Efficient *Bacillus subtilis* Cloning System using Bacteriophage Vector ϕ 105J9

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An efficient system for cloning in *Bacillus subtilis* is described which uses a newly constructed bacteriophage vector, ϕ 105J9. The phage genome contains cloning sites for the enzymes *Bam*HI, *Xba*I and *Sal*I, and can accommodate inserts of passenger DNA of at least 4 kbp. Recombinant phages, which can both plaque and lysogenize normally, are recovered after direct transfection of protoplasts in the presence of polyethylene glycol. Several fully functional sporulation genes and one biosynthetic gene from *B. subtilis* have been isolated from genomic libraries that were constructed with the new vector. The system may provide an alternative to some of the cloning methods currently available that use *Escherichia coli* as host.

INTRODUCTION

A range of efficient techniques is now available for gene cloning and *in vitro* genetic manipulation using *Escherichia coli* as host. However, gene cloning systems using other organisms are still in the early stages of development. With the exception of *E. coli*, *Bacillus subtilis* has been subjected to more genetic analysis than any other prokaryotic organism, mainly because of interest in the developmental process that leads to spore formation (see, for example, reviews by Piggot & Coote, 1976; Young & Mandelstam, 1979). Thus, a great deal of background information is available for this organism, including a comprehensive genetic map of the chromosome (Henner & Hoch, 1980). In addition, *B. subtilis* has two major advantages over *E. coli* as a host for genetic manipulation *in vitro*. Firstly, it is non-pathogenic. Secondly, *B. subtilis*, in contrast to *E. coli*, produces extracellular proteins and this is particularly useful for large-scale production and purification of the products of cloned genes.

Two methods have been used for the transformation of recombinant DNA molecules into *B. subtilis* recipients. The first relies on the natural state of competence that occurs in *B. subtilis* under certain physiological conditions. However, during uptake the transforming DNA undergoes various forms of processing by nucleases (Venema, 1979) so that the establishment of intact plasmid or phage DNA molecules occurs at low frequency. Thus, cloning methods using competent *B. subtilis* cells usually rely on rescue of the fragmented transforming molecules by recombination with a homologous vector resident in the recipient cell. This type of approach has been used with both plasmid (Gryczan *et al.*, 1980*b*) and phage (Kawamura *et al.*, 1979) vectors. The other method was developed by Chang & Cohen (1979) for use with plasmid DNA. They showed that protoplasts could be transformed at high frequencies ($> 10^7$ transformants per μ g DNA) with supercoiled plasmid DNA, although transformation frequencies were 1 to 3 orders of magnitude lower with relaxed circular plasmid DNA, which is produced, for example, after re-ligation of linear molecules.

There are several other disadvantages to the use of plasmids as cloning vehicles in *B. subtilis*. Firstly, it seems to be difficult to clone large (> 2.5 kbp) fragments of DNA with some plasmid vectors (Gryczan & Dubnau, 1982). Secondly, plasmids are generally maintained at a greater copy number than the host chromosome: the concomitant increase in gene dosage can have, at

least with some genes, deleterious effects on the host cell (Kawamura *et al.*, 1981; Banner *et al.*, 1983). Thirdly, homologous inserts of *B. subtilis* DNA are likely to be maintained on plasmids only in a recombination deficient (*Rec*⁻) background. *Rec*⁻ strains of *B. subtilis* are difficult to construct and to maintain, and, of particular importance to the study of the developmental process, they do not sporulate well (J. Errington, unpublished observations). Fourthly, even in a *Rec*⁻ background, instability of inserts in plasmids can occur (Tanaka, 1979; Uhlen *et al.*, 1981).

Fortunately, none of the above disadvantages apply to bacteriophage vectors in *B. subtilis*. Temperate phages such as $\phi 105$ have a chromosomal attachment site analogous to that of λ in *E. coli* (Rutberg, 1969) and are therefore stably maintained in a single copy relative to the *B. subtilis* chromosome. Moreover, inserts in bacteriophage $\phi 105$ in *B. subtilis* are completely stable even in a *Rec*⁺ background (Jenkinson & Mandelstam, 1983; J. Errington, unpublished observations).

To date, the most successful means of cloning in *B. subtilis* with bacteriophage vectors is the 'prophage transformation' method (Kawamura *et al.*, 1979), which has been used to clone a number of biosynthetic and sporulation genes of *B. subtilis* (Kawamura *et al.*, 1979, 1980; Iijima *et al.*, 1980; Ikeuchi *et al.*, 1983; Jenkinson & Mandelstam, 1983; Savva & Mandelstam, 1984). This method relies on the rescue of recombinant molecules by recombination with a homologous vector, which is present as a prophage in the recipient cell. However, the method has two main drawbacks. Firstly, lysogenic cells, which are used as the recipients in transformation, are several orders of magnitude less competent than non-lysogenic strains (Peterson & Rutberg, 1969; Yasbin *et al.*, 1973; Garro & Law, 1974). Consequently, relatively large amounts of DNA are required, and the yield of transformed cells is low. Secondly, the vast majority of transformed cells result from recombination at the chromosomal locus of the selected gene, rather than by insertion of the cloned gene into the prophage. It was therefore desirable to develop a system of cloning in *B. subtilis* that uses direct transfection of cells with phage DNA.

This paper describes the construction of a new bacteriophage vector, $\phi 105J9$, and its use in *B. subtilis* by direct, PEG-mediated transfection of protoplasts.

METHODS

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

Chemicals. These were obtained from BDH except where otherwise indicated. Phenol was prepared for use by extracting twice with an equal vol. of 10 $\times$ concentrated TE buffer (see below) and was stored under TE buffer; 8-hydroxyquinoline was added to confer a yellow colouration to the phenolic phase and prevent oxidation (P. Fort, personal communication). Chloroform was prepared for use by adding 1/25 vol. of iso-amyl alcohol.

Buffers. TE buffer contained: Tris/HCl (10 mM, pH 7.5) and EDTA (1 mM). STE buffer contained: NaCl (400 mM), Tris/HCl (10 mM, pH 7.5) and EDTA (1 mM). Tris/acetate electrophoresis buffer (TAE) contained: Tris (40 mM), acetic acid (18 mM), EDTA (2 mM) (pH 8.1). Tris/borate electrophoresis buffer (TBE) contained: 89 mM-Tris, 89 mM-boric acid, 2.5 mM-EDTA (pH 8.3). Gel loading buffer contained: 10% (w/v) sucrose, 0.8 M-urea, 20 mM-M-bromophenol blue in TBE buffer. Ligation buffer contained: Tris/HCl (50 mM, pH 7.8), MgCl₂ (10 mM), spermidine (Sigma, 1 mM), bovine serum albumin (BRL, nuclease free; 50 μ g ml⁻¹), dithiothreitol (Sigma, 20 mM) and ATP (Sigma, 1 mM). Kinase buffer contained: Tris/HCl (10 mM, pH 7.5), MgCl₂ (10 mM) and 2-mercaptoethanol (Sigma, 10 mM).

Phage $\phi 105$ preparation. Crude lysates and CsCl purified phage were prepared as described by Jenkinson & Mandelstam (1983), except that DNAase I treatment was omitted.

Preparation of phage DNA. Phage purified on a CsCl gradient as above (titre 10¹¹ to 10¹² p.f.u. ml⁻¹) was precipitated by adding 1/4 vol. of a solution containing: NaCl (2.5 M) and 20% (w/v) PEG (mol. wt 6000, BDH). After 30 min at 22 °C the phage precipitate was recovered by centrifuging in a Beckman microfuge for 2 min. The phage pellet was then resuspended in TE buffer to the original volume. A 1/2 vol. of phenol was added and the mixture was vortex-mixed for 30 s. After 20 min at 22 °C the vortex-mixing was repeated and the two phases were separated by centrifugation for 5 min as above. The upper (aqueous) phase was carefully removed to a fresh tube and the DNA was precipitated by adding 1/10 vol. 2.5 M-sodium acetate and 2 vols absolute ethanol. The tube was inverted several times and the clump of DNA was removed on a plastic micropipette tip. The DNA was washed in 80% ethanol then dried *in vacuo* and resuspended in TE buffer to a concentration of approximately 1 μ g μ l⁻¹. The DNA was stored at 4 °C.

Table 1. Bacterial strains, plasmids and phages

	Genotype	Source or reference*
Escherichia coli strains		
HB101	F ⁻ <i>hsdS20</i> (<i>r_B</i> , <i>m_B</i>) <i>supE44</i> <i>supF58 lacY1</i> (or <i>lacIZY76</i>) <i>galK2 galT22 metB1 trpR55</i>	Boyer & Roulland-Dussoix (1969)
JM103	Δ (<i>lac pro</i>) <i>thi strA supE</i> <i>endA sbcB15 F' traD36 proAB</i> <i>lac^R ZAM15</i>	
JM103(pUC13)	As above, Ap ^R	Messing (1983)
Bacillus subtilis strains		
Spo⁺ strains		
1L29	<i>trpC2</i> (ϕ 105D1::1t)	BGSC
1L22	<i>trpC2 thr-5</i> (pBD64 Cm ^R Km ^R)	BGSC
168	<i>trpC2</i>	Laboratory stock
CU267	<i>trpC2 ilvB2 leuB16</i>	S. A. Zahler
CU448	<i>trpC2 argA2 ilvA1 pheA2</i>	S. A. Zahler
MY2016	<i>hisH2 lys-1 rpoB2 sul</i>	Yudkin & Turley (1980)
RF2	<i>aroD120</i>	R. Farquhar (unpublished)
Spo⁻ strains		
20.1	<i>trpC2 rpoB2 spoIIIE20</i> (E1)†	Piggot (1973)
59.7	<i>trpC2 leuB16 spoIIIA59</i> (NG14.7)†	Piggot (1973)
67.1	<i>metC3 spoIVA67</i> (NG17.23)†	Piggot (1973)
69.4	<i>hisH2 spoIIA69</i> (NG18.6)†	Errington & Mandelstam (1983)
298.2	<i>pheA12 spoIIIC298</i> (P9)†	Coote (1972)
574	<i>hisH2 spoVA574</i>	Errington & Mandelstam (1984)
601.1	<i>spoIIB601</i>	M. Deadman (unpublished)

* BGSC, Bacillus Genetic Stock Center, The Ohio State University, Columbus, Ohio, USA; S. A. Zahler, Genetics & Development Dept, Cornell University, Ithaca, NY, USA; R. Farquhar, this laboratory; M. Deadman, this laboratory.

† Original isolation no. of sporulation mutation.

Preparation of plasmid DNA. Plasmid pBD64 DNA was prepared from its *B. subtilis* host using the method of Lovett & Keggins (1979) as described by Jenkinson & Deadman (1984). Plasmid DNA from *E. coli* was extracted by the alkaline lysis method of Birnboim & Doly (1979) as described by Maniatis *et al.* (1982). Supercoiled plasmid DNA was purified by centrifugation to equilibrium in a CsCl/ethidium bromide density gradient as described by Lovett & Keggins (1979).

Preparation of B. subtilis chromosomal DNA. Strain 168 was grown to late exponential phase (OD₆₀₀ 1.0) in 500 ml Penassay broth (Oxoid). The cells were harvested (5000 g, 5 min at 4 °C), washed in TES buffer and resuspended in 20 ml TES buffer containing lysozyme (Sigma, 100 µg ml⁻¹) and RNAase (Sigma, 20 µg ml⁻¹, heat shocked at 85 °C for 10 min before use). After 30 min at 37 °C, 20 ml fresh TES buffer, 2 ml pronase solution (Sigma, protease type VI, 10 mg ml⁻¹ in TES buffer; self digested at 37 °C for 1 h before use) and 1.2 ml sarkosyl (Ciba-Geigy, 30%) were added. After a further 30 min at 37 °C the clear lysate was extracted with equal volumes of phenol, then with a mixture of phenol and chloroform (1:1), then with chloroform. The DNA in the aqueous phase was precipitated by adding 1/10 vol. 2.5 M-sodium acetate and 2 vols ethanol. The precipitate was washed with 80% ethanol, and re-dissolved in TE buffer to a final concentration of 1 mg ml⁻¹.

Preparation of competent cells. Strains of *B. subtilis* were made competent using the method of Anagnostopoulos & Spizizen (1961) as modified by Jenkinson (1983); *E. coli* strain HB101 was made competent using a CaCl₂ method as described by Fort & Piggot (1984).

Protoplast transfection. Protoplasts of *B. subtilis* were prepared, and transfected with ϕ 105D1::1t DNA, using the method of Chang & Cohen (1979) as modified by Levi-Meyrueis *et al.* (1980) for transformation with plasmid DNA. After 1 min in the presence of PEG solution (Chang & Cohen, 1979) samples (0.1 to 0.5 ml) were added to 3 ml of molten isotonic phage overlay agar (prepared by mixing equal volumes of molten Oxoid tryptose blood agar base and 2 × SMM medium of Chang & Cohen, 1979). Sensitive indicator bacteria (0.1 ml) were added, from a late exponential phase culture in Penassay broth. The overlay was mixed quickly and poured on to the surface of a warm DM3 plate (protoplast regeneration medium of Chang & Cohen, 1979).

Restriction endonuclease digestion. Restriction endonucleases were obtained from Amersham or BRL and digestion was carried out as recommended by the suppliers.

Ligation conditions. Ligation was carried out in ligation buffer containing T4 DNA ligase (Boehringer) at a final concentration of 0.02 units μl^{-1} for DNA with 'sticky' ends or 0.1 units μl^{-1} for blunt ends.

Gel electrophoresis. DNA fragments in gel-loading buffer (see above) were separated on horizontal 0.7% (w/v) agarose (Sigma, type 11) gels submerged in TAE buffer, and were visualized by ethidium bromide fluorescence on a UV transilluminator (Ultra-Violet Products, Calif., USA). Reactions involving *Bam*H1 linker molecules (see below) were monitored by autoradiography following electrophoresis through 0.4 mm thick 7% (w/v) polyacrylamide vertical gels using TBE buffer (see above).

Southern transfer, hybridization and autoradiography. Fragments of $\phi 105J6$ DNA (see below), separated on agarose gels as described above, were transferred to nitrocellulose (Anderman, East Molesey, Surrey, UK) using the method of Southern (1975) as described by Maniatis *et al.* (1982). pBD64 DNA (0.3 μg) was labelled with [^{32}P]phosphate by nick translation (Rigby *et al.*, 1977) using a kit supplied by Amersham and 15 μCi [$\alpha\text{-}^{32}\text{P}$]dCTP [3000 Ci mmol^{-1} (111 TBq mmol^{-1}); Amersham]. Hybridization of labelled probe and nitrocellulose filter, washing, and preparation for autoradiography were performed as described by Maniatis *et al.* (1982).

Phosphorylation of *Bam*H1 linker molecules. *Bam*H1 linker molecules (New England Biolabs, Bishops Stortford, UK; 10-mer, 5'-OH) were prepared for ligation by phosphorylation in a 10 μl reaction mixture containing: 1 μg linkers, 10 μCi [$\gamma\text{-}^{32}\text{P}$]ATP (NEN, 3000 Ci mmol^{-1}) and T4 polynucleotide kinase (BRL, 6 units) in kinase buffer. After 30 min at 37 °C a further 6 units of kinase were added, along with unlabelled ATP (final concentration 1 mM), and the reaction volume was increased to 20 μl . After a further 30 min at 37 °C the reaction was terminated by heating at 65 °C for 10 min. A sample (1 μl) of phosphorylated linkers was then tested for efficient ligation and subsequent digestion with *Bam*H1, the reaction products being monitored by polyacrylamide gel electrophoresis and autoradiography as described above (P. Fort, personal communication).

Cloning fragments of plasmid pBD64 DNA into $\phi 105DI$: 1t. Plasmid pBD64 DNA (20 μg) was partially digested with *Alu*I (0.3 units) for 30 min at 37 °C in a 500 μl reaction volume. *Bam*H1 linkers (1 μg), prepared as described above, were blunt-end ligated to 5 μg of the mainly linearized pBD64 in a 30 μl reaction mixture at 15 °C for 16 h. The reaction was stopped by heating at 65 °C for 10 min, then NaCl was added (final concn 50 mM) and the DNA was digested with *Bam*H1 (20 units, 2 h at 37 °C). Ligation and digestion were monitored as described above. When digestion was complete the DNA was extracted once with an equal volume of phenol, and the low mol. wt digestion products of the *Bam*H1 linker molecules were removed by passage through a 5 ml column of Ultrogel AcA34 (LKB) equilibrated with STE buffer (P. Fort, personal communication). Fractions (200 μl) were eluted from the column with STE buffer and the high mol. wt DNA was detected as a small peak of radioactivity (fractions 11 to 13), which eluted before the main peak corresponding to the low mol. wt digestion products. Radioactivity in each fraction was measured by direct counting in a Wallac (LKB) liquid scintillation counter. $\phi 105DI$: 1t DNA (80 μg) was also partially digested with *Alu*I (0.5 units in a 400 μl reaction; 10 min at 37 °C) to give mainly singly cleaved molecules. Phosphorylated *Bam*H1 linkers (0.7 μg) were added to 10 μg of partially digested 105DI:1t DNA as described above.

pBD64 (0.5 μg) and $\phi 105DI$: 1t (5 μg) DNAs were ligated via their *Bam*H1 cohesive ends in a 25 μl reaction for 16 h at 15 °C. One-fifth of the ligation mixture was used to transform competent cells of strain ($\phi 105DI$: 1t) and after 30 min at 37 °C the cells were plated on nutrient agar containing chloramphenicol (5 $\mu\text{g ml}^{-1}$).

Construction of plasmid pSG1. The heterogeneous phage DNA (5 μg) containing a small amount (~10%) of $\phi 105J6$ DNA (see Results) was digested to completion with *Bam*H1 (10 units; 2 h at 37 °C), then precipitated with 2 vols propan-2-ol in the presence of 2 M-ammonium acetate to remove the restriction endonuclease (Maniatis *et al.*, 1982). The DNA was washed with 80% ethanol, dried *in vacuo* and resuspended in a 50 μl ligation mixture containing 30 ng *Bam*H1-cleaved pUC12 DNA. After 16 h at 15 °C, 5 μl of the ligation was used to transform competent cells of strain HB101. Ampicillin-resistant transformants were selected on nutrient agar containing ampicillin (50 $\mu\text{g ml}^{-1}$). After 24 h at 37 °C, the transformants were replica plated to nutrient agar containing chloramphenicol (10 $\mu\text{g ml}^{-1}$). Chloramphenicol-resistant transformants appeared after 24 h at 37 °C.

Cloning fragments from plasmid pSG1 into 105DI: 1t. Plasmid pSG1 DNA (30 μg) was digested to completion with *Sma*I (37.5 units, 2 h at 37 °C) followed by *Hind*III (30 units, 2 h at 37 °C). Samples of $\phi 105DI$: 1t DNA (40 μg) were digested to completion with *Hind*III (30 units, 2 h at 37 °C) or *Pvu*II (40 units, 2 h at 37 °C). Following removal of each restriction endonuclease as described above, a 100 μl ligation mixture was prepared containing: 30 μg pSG1 double digest, 10 μg $\phi 105DI$: 1t *Hind*III digest, and 24 μg $\phi 105DI$: 1t *Pvu*II digest. The reaction was incubated for 16 h at 15 °C then a 5 μl sample was used to transform competent cells of *B. subtilis* strain 168 ($\phi 105DI$: 1t) with selection for chloramphenicol resistance as described above.

Derivation of $\phi 105J9$. $\phi 105J8$ DNA (500 ng) was digested to completion with *Bam*H1, then re-ligated in a 50 μl reaction volume (16 h at 15 °C) and used to transfect protoplasts of strain 168 (non-lysogenic). Lysogenic cells from the centres of the resulting plaques were tested for loss of chloramphenicol resistance by inoculating them on nutrient agar plates containing chloramphenicol (5 $\mu\text{g ml}^{-1}$).

Construction of *B. subtilis* genomic libraries. Vector ($\phi 105J9$) DNA (20 μg) was ligated in a 25 μl reaction volume (4 h at 22 °C) to form long concatameric molecules via the phage cohesive ends (Scher *et al.*, 1977). After

inactivation of the ligase at 65 °C for 10 min, the DNA was digested to completion with *Bam*HI (20 units, 2 h at 37 °C). This resulted in the production of linear, genome-length molecules consisting of the two phage 'arms' joined back-to-back at the phage cohesive ends.

Chromosomal DNA from *B. subtilis* was prepared for ligation by digesting it to completion with *Bcl*I or *Bgl*II.

Target and vector DNAs were added to 50 or 100 µl ligation mixtures at concentrations of 3 ng µl⁻¹ and 10 ng µl⁻¹, respectively. After 16 h at 15 °C samples of the ligation mixture were used to transfect protoplasts of strain 168 as described above.

Recovery of the amplified pool of recombinant phage. After 24 h at 37 °C 1000 to 5000 plaques were visible in each overlay. The top agar layer of each plate was gently resuspended in 5 ml Penassay broth using a bent, sterile Pasteur pipette. The liquid and macerated pieces of top agar were then transferred to a sterile 15 ml centrifuge tube and left at 4 °C for 1 to 10 h. The agar and cell debris were removed by centrifugation (5000 *g* for 15 min) and the supernatant was carefully removed. The phage suspension was sterilized by vortex mixing for 30 s in the presence of 1/50 vol. CHCl₃ and was left at 4 °C overnight before use. The final titre of the suspension was generally from 10⁶ to 10⁷ p.f.u. ml⁻¹.

Screening the recombinant phage pools. Strains (non-lysogenic) were grown at 37 °C to mid exponential phase (OD₆₀₀ ~ 0.5) in 5 ml Penassay broth. Samples (0.2 ml) were mixed with 0.1 ml of each recombinant phage pool and immediately plated. Selection for phages carrying functional genes that complemented auxotrophic mutations was made by growth on nutrient agar for 16 h at 37 °C, followed by replica plating to lactate glutamate minimal agar (Piggot, 1973) supplemented with the appropriate amino acids. Phages carrying functional sporulation genes were identified by growth on Schaeffer's medium (Schaeffer *et al.*, 1965) for 20 h at 37 °C followed by selection for Spo⁺ using chloroform vapour (Hoch, 1971).

RESULTS

Direct transfection of protoplasts

Mature ϕ 105 DNA transfects competent cells of *B. subtilis* at relatively low frequency (about 10² infectious centres per µg DNA; Rutberg *et al.*, 1969). However, by treating protoplasts of *B. subtilis* strains with ϕ 105 DNA in the presence of PEG (see Methods) much higher frequencies of transfection were consistently obtained (between 10⁵ and 10⁶ infectious centres per µg DNA: data not shown). A similar transfection system has been described recently using the bacteriophage ϕ d07, which is related to ϕ 105 (Perkins & Dean, 1983).

Wild-type ϕ 105 is rather unsuitable as a cloning vector since it contains no unique restriction endonuclease cleavage sites that are suitable for the insertion of passenger DNA. However, several potentially useful derivatives of this phage have been obtained. For example, ϕ 105 dLys (Jenkinson & Mandelstam, 1983) contains a unique *Xba*I site; ϕ 105 dCm^r (Jenkinson & Deadman, 1984) contains a unique *Bgl*II site. Both of these vectors have been used successfully for further cloning with the prophage transformation method of Kawamura *et al.* (1979), but they are defective and require the addition of wild-type helper phage for infection. When tested in the protoplast system, DNA from both of these phages transfected at much lower frequency (< 10² per µg DNA, selecting Lys⁺ or chloramphenicol-resistance) than did the wild-type phage DNA. Presumably the initial infectious event in protoplasts almost always leads to lysis rather than lysogeny. Wild-type phage progeny released following the initial lytic cycle are able to re-infect the surrounding cells and give rise to a plaque, whereas those defective phage particles released following primary infection are unable to re-infect and are lost. In order to make use of the high transfection efficiency observed in protoplasts it was necessary to construct a non-defective phage vector.

Construction of a ϕ 105 cloning vector

ϕ 105DI: It was chosen as the starting material because it is a derivative of wild-type ϕ 105 from which 4 kbp of non-essential DNA has been deleted (Flock, 1977). *Bam*HI was the obvious restriction endonuclease cleavage site to attempt to introduce into the phage genome, since there are no sites for this enzyme in ϕ 105, and the cohesive ends produced by cleavage with *Bam*HI are compatible with those produced by several other restriction endonucleases (Roberts, 1982).

Preliminary attempts to introduce a *Bam*HI linker molecule directly into the ϕ 105DI:It genome were unsuccessful because there was no easy way to identify recombinant clones. As an

Fig. 1. Schematic diagram outlining the procedure used for the cloning of fragments from plasmid pBD64 into phage $\phi 105DI:1t$ using *Bam*HI linker molecules. The restriction map of plasmid pBD64 (except for the restriction sites for the enzyme *AluI*) has been described previously (Gryczan *et al.*, 1980a). The positions of *AluI* sites in pBD64 other than those shown have not been determined. The one letter abbreviations for restriction endonuclease cleavage sites in this figure and other figures are as follows: A, *AluI*; B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hind*III; M, *Sma*I; P, *Pvu*II; R, *Pst*I; S, *Sal*I; T, *Sst*I; V, *Ava*I; X, *Xba*I. Dotted lines show the approximate locations of the genes for chloramphenicol (Cm^R) and kanamycin (Km^R) resistance.

alternative approach, the *B. subtilis* plasmid cloning vector, pBD64 (Gryczan *et al.*, 1980a) was used to provide restriction fragments carrying selectable genes for chloramphenicol or kanamycin resistance that could be cloned into $\phi 105DI:1t$ via *Bam*HI linker molecules. Using the scheme described in Fig. 1 and in Methods, fragments of pBD64 carrying chloramphenicol and/or kanamycin resistance determinants were ligated via *Bam*HI linker molecules to $\phi 105DI:1t$ that had been partially digested with *AluI*. This enzyme cleaves $\phi 105DI:1t$ at >50 sites (data not shown) so insertions into the phage genome would be essentially at random. After transformation of a $\phi 105DI:1t$ lysogenic strain, eight chloramphenicol-resistant (Cm^R) colonies

Fig. 2. Restriction endonuclease cleavage maps of phages $\phi 105DI:1t$ and $\phi 105J6$, with the insert in $\phi 105J6$ enlarged below, and compared with the plasmid, pBD64, from which it was derived (see text). One letter abbreviations for restriction endonucleases are as in Fig. 1. The *EcoRI* map for $\phi 105$ has been described previously (Scher *et al.*, 1978; Bugaichuk *et al.*, 1984).

were obtained, presumably by rescue of the *cat* gene onto the $\phi 105DI:1t$ genome. Phage lysates obtained after induction with mitomycin C were then prepared from each transformant. One of these contained a high phage titre (2.7×10^8 p.f.u. ml⁻¹) and transduced *B. subtilis* to Cm^R at high frequency (1.2×10^6 Cm^R transductants ml⁻¹; compare with 5×10^3 ml⁻¹ for transduction by the defective Cm^R transducing phage, $\phi 105$ dCm^R; Jenkinson & Deadman, 1984). Unfortunately, not all of the plaque-forming particles transduced to Cm^R. Despite repeated single colony isolation, and growth in the presence of chloramphenicol, only 12 to 15% of plaque-forming particles gave Cm^R lysogenic cells. Moreover, when phage DNA was isolated, only a small proportion of it was cleaved by *Bam*H1. Nevertheless, it was possible to map the insert in the phage by Southern transfer and hybridization of nick translated, ³²P-labelled pBD64 DNA (Fig. 2).

The 2 kbp *Bam*H1 insert was towards the right hand side of the $\phi 105DI:1t$ genome within the 9.0 kbp *Eco*RI fragment (Scher *et al.*, 1978; Bugaichuk *et al.*, 1984). The increase in size of this *Eco*RI fragment was possibly more than 2 kbp, which suggests that there may have been a duplication of a small region of the $\phi 105$ genome flanking the insertion. This may explain why the phage is unstable; a single crossover within the duplicated regions could result in precise excision of the insert, along with the restoration of the phage genome to its original state.

Although the unstable phage, designated $\phi 105J6$, was not itself useful as a cloning vector, the 2 kbp *Bam*H1 insert carrying Cm^R was potentially useful as a target DNA in further attempts to construct a vector. It was therefore sub-cloned in *E. coli* strain HB101 into the unique *Bam*H1 site of plasmid pUC12 by selection for Cm^R, which is known to be expressed in *E. coli* (Horinouchi & Weisblum, 1982). pUC12 was a convenient vector in which to clone this fragment since the *Bam*H1 site in this plasmid is within a 'polylinker' region that includes unique restriction endonuclease sites for several other enzymes (Messing, 1983). The constructed plasmid, pSG1, which could easily be prepared on a large scale, provided a convenient source of Cm^R target DNA, flanked by two *Bam*H1 sites that could be removed using various pairs of enzymes and inserted into the $\phi 105DI:1t$ genome without any further use of linkers.

The scheme for using pSG1 to construct a fully functional $\phi 105$ cloning vector is shown in Fig. 3. Plasmid pSG1 was digested with *Hind*III and *Sma*I to give a fragment of 2 kbp containing the

Fig. 3. Schematic diagram showing the procedure used for cloning fragments from plasmid pSG1 into phage $\phi 105D1:1t$. The one letter abbreviations for restriction endonuclease cleavage sites are as in Fig. 1. The lower part of the figure shows, on a smaller scale, the $\phi 105D1:1t$ prophage with insertions of the Cm^R determinant at random between *HindIII* and *PvuII* sites.

cat gene and flanking *Bam*H1 sites on a molecule with one *Hind*III 'cohesive' end and one blunt end (*Sma*I). This was ligated to $\phi 105D1:1t$ digested with *Hind*III and *Pvu*II (which also gives blunt ends). Transformation of strain 168 ($\phi 105D1:1t$) enabled the Cm^R fragment to be rescued onto the prophage genome by crossing over involving the covalently linked *Hind*III and *Pvu*II fragments of $\phi 105D1:1t$ DNA. The first transformation experiment gave 290 Cm^R colonies, which were screened for plaque formation and ability to transfer Cm^R by transduction as 16 pools each containing 18 colonies. Phages that formed plaques on sensitive strains and transduced to Cm^R at high frequency were isolated from two different pools. One of the new phages, $\phi 105J8$, was completely stable: 100/100 plaques contained Cm^R lysogenic cells. The second phage, $\phi 105J7$, gave a small proportion (about 20%) of chloramphenicol-sensitive lysogenic cells, presumably again because of a small duplication of DNA sequences flanking the insertion. DNA was prepared from both phages, and restriction maps were constructed using single and double digestion with a variety of endonucleases (Fig. 4).

One of the Cm^R inserts ($\phi 105J8$) was near the middle of the phage genome, close to the region already deleted in $\phi 105D1:1t$; the other ($\phi 105J7$) was near one end of the phage in a region whose function has not been determined but which is clearly not essential for lytic growth or lysogeny. $\phi 105J8$ was chosen for further development because of its complete stability.

In order to circumvent the need to purify repeatedly the two phage arms from the 2 kbp Cm^R fragment prior to cloning into the *Bam*H1 site, $\phi 105J8$ DNA was cleaved with *Bam*H1, then re-

Fig. 4. Restriction endonuclease cleavage maps for bacteriophages $\phi 105$, $\phi 105DI:lt$ and their derivatives. The locations of the 2.0 kbp *Bam*HI inserts conferring Cm^R in $\phi 105J7$ and $\phi 105J8$ are indicated by the broken lines. For clarity, only those *Hind*III and *Pvu*II sites involved in the insertions are shown. The *Eco*RI sites on each phage are provided for reference and are as determined previously (Scher *et al.*, 1978; Bugaichuk *et al.*, 1984). One letter restriction endonuclease abbreviations are as in Fig. 1.

ligated and used to transfect protoplasts of strain 168 (non lysogenic). Of 20 plaques tested following transfection, only one still transduced to Cm^R , indicating that the remainder of phages had been generated by re-circularization without the Cm^R insert. One of the chloramphenicol-sensitive phages was isolated and its DNA was prepared. As expected the restriction map of the new phage, $\phi 105J9$, was identical to that of $\phi 105J8$, except there was a single *Bam*HI site and no 2 kbp insert (Fig. 4).

In addition to the unique *Bam*HI site in $\phi 105J9$ there is a unique *Xba*I site, also derived from the polylinker of pUC12, and a second *Sal*I site of similar origin. These enzymes could also be used for cloning, although there would be some loss of efficiency using *Sal*I. The second *Sal*I site, which is also present in wild type $\phi 105$, is likely to be located within an essential gene (J. Errington, unpublished results; H. F. Jenkinson & M. Deadman, personal communication).

Cloning in *Bacillus subtilis* using $\phi 105J9$

The scheme for using $\phi 105J9$ as a cloning vector in *B. subtilis* is shown in Fig. 5. $\phi 105J9$ DNA was treated with ligase to produce long concatameric molecules making use of the phage cohesive ends (Scher *et al.*, 1977). Subsequent digestion with *Bam*HI produced linear, genome length molecules consisting of the two phage 'arms' joined back-to-back. The vector DNA was then put into a ligation mixture at a relatively low concentration ($10 \text{ ng } \mu\text{l}^{-1}$) to favour the production of circular molecules rather than concatenates (Maniatis *et al.*, 1978). The target DNA used was chromosomal DNA prepared from *B. subtilis* strain 168 cleaved with *Bcl*I or *Bgl*II, both of which produce cohesive ends that are complementary to those produced by *Bam*HI. Generally target DNA was added to vector DNA in the ligation mixture to give a three-fold molar excess of target ends. These optimal conditions for ligation were determined empirically by testing the efficiency of cloning of the isolated 2 kbp *Bam*HI fragment from pSG1 which contains the intact Cm^R gene (data not shown). The ligation mixture containing approximately 500 ng vector DNA was used to transfect protoplasts made from strain 168 (non-lysogenic) and this usually gave rise to a total of approximately 10000 progeny p.f.u. After overnight incubation in an overlay plate containing indicator bacteria (*B. subtilis* strain 168) the amplified pool of recombinant phages was recovered from each overlay and contained about 10^7 p.f.u. ml^{-1} . In the control experiments described above about 30% of the plaques that appeared following transfection contained phage that transduced to Cm^R .

Fig. 5. Use of the phage vector, $\phi 105J9$, for the construction of genomic libraries in *B. subtilis*. The letter B over an arrow indicates a cleavage site for *Bam*HI; cos indicates the cohesive termini of $\phi 105$ DNA (Scher *et al.*, 1977).

Strains carrying the following 15 biosynthetic or sporulation mutations were then tested for complementation by each of the recombinant phage pools: *argA2*, *aroD120*, *hisH2*, *ilvA1*, *ilvB2*, *leuB16*, *lys-1*, *pheA2*, *spoIIA69*, *spoIIC298*, *spoIIE20*, *spoIIIA59*, *spoIIIB601*, *spoIVA67* and *spoVA574*. Positive results were obtained with four different mutations (about 100 to 200 *Aux*⁺ or *Spo*⁺ colonies) and in each case a high frequency transducing phage was subsequently isolated. DNA was prepared from each of the phages and digested with *Eco*RI (Fig. 6). As expected, only the 7.6 kbp *Eco*RI fragment containing the unique *Bam*HI site in $\phi 105J9$ was affected by the insertion of chromosomal DNA. Details of the recombinant phages isolated are shown in Table 2. All of the new phages formed plaques on a sensitive host and no instability of the cloned gene was observed.

In addition to these new phages, a fifth recombinant phage has been isolated which carries a *Bcl*I fragment that complements mutations in the *gerE* locus (W. S. James, personal communication). $\phi 105J9$ has also been used to sub-clone the *spoIID* locus from an unstable

Fig. 6. Agarose gel electrophoresis (0.7%) of DNA from $\phi 105$ and related phages following digestion with *Eco*RI: lanes 1 and 9, molecular weight marker fragments (λ , *Hind*III) with sizes in kbp; lane 2, $\phi 105$ wild-type; lane 3, $\phi 105D1$: 1t; lane 4, $\phi 105J9$; lane 5, $\phi 105J11$; lane 6, $\phi 105J12$; lane 7, $\phi 105J13$. In tracks 3 and 4 the band running at approximately 7.6 kbp is composed of two *Eco*RI fragments: fragment D and the deleted form of fragment B containing the *Bam*HI site (Scher *et al.*, 1978; Bugaichuk *et al.*, 1984). In tracks 5, 6, 7 and 8 only one fragment of 7.6 kbp (D) is present.

Table 2. Characteristics of recombinant transducing phages derived from $\phi 105J9$

Recombinant phages were isolated on the basis of transducing activity for various sporulation and auxotrophic mutations. The approximate size of the insert in each phage and the number of *Eco*RI sites were determined using data from Fig. 6.

Recombinant phage	Enzyme used to cleave chromosomal DNA	Approx. size of fragment inserted (kbp)	No. of <i>Eco</i> RI sites in insert	Mutation complemented
$\phi 105J11$	<i>Bcl</i> I	2.6	1	<i>spoII</i> C298
$\phi 105J12$	<i>Bgl</i> II	2.4	2	<i>spoII</i> A69
$\phi 105J13$	<i>Bcl</i> I	4.1	1	<i>lys-1</i>
$\phi 105J15$	<i>Bgl</i> II	3.3	0	<i>spoVA</i> 574

primary $\phi 105$ clone, which was obtained using the prophage transformation technique (I. Lopez-Diaz, personal communication). The cloning system has also been used to construct genomic libraries of *B. licheniformis* and *B. pumilis* DNA from which several DNA fragments that complement *B. subtilis* *spo* mutations have been obtained (S. Turner & A. J. Smith, unpublished results).

DISCUSSION

The system described in this paper has several advantages over the alternatives currently available for cloning genes in *B. subtilis*. The method is quick, requires relatively small amounts of DNA and affords a convenient direct selection for clones carrying fully functional transcriptional units. Moreover, the efficiency of the method, which uses *B. subtilis* as host, compares favourably with analogous methods that use *E. coli* as host. For example, the frequency of transfection with $\phi 105$ DNA in protoplasts (up to 10^6 p.f.u. per μg DNA) is at least as great as that obtained using competent cells of *E. coli* and λ DNA or with phage M13 DNA (10^5 to 10^6 p.f.u. per μg DNA; Zinder & Boeke, 1982). However, the system is not yet as efficient as *in vitro* packaging of λ DNA (10^7 to 10^8 p.f.u. per μg DNA; Sternberg *et al.*, 1977).

The usefulness of the *Bam*H1 cloning site in $\phi 105J9$ is well demonstrated by the results in Table 2. The reasons why complementing phages were obtained for only four of the 15 mutations tested was probably due to the location of cleavage sites for the endonucleases *Bgl*II and *Bcl*I. Clearly the presence of a site within a gene or between a gene and its promoter would prevent complementation from occurring. In addition, the distance between the cleavage sites flanking a gene is important since $\phi 105J9$ is unlikely to be able to accommodate DNA inserts much larger than 4 kbp because of packaging restrictions. The latter factor is particularly important when *Bgl*II is used, since this enzyme cleaves *B. subtilis* DNA relatively infrequently. It should, however, be possible to construct a fully representative *B. subtilis* genomic library by making use of the enzyme *Mbo*I, which also produces cohesive ends that are compatible with *Bam*H1 (Roberts, 1982). This enzyme has a tetranucleotide recognition sequence and therefore cleaves chromosomal DNA much more frequently than *Bcl*I or *Bgl*II. Partial digestion with *Mbo*I followed by isolation of fragments of 3 to 4 kbp would generate essentially random fragments of DNA for cloning, among which intact copies of most loci would be represented. Alternatively, essentially random chromosomal DNA fragments could be prepared for cloning by mechanical shearing (Maniatis *et al.*, 1978) followed by the addition of *Bam*H1 linker molecules; *Bam*H1 cleaves *B. subtilis* chromosomal DNA very infrequently (unpublished results). The *Xba*I and *Sal*I sites that have been introduced into $\phi 105J9$ are also useful for cloning or subcloning, although there would be some loss of efficiency using the latter enzyme, since there is a second *Sal*I site in the vector. It could therefore be useful to attempt to remove the second *Sal*I site by mutagenesis, as has been done in other cloning vectors, for example in the construction of the pUC plasmids (Vieira & Messing, 1982). The addition of further unique cloning sites would be more difficult since many of the commonly used enzymes, such as *Eco*RI, *Pst*I and *Hind*III, cut wild type $\phi 105$ at several sites (8, 15 and 15 respectively; Scher *et al.*, 1978; Bugaichuk *et al.*, 1984). However, there are several other potentially useful restriction endonucleases that recognise hexanucleotide sequences, cut at relatively few sites in $\phi 105$, and produce 4 bp cohesive ends (for example, *Kpn*I, *Nco*I, and *Sst*I; Bugaichuk *et al.*, 1984).

It should be possible to extend the cloning system for use with DNA from other organisms. Cloning of *Escherichia coli* DNA in *B. subtilis* would be useful in certain circumstances, since the lack of homology between *E. coli* and *B. subtilis* chromosomal DNA would enable plaque hybridization to be used to screen for specific *E. coli* inserts in the absence of background hybridization. *B. subtilis* might also be a more useful background than *E. coli* in which to clone and study genes from other Gram-positive organisms. It might be possible to extend the use of the system to the creation of genomic libraries of some higher organisms, although it would be necessary to increase the packaging potential of the vector for inserts larger than 4 kbp. Presumably DNA from the immunity region of $\phi 105$ could be deleted to give clear plaque vectors similar to those of λ (Blattner *et al.*, 1977). Deletions of this kind covering a 6 kbp region

are available (Flock, 1977) and it is unlikely that this represents the limit of a non-essential DNA that can be deleted from $\phi 105$. The plasmid pSG1 is clearly a useful probe for the identification of non-essential regions in the $\phi 105$ genome, and in this report two new regions of this type have been found. It is hoped to identify other non-essential regions by ligating phage and plasmid DNA's after cleavage with other combinations of restriction enzymes. Nevertheless, without any further modifications the vector, $\phi 105J9$, has been used successfully to clone an auxotrophic gene and several sporulation genes. This represents a significant advance in cloning technology using *B. subtilis* as host.

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