. The recombinant phage (ϕ 105d*lys*) contained DNA which was about 2 MDal smaller than the wild-type phage DNA, and the phage particles had no tails. The phage did not plaque but, when provided with tails *in vitro*, it transduced both *lys-1* and *lys-3* strains of *B. subtilis* to Lys⁺. The *lys*⁺ gene was located on a 2.5 MDal *Eco*RI restriction fragment. Subsequently this phage was used to clone, using a similar technique, the *spoIIIB* gene(s). The second recombinant phage, ϕ 105d*spoIIIB*, was also defective, i.e. without tails. The DNA was 1.5 MDal smaller than the wild-type phage DNA and the *spoIIIB*2⁺ gene was located on a 3 MDal *Eco*RI fragment. When provided with tails *in vitro*, phage ϕ 105d*spoIIIB* transduced cells of a *spoIIIB*2 recipient to Spo⁺. In these transductants the *spoIIIB*2 mutation was complemented, and the cells sporulated normally.

INTRODUCTION

From recent reviews (Young & Mandelstam, 1979; Piggot *et al.*, 1981) it appears that about 50 genetic loci have now been identified whose expression is required for spore formation and germination in *Bacillus subtilis*. By cloning the genes in these loci it should be possible (a) to determine what their protein products are and (b) to identify the regulatory molecules which might control their expression. To these ends some significant advances have recently been made using cloned sporulation genes on phage and plasmid vectors. Thus, Hirochika *et al.* (1981) have identified a protein which might be a product of the *spoOB* locus, and the forms of RNA polymerase which recognize the known nucleotide sequences of at least two *spo* genes (Moran *et al.*, 1982) have been identified (Losick & Pero, 1981).

From the map distances as measured by recombination index between mutations within a locus it appears that several of the *spo* loci probably contain more than one gene (Coote, 1972; Piggot & Coote, 1976; Lloyd-Jones, 1976; Yudkin & Turley, 1981). To determine precisely how many genes each locus contains, complementation analysis is required in conjunction with the genetic mapping. Using a protoplast fusion technique, Dancer & Mandelstam (1981) have shown by complementation that the *spoIVC* locus contains at least two cistrons. However this method was successful only with mutants blocked at stage IV and this is obviously a serious limitation. Gene cloning has now made it possible to carry out complementation analysis on other loci. Thus Hirochika *et al.* (1981) have cloned the *spoOB*⁺ gene on phage ϕ 11 and used it to complement mutations in the *spoOB* locus. More recently, Liu *et al.* (1982) have shown, using a cloned region of the *spoIIA* locus, that this locus probably contains at least two genes.

We decided to clone *spo* genes using the temperate *B. subtilis* phage ϕ 105 (Birdsell *et al.*, 1969) which contains DNA of size about 25 MDal and which has been used as a cloning vector for at least one *B. subtilis* vegetative gene (Iijima *et al.*, 1980). In this paper we describe the cloning of the *lys* and *spoIIIB* loci in phage ϕ 105, and the complementation of the *lys-1*, *lys-3* and *spoIIIB*2 mutations by the recombinant phages.

† Present address: Department of Oral Biology, University of Otago, Box 647, Dunedin, New Zealand.

Table 1. *Strains of B. subtilis used*

Strain designation	Genotype	Original isolation no./source/reference
168	<i>trpC2</i>	Laboratory stock
MY2016	<i>hisH2 lys-1 sul^R rif^R</i>	Laboratory stock
CU267	<i>leuB16 ilvB2 trpC2</i>	S. Zahler
SL989	<i>lys-3 metB10 recE4</i>	P. Piggot
MB79	<i>metC3 pheA12</i>	Laboratory stock
SG20	<i>metC3 lys-3</i>	Laboratory stock
1L11	<i>trpC2 (φ105)</i>	BGSC*
CU267	<i>leuB16 ilvB2 trpC2 (φ105)†</i>	Laboratory stock
89.9	<i>lys-1 spoVA89</i>	89; Hranueli <i>et al.</i> (1974)
2.3	<i>lys-1 spoIIIB2</i>	A3; Piggot (1973)
2.5	<i>rif^R spoIIIB2</i>	A3; Piggot (1973)
165.1	<i>trpC2 spoIVB165</i>	P7; Coote (1972)
299	<i>trpC2 spoOA299</i>	P10; Coote (1972)
34.4	<i>lys-1 tal-1 spoOA34</i>	3F1; Hranueli <i>et al.</i> (1974)
43.7	<i>pheA12 spoOA43</i>	NG6.21; Piggot (1973)
26.4	<i>lys-1 spoIIIA26</i>	E34; Piggot (1973)
59.7	<i>leuB16 trpC2 spoIIIA59</i>	NG14.7; Piggot (1973)

* Bacillus Genetic Stock Center, The Ohio State University, Columbus, Ohio, U.S.A.

† This strain was used as the stock lysogen after phage φ105 was transferred into it from strain 1L11. Other strains lysogenic for phage φ105 referred to in the text were all made from the above strains by plaquing the phage on to a lawn of the strain and picking from the centre of a plaque.

METHODS

Bacteria. The bacterial strains used are listed in Table 1.

Preparation of phage φ105 lysates. BHIB (Brain Heart Infusion Broth, Oxoid) containing 0.5% (w/v) Yeast Extract (Oxoid; 5 ml), was inoculated with a fresh colony of a φ105 lysogen, and the bacteria were grown with shaking at 37 °C to an $A_{600} \sim 0.4$ (about 2×10^8 cells ml⁻¹). Mitomycin C (Sigma; final concn 0.5 µg ml⁻¹) was then added, and after 20 min incubation the cells were pelleted by centrifugation, suspended in fresh warm aerated BHIB (5 ml) and shaken for 2 h by which time lysis had usually occurred. The cell debris was removed by centrifugation (5000 g, 5 min) and DNAase I (Sigma; final concn 10 µg ml⁻¹) was added to the supernatant. This was left for 5 min at 20 °C and then filtered through a 0.45 µm membrane filter and stored at 4 °C over chloroform (0.1 ml). Such a lysate typically contained 2×10^9 p.f.u. ml⁻¹.

Measurement of phage titre. Phage φ105 suspension (0.1 ml) was mixed with mid-exponential phase cells of indicator strain CU267 in BHIB (0.1 ml); molten agar [3 ml of one-third-strength Tryptose Blood Agar Base (TBAB, Difco)] was then added, and the mixture poured on to warm TBAB plates. Plaques were counted after incubating the plates at 30 °C for 16 h.

Phage φ105 purification. A culture of strain CU267 (lysogenic for phage φ105) in BHIB (250 ml) was grown to a density of about 2×10^8 cells ml⁻¹. Phage were induced with mitomycin C as above, and the cells suspended in fresh medium and allowed to lyse. To the clarified culture supernatant was added NaCl (5.8 g) and polyethylene glycol 6000 (22.5 g) (Yamamoto *et al.*, 1970), and these were dissolved by gently agitating the mixture. This was left at 4 °C for 2 h, and the precipitate was collected by centrifugation (10000 g, 20 min, 4 °C). The pellet was suspended in phage buffer (3 ml, consisting of 10 mM-Tris, 100 mM-NaCl, 10 mM-MgSO₄·7H₂O, pH 7.4) and layered on to the surface of a four-step CsCl gradient. The gradient was prepared by layering the following solutions of CsCl in a 13 × 51 mm Beckman Ultra-Clear centrifuge tube: density (ρ) 1.50 g ml⁻¹ (1.0 ml), 1.46 g ml⁻¹ (0.8 ml), 1.39 g ml⁻¹ (0.6 ml) and 1.17 g ml⁻¹ (0.6 ml). The gradient was centrifuged in a Beckman SW50.1 swing-out rotor at 110000 g for 2.5 h, and the phage band (about three-quarters of the way down the gradient) was removed through the side of the tube with a 19 gauge needle and syringe. The phage suspension (about 0.5 ml) was then dialysed for 16 h at 4 °C against phage buffer (5 l), and the contents of the dialysis tubing were made up to 1 ml with phage buffer containing 0.05% (w/v) gelatin for storage at 4 °C.

Phage PBSX purification. Phage PBSX was prepared from strain 168 in a similar way to that described above for phage φ105. It banded much higher up the gradient (buoyant density of PBSX = 1.375 g ml⁻¹; Okamoto *et al.*, 1968a) than did φ105 (buoyant density about 1.47 g ml⁻¹). Phage PBSX is a defective phage which is carried by *B. subtilis* strain 168. When induced, e.g. with mitomycin C, it packages chromosomal DNA randomly into the phage heads (Okamoto *et al.*, 1968b). The phage thus provides an easily purified source of chromosomal DNA which appears to be good starting material for cloning (see Hirochika *et al.*, 1981).

Transduction. Stationary-phage cells in BHIB (0.5 ml) were mixed with phage lysate (0.1 ml) and incubated with shaking at 37 °C for 30 min. The cells were harvested by centrifugation, washed twice with 10 ml Spizizen's minimal medium containing 0.5% (w/v) glucose (Anagnostopoulos & Spizizen, 1961), made to 1 ml in the same medium and 0.1 ml portions were plated on to lactate/glutamate minimal agar (Piggot, 1973) containing appropriate L-amino acid supplements (200 µg ml⁻¹ each). Plates were incubated for 2 d at 37 °C, and transductants were purified by streaking them on lactate/glutamate minimal agar. To select Spo⁺ transductants, 0.1 ml samples were plated on to Schaeffer's agar (Schaeffer *et al.*, 1965) in glass Petri plates which were incubated for 18 h at 37 °C. The agar was exposed to chloroform vapour for 2 to 5 h at 55 °C (Hoch, 1971) to select against asporogenous (recipient) cells. The plates were then incubated for a further 3 to 4 d at 37 °C by which time opaque, brown Spo⁺ colonies appeared on a translucent (recipient) background.

Preparation of phage DNA. Phage (0.4 ml, containing 10¹¹ p.f.u. for ϕ 105, or a roughly equivalent number of particles for PBSX) were mixed with EDTA [2 µl of a 4% (w/v) solution] and SDS [5 µl of a 20% (w/v) solution] and heated at 70 °C for 5 min. The solution was allowed to cool, and potassium acetate (50 µl of a 5 M-solution) was added to precipitate protein and SDS. After allowing the suspension to stand for 30 min on ice, it was centrifuged for 15 min (in a Beckman microfuge). The supernatant was decanted into a fresh sterile microtube, and 95% (v/v) ethanol (1 ml) was added at room temperature to precipitate DNA. The phage DNA was immediately picked out with a fine plastic tip and redissolved in Tris/EDTA buffer (10 mM-Tris, 1 mM-EDTA, pH 7.4; 0.3 ml). Alternatively, if the DNA was not visible as a clump, the mixture was centrifuged (1 min, in a microfuge), the pellet was drained, the inside of the tube dried, and Tris/EDTA buffer was added to dissolve the pellet.

Preparation of DNA from bacteria. Cell DNA for constructing strains and for mapping was prepared as described by Ward & Zahler (1973), except that 5 ml of culture was used and the final volume of the DNA solution was 1.5 ml.

Transformation. Transformation of competent cells was done by the method of Anagnostopoulos & Spizizen (1961) as described by Jenkinson (1983). Transformants were plated on lactate/glutamate minimal agar with appropriate supplements, unless selection was for Spo⁺ transformants, when Schaeffer's medium in glass plates was used as described above. When DNA from an agarose gel was used in transformation, rifampin (final concn 2.5 µg ml⁻¹) was included in the agar to prevent growth of contaminants from the agarose.

Restriction enzymes. These were obtained from BRL, and the buffers used were those recommended by BRL. Double digests using *Eco*RI and *Xba*I were carried out in *Eco*RI buffer. For double digests with *Kpn*I and *Eco*RI, *Kpn*I (in *Kpn*I buffer) was used first and then the buffer components of the mixture were adjusted before adding *Eco*RI. Digestions were performed at a concentration of 1 or 2 units of enzyme (µg DNA)⁻¹ for 3 h at 37 °C, and they were terminated by treatment at 65 °C for 20 min.

Ligation. Phage ϕ 105 DNA, digested with *Sau*3A, or *Eco*RI (10 µg DNA in 0.25 ml) was mixed with an equal volume of PBSX DNA (20 to 30 µg in 0.25 ml) digested with the appropriate enzyme and the mixture was cooled to 4 °C. Freshly prepared ligase buffer [0.5 ml, containing 120 mM-Tris, 20 mM-MgCl₂, 20 mM-dithiothreitol, 0.2 mM-ATP (sodium salt), pH 7.5] was then added, mixed by inversion, and followed by 5 units of T4 DNA ligase. The ligation was carried out at 15 °C for at least 16 h, and before using the DNA the ligation was checked by agarose gel electrophoresis.

Electrophoresis of DNA. For separation of DNA fragments, samples were made to 6.5% (w/v) in Ficoll (Sigma) and 0.001% in bromophenol blue, and loaded into the wells formed in a horizontal 0.7% or 0.8% agarose gel submerged in gel buffer (40 mM-Tris, 18 mM-acetic acid, 2 mM-EDTA, pH 8.1). Fragments too small to be separated efficiently in agarose were electrophoresed in polyacrylamide gels (5%, w/v) in a vertical apparatus, using the same buffer as above. Gels were stained with ethidium bromide (1 µg ml⁻¹) and visualized on a UV transilluminator.

Induction of sporulation. Cells were induced to sporulate by the resuspension method of Sterlini & Mandelstam (1969).

Measurement of sporulation marker events. Alkaline phosphatase was assayed as described by Glenn & Mandelstam (1971) using Tris/HCl buffer (1 M, pH 8). Glucose dehydrogenase activity was measured as described by Sadoff (1966). The numbers of spores surviving treatment with heat or with lysozyme were estimated as described by Jenkinson *et al.* (1980).

Chemicals. Acrylamide was obtained from BDH, and all other chemicals, unless stated, were from Sigma.

RESULTS

Isolation of phage ϕ 105dlys

To try to isolate specialized transducing phage ϕ 105 carrying the *lys* gene of *B. subtilis* (and possibly other closely linked sporulation genes in the *spoVA* or *spoIIA* loci), the prophage transformation method described by Kawamura *et al.* (1981) was used. For this, DNA from phage ϕ 105 (about 10 µg), partially digested with *Sau*III enzyme, was mixed (see Methods)

with PBSX DNA (20 µg, partially digested with *Sau*III A to give fragments mainly of size 3 to 7 MDal), ligated and used to transform competent cells (1×10^8 cells ml⁻¹) of strain MY2016 *hisH2 lys-1* (ϕ105) to Lys⁺. Transformants (112 in total were obtained) were picked on to lactate/glutamate minimal agar containing L-histidine and grown for 18 h at 37 °C. It was expected that most of these colonies would be true transformants in which the original *lys-1* mutation had been lost by homologous recombination. However, some might have become phenotypically Lys⁺ through complementation of *lys-1* by *lys*⁺ integrated into the resident prophage.

Complemented strains, if present, would be a possible source of transducing phages carrying the *lys*⁺ gene. Accordingly, the 112 colonies were pooled in BHIB (10 ml), phage was induced with mitomycin C (see Methods) and a lysate was obtained which had a titre of 1.5×10^9 p.f.u. ml⁻¹. This was then used to transduce strain MY2016 *lys-1* (non-lysogenic for ϕ105) to Lys⁺. Lys⁺ colonies appeared at a frequency of about 2×10^5 per ml of transduced culture. Five of these transductants were picked and found to be immune to superinfection by phage ϕ105. They were therefore assumed to have been lysogenized by ϕ105 carrying *lys*⁺. However, when lysates were prepared from them and used in turn to transduce strain MY2016 to Lys⁺, no transductants were obtained. Furthermore, the lysates had no plaque-forming activity.

Since each of the five Lys⁺ transductants picked was immune to superinfection, it was reasonable to assume that they contained phage which was defective in either induction or infection. To determine whether these lysates did, in fact, contain phage that required plaque-forming phage as helper to transduce, lysate (0.1 ml) made from one of the five transductants was mixed with wild-type phage ϕ105 lysate (0.1 ml, 1×10^8 p.f.u.). The mixture was used to transduce stationary-phage cells of strain MY2016 (0.5 ml, 1×10^9 c.f.u.) to Lys⁺, and 7.2×10^3 Lys⁺ transductants ml⁻¹ were obtained. The number of transductants depended upon the amount of wild-type phage lysate added, none being obtained when the wild-type phage alone was used. Thus, the high numbers of transductants obtained previously, using the lysate prepared from the initial transformants, must have been due to the presence in the lysate of wild-type phage.

One Lys⁺ transductant was purified, designated strain SG18, and after induction of the cells with mitomycin C, phage were prepared by CsCl gradient centrifugation. Only one phage band was visible at the bottom of the gradient (PBSX phage is always induced and it forms a band at the top of the gradient) and this had a higher density than the wild-type phage ϕ105 (about 1.50 g ml⁻¹ compared with about 1.47 g ml⁻¹ for wild-type phage). Electron microscopic observation of the phage in this band showed that only phage heads were present, and no tails were visible. This defective recombinant phage which carried the *lys* gene was denoted ϕ105d*lys*.

Nature of the helper activity

These results suggested that the inability of phage ϕ105d*lys* to transduce Lys⁻ cells to Lys⁺ may have been due to the lack of functional tails. This seemed more probable when it was found that vortex-mixing of the wild-type phage for several minutes before using it as helper in transduction increased the number of Lys⁺ transductants obtained even though it decreased the number of p.f.u. (results not shown). To check, therefore, that the helper activity was not just due to the addition of infectious particles, wild-type phage ϕ105 suspension in phage buffer (1 ml, 3×10^{10} p.f.u.) was vortex-mixed for 3 min to remove tails, layered on to CsCl (2 ml, density 1.39 g ml⁻¹) and then centrifuged at 110000 *g* (Beckman SW50.1 rotor) for 3 h. It was assumed that under these conditions intact phage would collect almost at the bottom of the tube while any free tails would remain at the diffuse region between the CsCl solution and phage buffer. After centrifugation, fractions (0.5 ml) were removed from the tube and assayed for (a) plaque-forming and (b) helper activities. Helper activity was almost completely confined to the fraction containing the tails and this fraction had less than 0.1 % of the total plaque-forming activity (Fig. 1). On the other hand, virtually no helper activity was associated with the plaque-forming phage (Fig. 1). Thus the helper activity in wild-type phage ϕ105 lysates could be attributed to the presence of free tails which presumably assemble *in vitro* on to the ϕ105d*lys* phage, enabling it to infect cells.

Fig. 1. Plaque-forming and helper activities of wild-type phage $\phi 105$ after centrifugation of vortex-mixed phage through CsCl. Phage $\phi 105$ was vortex-mixed for 3 min to remove tails, and 1 ml of suspension was layered on to the surface of CsCl (2 ml; density 1.39 g ml^{-1}). After centrifugation (30000 r.p.m., 3 h, Beckman SW50.1), fractions (0.5 ml) were removed from the tube and assayed for plaque-forming (open blocks) and helper (hatched blocks) activities. The plaque-forming activity was measured as described in Methods using strain CU267 as host. Helper activity was measured by mixing 0.01 ml of each fraction with 0.05 ml phage $\phi 105\text{dlys}$ lysate prepared from strain SG18, and using this to transduce strain MY2016 (*hisH2 lys-1*) to Lys⁺. Varying the time of pre-incubation of CsCl sample with phage $\phi 105\text{dlys}$ from 5 to 60 min did not seem to affect significantly the numbers of transductants obtained. Fraction 3 corresponds to the region at the top of the CsCl where phage tails are concentrated.

Complementation of *lys-1* and *lys-3* mutations

It was obviously necessary to check that the Lys⁺ transductants of strain MY2016 arose through complementation of the *lys-1* mutation and not through recombination in the *lys* region of the chromosome. The simplest way of doing this was to show by transformation that these transductants, though phenotypically Lys⁺, still contained the *lys-1* mutation. Accordingly, DNA was prepared from five of the transductants and each preparation was used to transform strain MB79 (*metC3 pheA12*) to Met⁺. In an experiment of this type up to 5% of the Met⁺ transformants should also have acquired the *lys-1* mutation by congression (Nester *et al.*, 1963). Transformants (100 from each transformation) were picked and scored separately for congression of *lys* and *phe*⁺ (since this latter allele from the Phe⁺ donor DNA should also be transferred at a similar frequency). The expected results were obtained for all five of the DNA preparations; that is between two and six of the Met⁺ transformants were Lys⁻, and between two and six were Phe⁺. This confirmed that the original *lys-1* mutation was present in Lys⁺ transductants and had not been lost by recombination.

To determine whether phage $\phi 105\text{dlys}$ also complemented the *lys-3* mutation, strain SL989 (*lys-3 metB10 recE4*) was used as a recipient in transduction. This strain carries, in addition to *lys-3*, the *recE4* mutation (Dubnau *et al.*, 1973) which prevents homologous recombination from occurring. Stationary-phase cells (0.5 ml) of strain SL989 were mixed with phage $\phi 105\text{dlys}$ lysate (0.1 ml) and wild-type $\phi 105$ helper phage (4×10^7 p.f.u.), and after selection was made on lactate/glutamate minimal agar containing L-methionine, 6.3×10^2 Lys⁺ transductants ml⁻¹ were obtained. Since the recipient strain SL989 was recombination-deficient, the Lys⁺ transductants could only have arisen by complementation of the *lys-3* mutation.

The *spoVA* locus is close to *lys* (Piggot & Coote, 1976) and therefore it was possible that phage $\phi 105\text{dlys}$ might also have carried the sporulation genes in this locus. The phage was used in an

Fig. 2

Fig. 3

Fig. 2. Restriction endonuclease analysis of phage $\phi 105$ DNA and phage $\phi 105dlys$ DNA using *EcoRI* and *XbaI*. The figure is a composite of two gels: the upper (0.8% agarose) gel shows *EcoRI* fragments A to F (see text); the lower inset (5% polyacrylamide) gel shows fragments G, H and I. Both gels were stained with ethidium bromide. Lane 1, molecular weight standards with fragment sizes given in MDal [upper gel, phage λ *HindIII* fragments; lower gel, *Staphylococcus aureus* Imp 18 plasmid (kindly provided by K. G. H. Dyke) *HinfI* fragments]; lane 2, $\phi 105$ digested with *EcoRI*; lane 3, $\phi 105dlys$ with *EcoRI*; lane 4, $\phi 105dlys$ with *EcoRI* plus *XbaI*; lane 5, $\phi 105$ with *EcoRI* plus *XbaI*; lane 6, $\phi 105$ with *XbaI*; lane 7, $\phi 105dlys$ with *XbaI*.

Fig. 3. Agarose gel electrophoresis (0.8% agarose) of the fragments obtained after *EcoRI* digestion of DNA from phage $\phi 105$ (lane 1), $\phi 105dlys$ (lane 2) and $\phi 105dsplIIIB$ (lane 3).

attempt to transduce strain 89.9 (*lys-1 spoVA89*) to Spo^+ , but no Spo^+ transductants were obtained. This result, however, does not show definitively that phage $\phi 105\text{dlys}$ does not carry *spoVA89*⁺ because the *spo-89* mutation might be dominant. Therefore, in another experiment, DNA was prepared from the phage and used to transform strain 89.9 to Lys^+ . The mutation *spo-89* is about 60% linked by transformation to *lys-1* (unpublished results), so if phage $\phi 105\text{dlys}$ carried *spo-89*⁺ then approximately 60% of the Lys^+ transformants would be expected to be Spo^+ . In fact, out of 5.3×10^3 transformants obtained, none was Spo^+ , showing that the phage did not carry that part of the *spoVA* locus defined by the mutation *spo-89*.

Restriction endonuclease mapping of $\phi 105$ wild-type and $\phi 105\text{dlys}$ DNAs

Phage $\phi 105$ DNA has been shown by Perkins *et al.* (1978) and by Scher *et al.* (1978) to be cleaved into nine fragments by the enzyme *EcoRI*. These were arranged in a linear map by Perkins *et al.* (1978) and lettered B to J. This convention has been retained in this paper and Fig. 2 (lane 2) shows the fragments obtained after digestion of wild-type phage DNA with *EcoRI*. Band A is the sum of fragments C and D which have partially annealed owing to their sticky ends (Perkins *et al.*, 1978).

Fragment J (about 0.2 MDal) of Scher *et al.* (1978) does not appear in the digest, and absence of this band was also noted by Iijima *et al.* (1980). When $\phi 105\text{dlys}$ DNA was cleaved with *EcoRI*, the 3.6 MDal, 0.62 MDal and 0.26 MDal fragments (E, G and I bands) were missing, and one new fragment (2.5 MDal appeared (denoted band L in Fig. 2, lane 3). The sum of the molecular weights of the *EcoRI* fragments obtained from wild-type phage DNA was 25.1 MDal; the sum of the fragments from $\phi 105\text{dlys}$ DNA was 23.1 MDal.

To show that fragment L contained the *lys* gene, $\phi 105\text{dlys}$ DNA (1 μg) was digested with *EcoRI*, the fragments were separated by agarose gel electrophoresis, and band L was excised from the partially stained gel. When homogenized in transformation medium and mixed with competent cells of strain MY2016 (1 ml, about 1×10^8 c.f.u.), 5.2×10^4 Lys^+ transformants ml^{-1} were obtained. No more than 20 Lys^+ colonies ml^{-1} were obtained using any other of the *EcoRI* fragments.

Phage $\phi 105$ DNA has no sites for the enzymes *BamHI* (Iijima *et al.*, 1980), *BglII*, *XbaI*, *XhoI* or *SstII*. However, one of these enzymes, *XbaI*, cut $\phi 105\text{dlys}$ DNA at a single site, giving two fragments of sizes 7.2 MDal and 15.8 MDal (Fig. 2, lane 7). This single new *XbaI* site must reside in fragment L, and to show this, $\phi 105\text{dlys}$ DNA was digested with both *XbaI* and *EcoRI*. This resulted in the reduction in size of band L from 2.5 MDal to about 2.4 MDal (Fig. 2, lane 4). Since the smaller of the two fragments generated by single digestion with *XbaI* was 7.2 MDal, this result indicates that the *XbaI* site is within fragment L and to the right hand side of it (Fig. 4).

Several other restriction enzymes were used to try to construct a simple restriction map of the phages, but *PvuII*, *PstI*, *HindIII* and *HincII* (each of which recognizes a hexanucleotide sequence) cut the DNA at more than eight sites. However, the enzyme *KpnI* which cut the DNA of the wild-type phage into four fragments (gel not shown), cut $\phi 105\text{dlys}$ DNA into only three. The deleted region in the wild-type phage thus contained a *KpnI* site. The results obtained with single and double digests of *KpnI* and *EcoRI* position the *KpnI* sites in the wild-type and *dlys* phage DNAs as shown in Fig. 4.

Orientation of *lys-1*⁺ and *lys-3*⁺ on $\phi 105\text{dlys}$ DNA

In order to see if treatment with *XbaI* reduced Lys^+ transforming activity of the *dlys* phage DNA (i.e. whether or not the *XbaI* site was close to or within the *lys* gene), the DNA was treated with *XbaI* and *EcoRI*, or with *EcoRI* or left untreated, and used to transform competent cells of either strain MY2016 *lys-1* or strain SG20 *lys-3*, to Lys^+ . With the latter as recipient, treatment of the phage DNA with either *EcoRI* alone, or with *EcoRI* and *XbaI* together, reduced the number of Lys^+ transformants obtained to about 10% of the number obtained using the control (unrestricted) DNA (Table 2). However, with MY2016 (*lys-1*) as recipient, treatment of the DNA with *EcoRI* reduced the transforming activity to about 3%, and treatment with both enzymes reduced this further to about 1% of the unrestricted DNA value (Table 2). These results are taken to indicate that *lys-1* is closer to the *EcoRI* site at the right-hand end of fragment L than

Table 2. *Effect of EcoRI and XbaI treatment on the transforming activity of phage $\phi 105dlys$ DNA*

Phage DNA (1 μ g) was incubated with enzyme or left untreated and then used to transform strain MY2016 or strain SG20 to Lys⁺.

DNA incubated with:	No. of Lys ⁺ transformants ml ⁻¹	
	Recipient MY2016 (selection for <i>lys-I</i> ⁺)	Recipient SG20 (selection for <i>lys-3</i> ⁺)
Nothing	2.4 $\times 10^5$	1.52 $\times 10^5$
<i>EcoRI</i>	7.3 $\times 10^3$	2.6 $\times 10^4$
<i>XbaI</i> + <i>EcoRI</i>	2.2 $\times 10^3$	1.4 $\times 10^4$

is *lys-3* (Fig. 4), because the closer a marker is to the end of a transforming fragment, the less likely it is to transform. Thus, since *XbaI* treatment reduces further the transforming activity of *lys-I*⁺ but not of *lys-3*⁺, treatment with *XbaI* must bring *lys-I* even closer to the end of fragment L (Fig. 4).

Cloning of spoIIIB gene(s) using $\phi 105dlys$

The introduction of the *lys* gene into phage $\phi 105$ generated a phage that might be considered useful as a general vector for cloning homologous DNA in *B. subtilis*. This is because (1) the phage DNA was about 2 MDal smaller than that of the wild-type phage, and (2) a single restriction site was introduced into a sequence that is not essential for the phage. On this basis, it should be possible to clone an additional fragment of DNA up to 2 MDal in size into the *XbaI* site, transfect cells of a suitable recipient and then to select transducing phage carrying the gene which it is intended to clone. However, $\phi 105$ DNA is poorly infectious (Flock & Rutberg, 1974) since it is degraded during transfection, and *XbaI* was found to cut PBSX DNA slowly and incompletely. For these reasons, and because we had been unsuccessful on a number of occasions with $\phi 105$ wild-type phage, we attempted to use $\phi 105dlys$ as a starting point to clone *spo* genes by the technique of prophage transformation described above. From a number of Spo⁻ recipient strains tried in transformations, a recombinant phage was purified which complemented the *spo-2* mutation in the *spoIIIB* locus.

The details of the method are as follows. DNA preparations from $\phi 105dlys$ (10 μ g) and from PBSX (20 μ g) were cleaved with *EcoRI*, ligated (see Methods) and added to a culture (1 ml, 1×10^8 c.f.u.) containing competent cells of strain 2.3 (*spoIIIB2*) which were lysogenic for $\phi 105dlys$. Spo⁺ transformants were selected and the total number obtained (156) were picked on to nutrient agar, grown at 37 °C for 16 h and the colonies pooled in BHIB (10 ml). Phage were induced with mitomycin C, and the lysate (0.1 ml), mixed with wild-type phage lysate as helper (0.05 ml, about 5×10^7 p.f.u.), was used to transduce cells of strain 2.3 *spoIIIB2* to Spo⁺. Approximately 350 Spo⁺ transductants were obtained, and several of these were streaked onto lactate/glutamate minimal agar.

One of these transductants was purified, designated strain 2.4, and phage were prepared from this strain by mitomycin C induction and CsCl gradient centrifugation. The phage formed a single band in the gradient and the phage from this band transduced strain 2.3 *lys-I spoIIIB2* to Spo⁺, provided that tails of helper phage were added as described previously. The phage failed to transduce this strain to Lys⁺, or to transduce strain SG20 (*lys-3*) to Lys⁺, so it appeared that the *lys* gene had been inactivated or lost.

To check that the Spo⁺ transductants contained the original *spo* mutation several of them were picked, DNA was prepared from each of them and this was used to transform strain MB79 to Phe⁺. For reasons already given, one would expect that 2 to 5% of the Phe⁺ transformants should be Spo⁻. In one transformation 3% (48/1600) of the Phe⁺ transformants were Spo⁻, and in another 2.2% (44/2020) were Spo⁻, showing that the Spo⁺ transductants carried the original *spo-2* mutation. The recombinant phage carrying *spoIIIB* was denoted $\phi 105dspoIIIB$.

Fig. 4. Restriction endonuclease cleavage maps of DNA from phage $\phi 105$, phage $\phi 105dlys$ and phage $\phi 105dspoIIIB$ digested with *EcoRI*, *XbaI* or *KpnI*. The sizes of the fragments are in MDal, and they are lettered for identification (see Fig. 2 and text). The *EcoRI* fragment order of phage $\phi 105$ DNA is from Perkins *et al.* (1978).

Phenotypic properties of strain 2.4($\phi 105dspoIIIB$)

The complemented mutant strain 2.4 was found to sporulate normally, producing the wild-type (Spo^+) amount of glucose dehydrogenase and the wild-type number of heat-resistant and lysozyme-resistant spores at t_{10} (3.2×10^8 spores ml^{-1}). The original *spoIIIB* strain (2.3) produced no glucose dehydrogenase (Piggot, 1973) and it formed only about 3×10^5 spores ml^{-1} at t_{10} .

Restriction endonuclease analysis of $\phi 105dspoIIIB$ DNA

The *EcoRI* digest pattern of DNA extracted from the *spoIIIB*⁺-transducing phage differed from the $\phi 105dlys$ DNA (Fig. 3, lanes 2 and 3) in that fragment L had been lost and replaced by a new fragment of 3 MDal, denoted S. The sum total of the *EcoRI* fragments was 23.6 MDal; thus the $\phi 105dspoIIIB$ phage DNA was still smaller than that in the original wild-type phage by about 1.5 MDal. No new single restriction site was generated in the recombination, e.g. *BamHI* and *BglII* still did not cut the DNA, and furthermore the *XbaI* site was lost (results not shown). The *EcoRI* restriction map of $\phi 105dspoIIIB$ DNA is shown in Fig. 4.

Transforming activity of $\phi 105dspoIIIB$ DNA

The DNA extracted from the phage transformed strain 2.3 *lys-1 spoIIIB2* to Spo^+ (as would be expected from the transduction results), but not to Lys^+ . Neither did the DNA transform strain SG20 (*lys-3*) to Lys^+ , showing that the *lys* gene had been lost. The Spo^+ transforming activity was located in *EcoRI* fragment S (Table 3), since only this band, when excised from a partially stained agarose gel, transformed strain 2.5 *rij*^R *spoIIIB2* with high frequency to Spo^+ . The high background numbers of Spo^+ colonies in Table 3 were due to some overloading and consequently some smearing in the gel, and thus slight cross-contamination of the DNA fragments.

The *spoIIIB* locus is close to several other *spo* loci (Piggot, 1973; Piggot & Coote, 1976); therefore, it was worth checking if the phage DNA would transform to Spo^+ any strains harbouring *spo* mutations close to *spoIIIB*. None of the strains listed in Table 1 having mutations in the *spoIIIA*, *spoIVB* or *spoOA* loci was transformed to Spo^+ by $\phi 105dspoIIIB$ DNA.

The possibility was considered that it was not in fact the *spoIIIB* gene(s) that had been cloned, but some intergenic suppressor of the mutation in strain 2.3 (*spo-2* is the only mutation so far identified in *spoIIIB*). To try to rule out this possibility, two Spo^+ colonies, obtained by transforming strain 2.3 *lys-1 spoIIIB2* with phage $\phi 105dspoIIIB$ DNA, were purified. DNA was

Table 3. *Identification of the EcoRI fragment of phage ϕ 105dsplIB DNA which contains the spoIIIB2⁺ gene*

Phage DNA (approximately 2 μ g) was digested with *EcoRI* and subjected to agarose gel electrophoresis. The gel was stained with ethidium bromide (1 μ g ml⁻¹; 5 min), visualized on a UV transilluminator, and the partially stained bands were excised. The agarose slices were homogenized in transformation medium (0.8 ml) using a syringe fitted with a 19 gauge needle. Competent cells of strain 2.5 rif^R *splIB2* (0.1 ml, 1×10^8 c.f.u.) were then added, and after 90 min incubation at 37 °C, samples (0.1 ml) of culture, suitably diluted, were plated on to Schaeffer's medium in glass Petri plates. After incubating the plates for 18 h at 37 °C, selection was made for Spo⁺ transformants as described in Methods.

<i>EcoRI</i> fragment	No. of Spo ⁺ transformants ml ⁻¹
7.5 MDal (B)	3.0×10^3
5.7 MDal (C)	1.2×10^3
4.8 MDal (D)	2.1×10^3
3.0 MDal (S)	2.6×10^5
2.1 MDal (F)	2.1×10^2
None (control)	1.2×10^1

prepared from each of them, and the preparations were used to transform strain MB79 to Phe⁺. If the *spl* mutation in the Spo⁺ transformants was merely being suppressed, then among the Phe⁺ transformants there should be congressants carrying the original *splIB2* mutation. Out of 4160 transformants counted, none was Spo⁻, so it seemed reasonable to conclude that the Spo⁺ phenotype of the transformants obtained using phage ϕ 105dsplIB DNA was due to recombination within the *splIB* gene(s).

DISCUSSION

The temperate phages ρ 11 and ϕ 105 have been used to clone both vegetative and early sporulation genes of *B. subtilis* (Kawamura *et al.*, 1980; Iijima *et al.*, 1980; Hirochika *et al.*, 1981). Phage ϕ 105 is potentially more useful than ρ 11 because its DNA is less than half the size of that in ρ 11. Consequently, the number of restriction fragments generated by digestion of the DNA with commonly used restriction enzymes is smaller, and it is easier to define and isolate the fragment containing a putative cloned gene (see text).

The recombinant phage ϕ 105dlys first isolated had evidently lost some genes required for synthesis or assembly of the tail components. However, the phage appears to have all the functions necessary for integration, excision and replication of the prophage. A recombinant phage ϕ 105dmetB isolated by Iijima *et al.* (1980) was also found to lack tails, but this phage existed only in the prophage state together with the wild-type phage in a double lysogen. Both the ϕ 105dlys and ϕ 105dsplIB phages described in this paper were able to transduce when provided with tails, and the transductants obtained were single lysogens. This was evident from CsCl gradients of phage obtained from lysogens induced with mitomycin C, since the ϕ 105 phage band contained only transducing and not plaque-forming phage.

The isolation of ϕ 105dlys and ϕ 105dsplIB establishes that the *lys-1*, *lys-3* and *splIB2* mutations can be complemented. This occurs in the absence of the *recE4* gene product which is known to be required for recombination (Mazza & Galizzi, 1978) in *B. subtilis*. The transductants arising from infection with ϕ 105dlys or ϕ 105dsplIB were diploid for the gene involved because (a) the original mutations were shown to be still present [this excludes the possibility that the observed phenotypes (Lys⁺ or Spo⁺) were the result of recombination], (b) the transductants, when induced, gave rise to transducing phages carrying the *lys*⁺ or *spl*⁺ genes, (c) all Lys⁺ or Spo⁺ transductants which were tested were immune to superinfection by wild-type ϕ 105 phage, and (d) no deletion or loss of *lys*⁺- or *spl*⁺-transducing activity of these recombinant phages has been observed, even after numerous sub-cultures of *recE4*⁺ lysogens. This establishes the phage as a stable vector for clonal and complementation analysis.

Dancer & Mandelstam (1981) have shown that by fusing protoplasts of Spo⁻ and Spo⁺ strains at about stage III in sporulation, mutations in any of the stage IV loci could be complemented by

the chromosome carrying the *spoIV*⁺ allele. However, there was no complementation of mutations in any *spoIII* or *spoV* loci. They considered that this lack of complementation could have been due to one of the following: (a) dominance of the *spo* mutation; (b) a limitation of the method, e.g. the time of fusion (stage III) might have been too late; or (c) a requirement for expression of the *spo* gene in the forespore compartment (see Lencastre & Piggot, 1979). The results in this paper show clearly that the *spoIIB2* mutation can be complemented. This rules out, therefore, dominance of *spoIIB2* as a reason for the failure to obtain complementation in the fusion experiments (Dancer & Mandelstam, 1981). Instead, it is more likely that the *spoIIB* gene(s) is expressed before the earliest time (3 h after induction of sporulation) at which it is practicable to obtain spores in protoplast fusion experiments. The remaining possibility, that the gene is expressed in the forespore compartment, cannot be ruled out, but is unlikely in view of the results of Lencastre & Piggot (1979).

This paper further establishes phage $\phi 105$ as a suitable vehicle for the cloning and expression of homologous DNA in *B. subtilis*. Using this vector it should now be possible to carry out complementation tests on the *spoIII* or *spoV* loci which could not be tested in the protoplast fusion system.

We are grateful to Miss Mary Blower for her excellent technical assistance, and to Professor D. J. Tipper for his advice and most helpful discussions in the early stages of this work. This work was supported by the Science and Engineering Research Council.

REFERENCES

- ANAGNOSTOPOULOS, C. & SPIZIZEN, J. (1961). Requirements for transformation in *Bacillus subtilis*. *Journal of Bacteriology* **81**, 741–746.
- BIRDSELL, D. C., HATHAWAY, G. M. & RUTBERG, L. (1969). Characterization of temperate *Bacillus* bacteriophage $\phi 105$. *Journal of Virology* **4**, 264–270.
- COOTE, J. G. (1972). Sporulation in *Bacillus subtilis*. Genetic analysis of oligosporogenous mutants. *Journal of General Microbiology* **71**, 17–27.
- DANCER, B. N. & MANDELSTAM, J. (1981). Complementation of sporulation mutations in fused protoplasts of *Bacillus subtilis*. *Journal of General Microbiology* **123**, 17–26.
- DUBNAU, D., DAVIDOFF-ABELSON, R., SCHER, B. & CIRIGLIANO, C. (1973). Fate of transforming deoxyribonucleic acid after uptake by competent *Bacillus subtilis*: phenotypic characterization of radiation-sensitive recombination-deficient mutants. *Journal of Bacteriology* **114**, 273–286.
- FLOCK, J. & RUTBERG, L. (1974). Mature DNA from temperate *Bacillus* phage $\phi 105$ requires primary recombination to be infectious in transfection. *Molecular and General Genetics* **131**, 301–311.
- GLENN, A. R. & MANDELSTAM, J. (1971). Sporulation in *Bacillus subtilis* 168. Comparison of alkaline phosphatase from sporulating and vegetative cells. *Biochemical Journal* **123**, 129–138.
- HIROCHIKA, H., KOBAYASHI, Y., KAWAMURA, F. & SAITO, H. (1981). Cloning of sporulation gene *spoOB* of *Bacillus subtilis* and its genetic and biochemical analysis. *Journal of Bacteriology* **146**, 494–505.
- HOCH, J. A. (1971). Selection of cells transformed to prototrophy for sporulation markers. *Journal of Bacteriology* **105**, 1200–1201.
- HRANUELI, D., PIGGOT, P. J. & MANDELSTAM, J. (1974). Statistical estimate of the total number of operons specific for *Bacillus subtilis* sporulation. *Journal of Bacteriology* **119**, 684–690.
- IJIMA, T., KAWAMURA, F., SAITO, H. & IKEDA, Y. (1980). A specialized transducing phage constructed from *Bacillus subtilis* phage $\phi 105$. *Gene* **9**, 115–126.
- JENKINSON, H. F. (1983). Altered arrangement of proteins in the spore coat of a germination mutant of *Bacillus subtilis*. *Journal of General Microbiology* **129**, 1945–1958.
- JENKINSON, H. F., KAY, D. & MANDELSTAM, J. (1980). Temporal dissociation of late events in *Bacillus subtilis* sporulation from expression of genes that determine them. *Journal of Bacteriology* **141**, 793–805.
- KAWAMURA, F., SAITO, H., HIROCHIKA, H. & KOBAYASHI, Y. (1980). Cloning of sporulation gene, *spoOF*, in *Bacillus subtilis* with $\phi 11$ phage vector. *Journal of General and Applied Microbiology* **26**, 345–355.
- KAWAMURA, F., SHIMOTSU, H., SAITO, H., HIROCHIKA, H. & KOBAYASHI, Y. (1981). Cloning of *spoO* genes with bacteriophage and plasmid vectors in *Bacillus subtilis*. In *Sporulation and Germination*, pp. 109–113. Edited by H. S. Levinson, A. L. Sonenshein & D. L. Tipper. Washington, D.C.: American Society for Microbiology.
- LENCASTRE, H. D. & PIGGOT, P. J. (1979). Identification of different sites of expression for *spo* loci by transformation of *Bacillus subtilis*. *Journal of General Microbiology* **114**, 377–389.
- LIU, H.-M., CHAK, K. F. & PIGGOT, P. J. (1982). Isolation and characterization of a recombinant plasmid carrying a functional part of the *Bacillus subtilis* *spoIIA* locus. *Journal of General Microbiology* **128**, 2805–2812.

- LLOYD-JONES, G. (1976). *Genetics and biochemistry of bacterial sporulation*. M.Sc. thesis, University of Oxford, U.K.
- LOSICK, R. & PERO, J. (1981). Cascades of sigma factors. *Cell* **25**, 582-584.
- MAZZA, G. & GALIZZI, A. (1978). The genetics of DNA replication, repair and recombination in *Bacillus subtilis*. *Microbiologica* **1**, 111-135.
- MORAN, C. P., JR, LANG, N., LEGRICE, S. F. J., LEE, G., STEPHENS, M., SONENSHEIN, A. L., PERO, J. & LOSICK, R. (1982). Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. *Molecular and General Genetics* **186**, 339-346.
- NESTER, E. W., SCHAFER, M. & LEDERBERG, J. (1963). Gene linkage in DNA transfer: a cluster of genes concerned with aromatic biosynthesis in *Bacillus subtilis*. *Genetics* **48**, 529-551.
- OKAMOTO, K., MUDD, J. A., MANGAN, J., HUANG, W. M., SUBBAIAH, T. V. & MARMUR, J. (1968*a*). Properties of the defective phage of *Bacillus subtilis*. *Journal of Molecular Biology* **34**, 413-428.
- OKAMOTO, J., MUDD, J. A. & MARMUR, J. (1968*b*). Conversion of *Bacillus subtilis* DNA to phage DNA following mitomycin C induction. *Journal of Molecular Biology* **34**, 429-437.
- PERKINS, J. B., ZARLEY, C. D. & DEAN, D. H. (1978). Restriction endonuclease mapping of bacteriophage $\phi 105$ and closely related temperate *Bacillus subtilis* bacteriophages $\rho 10$ and $\rho 14$. *Journal of Virology* **28**, 403-407.
- PIGGOT, P. J. (1973). Mapping of asporogenous mutations of *Bacillus subtilis*: a minimum estimate of the number of sporulation operons. *Journal of Bacteriology* **114**, 1241-1253.
- PIGGOT, P. J. & COOTE, J. G. (1976). Genetic aspects of bacterial endospore formation. *Bacteriological Reviews* **40**, 908-962.
- PIGGOT, P. J., MOIR, A. & SMITH, D. A. (1981). Advances in the genetics of *Bacillus subtilis* differentiation. In *Sporulation and Germination*, pp. 29-39. Edited by H. S. Levinson, A. L. Sonenshein and D. J. Tipper. Washington, D.C.: American Society for Microbiology.
- SADOFF, H. L. (1966). Glucose dehydrogenase-soluble. I. *Bacillus cereus*. *Methods in Enzymology* **9**, 103-107.
- SCHAEFFER, P., IONESCO, H., RYTER, A. & BALASSA, G. (1965). La sporulation de *Bacillus subtilis*: étude génétique et physiologique. *Colloques internationaux du Centre national de la recherche scientifique* **124**, 553-563.
- SCHER, B. M., LAW, M. F. & GARRO, A. J. (1978). Correlated genetic and *EcoRI* cleavage map of *Bacillus subtilis* bacteriophage $\phi 105$ DNA. *Journal of Virology* **28**, 395-402.
- STERLINI, J. M. & MANDELSTAM, J. (1969). Commitment to sporulation in *Bacillus subtilis* and its relationship to development of actinomycin resistance. *Biochemical Journal* **113**, 29-37.
- WARD, J. B., JR & ZAHLE, S. A. (1973). Genetic studies of leucine biosynthesis in *Bacillus subtilis*. *Journal of Bacteriology* **116**, 719-726.
- YAMAMOTO, K. R., ALBERTS, B. M., BENZINGER, R., LAWHORNE, L. & TREIBER, G. (1970). Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. *Virology* **40**, 734-744.
- YOUNG, M. & MANDELSTAM, J. (1979). Early events during bacterial endospore formation. *Advances in Microbial Physiology* **20**, 103-162.
- YUDKIN, M. D. & TURLEY, L. (1981). Mapping of six mutations in the *spoIIA* locus of *Bacillus subtilis* and studies of their response to a nonsense suppressor. *Journal of General Microbiology* **124**, 255-261.