Construction of Improved Bacteriophage ϕ 105 Vectors for Cloning by Transfection in *Bacillus subtilis*

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A series of improved phage vectors have been constructed, based on *Bacillus subtilis* bacteriophage ϕ 105, which can be used to clone genes in *B. subtilis* by direct transfection of protoplasts. The new vectors, designated ϕ 105J23, ϕ 105J24, ϕ 105J27 and ϕ 105J28, show frequencies of plaque formation that are equal to those of wild-type ϕ 105. This represents at least a 10-fold improvement over ϕ 105J9, the vector used in previous cloning experiments. Two of the new vectors ϕ 105J27 and ϕ 105J28 incorporate a mutation, *cts-52*, that renders the prophage temperature inducible. This has made it possible to devise a rapid small-scale procedure for screening progeny phage for the presence of inserted DNA. The usefulness of the new vectors is illustrated in the accompanying paper by cloning more than 20 *B. subtilis* sporulation genes.

INTRODUCTION

A variety of approaches have been used to clone genes in *Bacillus subtilis* (reviewed by Errington, 1987). Although the use of plasmid vectors has been successful in some instances (Segall & Losick, 1977; Bonamy & Szulmajster, 1982) serious limitations have been encountered with many of the standard plasmid vectors. The increase in gene copy number which results when chromosomal genes are cloned into multicopy plasmids may result in deleterious effects on the host cell (Kawamura *et al.*, 1981; Banner *et al.*, 1983). Difficulties have also been encountered when attempting to clone fragments of DNA larger than about 2.5 kbp in certain plasmid vectors (Gryczan & Dubnau, 1982). Plasmids containing inserts often exhibit structural or segregational instability (see, for example, Bron & Luxen, 1985), even in recipients which are Rec⁻ (Tanaka, 1979; Uhlen *et al.*, 1981; Hahn & Dubnau, 1985).

The use of temperate bacteriophages such as ϕ 105 and ρ 11 as cloning vectors provides a means of circumventing many of these difficulties. These phages integrate into the chromosome of *B. subtilis* in a manner analogous to that of phage λ in *Escherichia coli* (Rutberg, 1969) so that genes inserted into the prophage are stably maintained at a copy number of one per chromosome, and this is true even in Rec⁺ strains (Jenkinson & Mandelstam, 1983; Errington, 1984). Initially, prophage transformation (Kawamura *et al.*, 1979) was used to clone genes in *B. subtilis* bacteriophage vectors. This method relies on the rescue of transformed fragments of phage and chromosomal DNA by recombination with the homologous prophage in the host cell chromosome. Following recombination, the chromosomal DNA may be incorporated into the resident prophage either by replacement or by insertion, which often results in a defective phage (Kawamura *et al.*, 1979). Unfortunately, the vast majority of transformed cells have undergone recombination at the chromosomal locus of the gene rather than insertion of the chromosomal gene into the prophage. Furthermore, relatively large amounts of DNA are required, because lysogenic recipients are about two orders of magnitude less competent in transformation than

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non-lysogenic strains (Peterson & Rutberg, 1969; Yasbin *et al.*, 1973; Garro & Law, 1974) and because extensive processing of the transforming DNA occurs during uptake (Venema, 1979). Nonetheless, prophage transformation has been successfully used to clone a number of biosynthetic and sporulation genes (Kawamura *et al.*, 1979, 1980; Iijima *et al.*, 1980; Ikeuchi *et al.*, 1983; Jenkinson & Mandelstam, 1983; Savva & Mandelstam, 1984).

Errington (1984) has described a more efficient cloning method based on direct transfection of *B. subtilis* protoplasts with a new vector, $\phi 105J9$. This vector was derived from phage $\phi 105DI:1t$, which carries a 4 kbp deletion of non-essential DNA (Flock, 1977), by insertion of unique cloning sites for *Bam*HI and *Xba*I. A number of fully functioning sporulation and biosynthetic genes were isolated from *B. subtilis* genomic libraries constructed with the $\phi 105J9$ vector. However, the construction of comprehensive genomic libraries in $\phi 105J9$ has proved to be difficult as the number of plaque-forming units obtained with this phage is between 10 and 100 times lower than that obtained with the wild-type phage.

This paper describes the construction of a series of modified phage vectors, also derived from $\phi 105DI:1t$, in which the cloning sites are inserted at a different position within the phage genome. The new vectors plaque as efficiently as the wild-type. The vector was further developed to incorporate a temperature-inducible lesion, which allows the induction of the lysogenic phage by shifting the growth temperature. An accompanying paper describes the application of one of the new vectors, $\phi 105J27$, for efficient cloning of *B. subtilis* sporulation genes (Errington & Jones, 1987).

METHODS

Bacterial strains, plasmids and phages. These are listed in Table 1. Phages $\phi 105J78$, $\phi 105J83$, $\phi 105J86$, $\phi 105J92$ and $\phi 105J95$ are described in the accompanying paper (Errington & Jones, 1987).

Phage $\phi 105$ preparation and assay. Crude lysates and phage purified by treatment on CsCl gradients were prepared as described by Jenkinson & Mandelstam (1983) except that DNAase I treatment was omitted. Lysogenic strains containing temperature-inducible phages were grown at 30 °C and induced by exposure to heat (10 min at 48 °C followed by 1 to 2 h at 37 °C). The quality of extracted DNA from temperature-inducible phages was improved by treatment of the resuspended phage pellet with DNAase I (Sigma, type IV, 25 $\mu\text{g ml}^{-1}$) and RNAase (Sigma, type I-A, 25 $\mu\text{g ml}^{-1}$) for 15 min at 22 °C prior to phage purification on a CsCl step gradient (Jenkinson & Mandelstam, 1983). Phages were titrated by standard phage assay methods in tryptose blood agar base (Oxoid) made up at one-third the recommended concentration, overlaid on Oxoid nutrient agar.

Preparation of phage, plasmid and chromosomal DNAs. Phage DNA was prepared from phage purified on CsCl gradients by extraction with phenol as described previously (Errington, 1984). The plasmid pSGMU1 was prepared from its *E. coli* host on a CsCl gradient 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). Chromosomal DNA from *B. subtilis* 168 was prepared as described previously (Errington, 1984).

Transformation methods. Strains of *B. subtilis* were made competent by the method of Anagnostopoulos & Spizizen (1961) as modified by Jenkinson (1983). Protoplasts of *B. subtilis* were prepared and transformed with phage DNA by the method of Chang & Cohen (1979) as modified by Levi-Meyrueis *et al.* (1980). Protoplast transfections with DNA from phage $\phi 105$ were carried out as described previously (Errington, 1984).

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 a ligation buffer (Errington, 1984) containing T4 DNA ligase (Boehringer) at a final concentration of 0.02 units μl^{-1} for DNA with sticky ends and 0.1 units μl^{-1} for blunt ends.

Cloning in *E. coli*. Transformation of competent *E. coli* strains and use of the plasmid vectors pUC18 and pSGMU2 are described elsewhere (Fort & Piggot, 1984; Fort & Errington, 1985).

Cloning fragments from plasmid pSGMU1 into $\phi 105DI:1t$. Plasmid pSGMU1 DNA (4 μg) was digested to completion with *Eco*RI and *Hind*III (6 units, 2 h at 37 °C). The resulting 5' protruding single-stranded ends of the restriction fragments were then filled in to give blunt ends in a 20 μl reaction volume containing all four deoxyribonucleoside triphosphates (15 μM each), Tris/HCl (10 mM, pH 7.5), MgCl_2 (10 mM) and Klenow polymerase (Amersham, 8 units). After 10 min at 22 °C the reaction was terminated by phenol extraction and ethanol precipitation. Phage $\phi 105DI:1t$ DNA (3 μg) was digested to completion with *Sma*I (8 units, 2 h at 37 °C). Following removal of the restriction endonucleases, a 20 μl ligation mixture was prepared containing 30 μg pSGMU1 and 10 μg $\phi 105DI:1t$ digested as indicated above. The reaction mixture was incubated for 18 h at 15 °C and 2 μl and 5 μl samples were used to transform competent cells of *B. subtilis* strain CU267($\phi 105DI:1t$). After 30 min at 37 °C the cells were plated on nutrient agar containing chloramphenicol (5 $\mu\text{g ml}^{-1}$).

Table 1. *Bacterial strains, bacteriophages and plasmids*

<i>B. subtilis</i> strains	Relevant characteristic/origin	Reference/origin
168	<i>trpC2</i>	Laboratory stock
CU267	<i>trpC2 ilvB2 leuB16</i>	S. A. Zahler*
Bacteriophages		
$\phi 105$	Wild-type	Rutberg (1969)
$\phi 105\text{MU1}$	Temperature-inducible (<i>cts-52</i>) derivative of wild-type $\phi 105$	H. F. Jenkinson & D. J. Tipper† (unpublished results)
$\phi 105\text{DI:lt}$	4 kbp deletion of non-essential DNA from wild type $\phi 105$	Flock (1977)
$\phi 105\text{J9}$	Cloning vector	Errington (1984)
$\phi 105\text{J21}$	Insertion of 2 kbp fragment of DNA carrying <i>cat</i> ⁺ gene in $\phi 105\text{DI:lt}$ BamHI cleavage and religation of $\phi 105\text{J21}$ and $\phi 105\text{J22}$, respectively (<i>cat</i> ⁻) <i>cts-52</i> derivatives of $\phi 105\text{J23}$ and $\phi 105\text{J24}$, respectively	This paper
$\phi 105\text{J22}$		
$\phi 105\text{J23}$		
$\phi 105\text{J24}$		
$\phi 105\text{J27}$		
$\phi 105\text{J28}$		
Plasmids		
pUC18	<i>bla</i> ⁺ , cloning vector	Norranders <i>et al.</i> (1983)
pSGMU1	<i>bla</i> ⁺ , <i>cat</i> ⁺	Errington (1984)
pSGMU2	<i>bla</i> ⁺ , <i>cat</i> ⁺	Fort & Errington (1985)
pSGMU33	3.2 kb <i>EcoRI</i> fragment from $\phi 105\text{MU1}$ cloned in pUC18	This paper
pSGMU35	1.1 kbp <i>EcoRI</i> <i>HindIII</i> fragment from pSGMU33 cloned in pSGMU36	This paper

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Derivation of phages $\phi 105\text{J23}$ and $\phi 105\text{J24}$. DNA extracted from $\phi 105\text{J21}$ or $\phi 105\text{J22}$ (1 μg) was digested to completion with *Bam*HI, religated in a 50 μl reaction volume (18 h at 15 °C) and used to transfect protoplasts of strain CU267 (non-lysogenic). Lysogenic cells from the centres of the resulting plaques were tested for loss of chloramphenicol resistance by streaking on nutrient agar (Oxoid) containing chloramphenicol (5 $\mu\text{g ml}^{-1}$).

Rapid small-scale procedure for isolating phage DNA. Small-scale phage lysates (5 ml) were prepared by temperature induction of lysogens as described above except that 30 min after the shift in temperature, DNAase (1 μl of a stock solution, 1 mg ml^{-1} , stored in 150 mM-NaCl, 10% (v/v) glycerol at -20 °C) and RNAase (5 μl of a stock solution, 10 mg ml^{-1} , stored in 50 mM-Tris/HCl, 5 mM-EDTA, 100 mM-NaCl at -20 °C) were added to digest nucleic acids released from the host upon lysis. A 1 ml sample of lysate was centrifuged (Beckman microfuge, 5 min) to remove cell debris and unlysed cells and to the clear supernatant was added 500 μl of a solution containing polyethylene glycol (30%, w/v) and NaCl (1.2 M). After at least 1 h on ice, the precipitated phage was recovered by centrifugation (Beckman microfuge, 2 min) taking care to remove as much of the supernatant as possible. The phage pellet was resuspended in 50 μl of a solution containing: Tris/HCl (10 mM, pH 7.5); cyclohexanediaminetetraacetic acid (CDTA, 5 mM); 2-mercaptoethanol (15 mM) and sodium dodecyl sulphate (0.25% w/v). The mixture was vortex mixed briefly, heated at 70 °C for 10 min then cooled to 22 °C. Potassium acetate (12.5 μl of a 5 M solution) was added and the tube was placed on ice for 30 min. Insoluble material was removed by centrifugation (Beckman microfuge, 5 min) and the phage DNA in the supernatant was precipitated by adding 2 vols ethanol. The precipitate was recovered by centrifugation (as above) and redissolved in 50 μl TC buffer (10 mM-Tris/HCl, pH 7.5; 1 mM-CDTA) containing 2-mercaptoethanol (15 mM). The DNA was extracted with 1 vol. phenol, then reprecipitated with ethanol in the presence of 0.3 M-sodium acetate. The precipitate was recovered by centrifugation, washed with 80% (v/v) ethanol then dried *in vacuo* and redissolved in 10 μl TC buffer. This procedure routinely yielded about 2 μg of phage DNA, enough for two or three restriction digestions.

RESULTS AND DISCUSSION

Construction of modified $\phi 105$ cloning vectors

Although the bacteriophage cloning vector $\phi 105\text{J9}$ has been used successfully to clone a number of sporulation genes (Errington, 1984; Lopez-Diaz *et al.*, 1986; James & Mandelstam,

Fig. 1. Restriction maps of phage $\phi 105DI:1t$ and plasmid pSGMU1. *EcoRI* and *SmaI* restriction sites and the one-letter designations and sizes (kbp) of the resultant fragments are shown for the phage (Bugaichuk *et al.*, 1984). The thick arrow indicates the location of a 4 kbp deletion of non-essential DNA in this phage (Flock, 1977).

Plasmid pSGMU1 (Errington, 1984), shown on an expanded scale, contains a chloramphenicol-resistance determinant, *car⁺*, as a 2.0 kbp *Bam*HI fragment of DNA inserted into the polylinker region of plasmid pUC13. The origin of replication (*ori*) and ampicillin-resistance gene (*bla⁺*) of the vector are also shown.

1985; Turner & Mandelstam, 1986; A. J. Smith & J. Mandelstam, unpublished results) it suffers from the disadvantage that the number of plaque-forming units produced after induction of the phage is significantly lower than that produced by the wild-type phage. In an effort to obtain a modified vector able to plaque at the same frequency as the wild-type phage, a procedure similar to that used to construct the vector $\phi 105J9$ (Errington, 1984) was used to insert a cloning site into an alternative position within the genome of $\phi 105DI:1t$ (Fig. 1). Plasmid pSGMU1 was digested with *Hind*III and *Eco*RI to produce two fragments (see Fig. 1). One of these fragments contained the *car⁺* gene, flanking *Bam*HI sites and polylinker regions on a molecule of 2 kbp with one *Hind*III and one *Eco*RI cohesive end. The cohesive ends were filled in to produce blunt ends and the fragments were then blunt-end ligated to fragments of $\phi 105DI:1t$ digested with *Sma*I, which restricts $\phi 105DI:1t$ DNA at six sites (Bugaichuk *et al.*, 1984). Transformation of strain CU267($\phi 105DI:1t$) enabled the fragment containing the *car⁺* gene flanked by the appropriate phage fragments to be rescued onto the resident prophage genome by crossing over. The first transformation experiment produced 150 colonies; these were divided into 15 pools, each containing 10 colonies, which, after induction of the phage, were screened for plaque formation and the ability to transduce chloramphenicol resistance. Lysates produced from pooled chloramphenicol-resistant colonies were filtered and diluted 10^6 -fold. Ten of the pooled lysates produced plaques at a dilution of 10^{-6} and eight of these pools produced phages that transduced sensitive host strains to chloramphenicol resistance at the same dilution. Fifty plaques from each successful pool were tested and all contained chloramphenicol-resistant lysogenic cells.

DNA was prepared from phages isolated from the eight different pools and restriction maps were constructed following digestion with *Eco*RI or *Sma*I (Fig. 2). The *Eco*RI digestions of all eight phages showed that one of the co-migrating fragments of 7.6 kbp (fragments D and B'; Fig. 1) had been replaced by a larger fragment of about 9.6 kbp. None of the other *Eco*RI fragments was affected.

Fig. 2. Agarose gel electrophoresis (0.7%, w/v) of DNA from phages $\phi 105DI:1t$ (tracks 2 and 5), $\phi 105J21$ (tracks 3 and 6) and $\phi 105J22$ (tracks 4 and 7) digested with *EcoRI* (tracks 2 to 4) or *SmaI* (tracks 5 to 7). Lane 1 contains *M*, markers (phage λ DNA digested with *HindIII*) with their sizes (kbp) indicated at the left.

Two types of pattern were observed in the *SmaI* digestions. One type of phage (designated $\phi 105J21$) appeared to have lost one of the co-migrating fragments of about 4.6 kbp (fragments E and C'), and a new fragment of about 6.6 kbp was present. There were two possible locations for the *cat*⁺-encoding insertion in this phage type: either between *SmaI* fragments D and E or between fragments F and C'. *HindIII* digestions demonstrated that the former alternative was correct (data not shown). The other type of phage (designated $\phi 105J22$) had lost the 6.2 kbp fragment (D) and a new fragment of about 8.2 kbp was present. The only possible location for this insertion was also between *SmaI* fragments D and E. The differences between the restriction patterns of the two phage types are due to the orientation of the 2.0 kbp insertion, which has an asymmetric *SmaI* site.

The 2.0 kbp *BamHI* fragment of DNA carrying the *cat* gene was removed from phages $\phi 105J21$ and $\phi 105J22$ by cleavage with *BamHI* followed by religation and transfection of protoplasts of strain CU267 (non-lysogenic). This procedure, like that described for the construction of $\phi 105J9$ (Errington, 1984), leaves behind in the prophage the polylinker restriction sites that flanked the *cat*⁺ gene insert. Of the 60 plaques tested from each ligation about 20% contained chloramphenicol-resistant lysogenic bacteria. The remainder of the phages were presumably generated by re-circularization of the phage DNA without the insert containing the *cat* gene.

Fig. 3. Detailed restriction map of the new phage vectors $\phi 105J23$ and $\phi 105J24$. Some minor adjustments to the map described by Bugaichuk *et al.* (1984) have been made in view of more recent data (results not shown). The arrow indicates the approximate location of a 4 kbp deletion of non-essential DNA in these phages, which are derived from phage $\phi 105DI:1t$ (Flock, 1977). The order of restriction sites in the polylinker regions inserted into phage $\phi 105J23$ and $\phi 105J24$ is shown on an expanded scale, *Bam*HI (B), *Eco*RI (E), *Hind*III (H), *Kpn*I (K), *Pst*I (P), *Sal*I (Sa), *Sma*I (Sm), *Sst*I (Ss), *Xba*I (X). Phages $\phi 105J27$ and $\phi 105J28$ have the same restriction patterns as those of $\phi 105J23$ and $\phi 105J24$, respectively.

DNA was then prepared from the two chloramphenicol-sensitive phage types. Single and double digestions with *Bam*HI and *Hind*III confirmed the structures of the new phages $\phi 105J23$ and $\phi 105J24$ (not shown). Both of these phages had lost the 2 kbp insert and had a single *Bam*HI site. The unique *Bam*HI and *Xba*I sites, and the second *Sal*I site, available for cloning in $\phi 105J23$ and $\phi 105J24$, are similar to those present in the $\phi 105J9$ vector constructed previously (Errington, 1984). However the frequencies of plaque formation by the phages $\phi 105J21$, J22, J23 and J24 were significantly higher than those obtained with $\phi 105J9$. In three independent assays, the new $\phi 105$ vectors were observed to plaque at frequencies similar to those obtained with the $\phi 105$ wild-type and $\phi 105DI:1t$ phages (1 to 5×10^9 p.f.u. ml⁻¹). This was between 10- and 100-fold higher than the frequency obtained with $\phi 105J9$ (between 5×10^7 and 2×10^8 p.f.u. ml⁻¹, data not shown). A similar improvement over $\phi 105J9$ was observed in the frequencies of transfection of protoplasts in terms of progeny phage per μ g transfecting DNA. Use of these new vectors should therefore enhance the efficiency of the cloning system by about an order of magnitude.

Detailed restriction maps of the new vectors are shown in Fig. 3. The new vectors also include two extra restriction sites, *Sst*I and *Sma*I, that were not incorporated into $\phi 105J9$. Although $\phi 105$ has additional sites for these enzymes, their inclusion facilitates the complete removal of

Fig. 4. Transfer of the *cts-52* allele onto the $\phi 105$ prophage by integration and excision of plasmid pSGMU35. This plasmid contains a 1.1 kbp *Hind*III–*Eco*RI fragment of DNA from $\phi 105$ *cts-52* that includes the *cts-52* mutation. It also carries a chloramphenicol-resistance determinant, *cat*⁺, and it can replicate autonomously in *E. coli* but not in *B. subtilis* (Fort & Errington, 1985). In a recipient strain lysogenic for phage $\phi 105$, chloramphenicol-resistant transformants can arise by integration of the plasmid via a single-crossover event (Duncan *et al.*, 1978; Haldenwang *et al.*, 1980) involving the cloned sequence in plasmid pSGMU35 (open box) and its homologue on the $\phi 105$ prophage (filled box). After removal of the selective pressure, excision of the inserted plasmid can occur by a reversal of the integration mechanism. The resultant prophage may carry either the *cts-52* allele or its wild-type homologue, depending upon the location of the single crossover (Gutterson & Koshland, 1983).

cloned DNAs from flanking $\phi 105$ sequences, for subsequent subcloning or restriction analysis. The location of this cloning site at a *Sma*I site 6.2 kbp from the left end of the phage defines a previously unknown region of the phage genome that is apparently non-essential for lytic growth or lysogeny.

Temperature inducible derivatives of the new vectors

Armentrout & Rutberg (1971) have described $\phi 105$ mutants that are temperature inducible (*cts*). Lysogens carrying *cts* prophages grow normally at 30 °C but at temperatures above 42 °C lytic phage growth is induced. The location of the *cts* mutations, which presumably identify a phage repressor gene, has been mapped to a 1.1 kbp *Hind*III–*Eco*RI fragment of $\phi 105$ (Dhaese *et al.*, 1984). Incorporation of the *cts-52* allele into the new vectors would facilitate the preparation of phage DNA and the screening of recombinants. Temperature induction of phage has several advantages over induction by mitomycin C. The latter is highly toxic and it also induces the cryptic prophage PBSX (Okamoto *et al.*, 1968), which must be separated from $\phi 105$ phage particles by centrifugation on a CsCl density gradient (Jenkinson & Mandelstam, 1983). Progeny obtained following transfection with *cts* vectors can be screened for the presence of inserts by a convenient small-scale procedure (see below and in Methods).

A marker replacement technique, similar to that described by Gutterson & Koshland (1983), was used to transfer the *cts-52* allele to various phage vectors. The 3.2 kbp *Eco*RI fragment, F, from $\phi 105$ MU1 was cloned into the unique *Eco*RI site of plasmid pUC18 to yield pSGMU33,

Fig. 5. Agarose gel electrophoresis (0.6%, w/v) of *Sst*I, *Sal*I double restriction digestions of phage DNA 'mini-preps'. DNA was prepared from 1 ml samples of crude phage lysates as described in Methods, and doubly-digested with *Sal*I and *Sst*I. To the left are indicated the molecular sizes (kbp) of the marker fragments produced by digestion of phage λ DNA with *Hind*III (track 1). To the right are the sizes of the fragments of DNA common to the ϕ 105 derivatives. The 7.8 kbp fragment that is present in some lanes is the result of annealing of the 6.3 and 1.5 kbp fragments via the phage cohesive ends. The smear at the bottom of the picture represents contaminating fragments of host chromosomal DNA and some RNA. The following phages are shown: ϕ 105J27 (lane 2), ϕ 105J78 (3), ϕ 105J83 (4), ϕ 105J86 (5), ϕ 105J92 (6), ϕ 105J95 (7).

which was isolated as a plasmid containing an insert of the correct size and *Eco*RI, *Hind*III restriction pattern. The 1.1 kbp *Eco*RI–*Hind*III fragment from plasmid pSGMU33 was then subcloned into the integration plasmid pSGMU2 (Fort & Errington, 1985) between its unique *Hind*III and *Eco*RI sites to yield plasmid pSGMU35. Although plasmid pSGMU2 cannot replicate autonomously in *B. subtilis* it can be transformed successfully into *B. subtilis* strains following selection for chloramphenicol resistance if a fragment of DNA has been inserted into it that has homology to the *B. subtilis* chromosome. This has been shown to result from integration of the plasmid by a single-crossover event that leads to duplication of the cloned region of homology on each side of the inserted plasmid as shown in Fig. 4 (Duncan *et al.*, 1978; Haldenwang *et al.*, 1980). Derivatives of strain CU267 lysogenic for phages ϕ 105J23 and ϕ 105J24 were transformed with plasmid pSGMU35 DNA and plated at 30 °C with selection for chloramphenicol resistance. In both cases about 10^3 transformants were obtained. Fifty independent colonies from each cross were all temperature-insensitive, suggesting that integration of the plasmid does not disrupt expression of the phage repressor gene (see Piggot *et al.*, 1984, for example, for a full account of the use of integrational plasmids to analyse transcription units). A single chloramphenicol-resistant colony from each transformation was grown in BHIB under non-selective conditions for about 30 generations to allow segregation of chloramphenicol-sensitive individuals by a reversal of the integration mechanism. It was

expected that a proportion of the chloramphenicol-sensitive segregants would retain the *cts-52* allele, depending upon which side of the marker the single-crossover event had occurred. The cultures were therefore heat-induced and the filter-sterilized supernatant was tested for the production of clear plaques on strain CU267 grown at 42 °C. In both cases about 10^4 clear p.f.u. ml⁻¹ were obtained. Phage from single clear plaques were isolated and replated at 30 °C to enable lysogens to be obtained, and these were chloramphenicol sensitive as expected. The temperature-inducible derivatives of ϕ 105J23 and ϕ 105J24 were designated ϕ 105J27 and ϕ 105J28 respectively. Both derivatives were indistinguishable from their respective parents in terms of restriction enzyme analysis.

Rapid small-scale procedure for screening progeny phage

Incorporation of the *cts-52* allele into the cloning vectors has made it possible to devise a rapid, small-scale procedure to determine whether progeny phage contain inserts (see Methods). To illustrate the results that may be obtained with the new procedure, it was applied to strains containing the vector prophage ϕ 105J27 and to some of the derivatives described in the accompanying paper (Errington & Jones, 1987). Fig. 5 shows an agarose gel of these small-scale DNA preparations that had been doubly digested with *Sst*I and *Sal*I. These enzymes cleave on the left and right sides of the *Bam*HI cloning site in ϕ 105J27 respectively (Fig. 3). These enzymes therefore release the cloned insert and they also generate vector fragments of approximately 17, 6.3, 5.4, 3.2, 2.0 and 1.5 kbp, which provide useful internal *M_r* markers. (The fragment of 7.8 kbp results from annealing of the 6.3 and 1.5 kbp fragments via the phage cohesive ends.) Clearly a comparison of the track containing vector DNA with the others indicates that the new derivatives all contain DNA inserts. This procedure should be particularly useful where no direct selection for the presence of an insert is possible.

The general advantages of cloning in *B. subtilis* with the ϕ 105 transfection system have been discussed fully (Errington, 1984). The improved effectiveness of the new vectors is illustrated by the results presented in the accompanying paper (Errington & Jones, 1987), which describes the use of one of the new vectors, ϕ 105J27, to construct genomic libraries from which DNA from more than 20 different *B. subtilis* sporulation loci have been isolated.

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REFERENCES

- ANAGNOSTOPOULOS, C. & SPIZIZEN, J. (1961). Requirements for transformation in *Bacillus subtilis*. *Journal of Bacteriology* **81**, 741–746.
- ARMENTROUT, R. W. & RUTBERG, L. (1971). Heat induction of prophage ϕ 105 in *Bacillus subtilis*: replication of the bacterial and bacteriophage genomes. *Journal of Virology* **8**, 455–468.
- BANNER, C. D. B., MORAN, C. P., JR & LOSICK, R. (1983). Deletion analysis of a complex promoter for a developmentally regulated gene from *Bacillus subtilis*. *Journal of Molecular Biology* **168**, 351–365.
- BIRNBOIM, H. C. & DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513–1523.
- BONAMY, C. & SZULMAJSTER, J. (1982). Cloning and expression of *Bacillus subtilis* sporulation genes. *Molecular and General Genetics* **188**, 202–210.
- BRON, S. & LUXEN, E. (1985). Segregational instability of pUB110-derived recombinant plasmids in *Bacillus subtilis*. *Plasmid* **14**, 235–244.
- BUGAICHUK, U. D., DEADMAN, M., ERRINGTON, J. & SAVVA, D. (1984). Restriction enzyme analysis of *Bacillus subtilis* bacteriophage ϕ 105 DNA. *Journal of General Microbiology* **130**, 2165–2167.
- CHANG, S. & COHEN, S. N. (1979). High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. *Molecular and General Genetics* **168**, 111–115.
- DHAESE, P., HUSSEY, C. & VAN MONTAGU, M. (1984). Thermo-inducible gene expression in *Bacillus subtilis* using transcriptional regulatory elements from temperate phage ϕ 105. *Gene* **32**, 181–194.
- DUNCAN, C. H., WILSON, G. A. & YOUNG, F. E. (1978). Mechanism of integrating foreign DNA during transformation of *Bacillus subtilis*. *Proceedings of the National Academy of Sciences of the United States of America* **75**, 3664–3668.
- ERRINGTON, J. (1984). Efficient *Bacillus subtilis* cloning system using bacteriophage vector ϕ 105J9. *Journal of General Microbiology* **130**, 2615–2628.

- ERRINGTON, J. (1987). Generalized cloning vectors for *Bacillus subtilis*. In *Vectors: a Survey of Molecular Cloning Vectors and Their Uses*. Edited by D. Denhardt & R. Rodriguez. Boston: Butterworths (in the Press).
- ERRINGTON, J. & JONES, D. (1987). Cloning in *Bacillus subtilis* by transfection with bacteriophage vector $\phi 105J27$: isolation and preliminary characterization of transducing phages for 23 sporulation loci. *Journal of General Microbiology* **133**, 493–502.
- FLOCK, J.-I. (1977). Deletion mutants of temperate *Bacillus subtilis* bacteriophage $\phi 105$. *Molecular and General Genetics* **155**, 241–247.
- FORT, P. & ERRINGTON, J. (1985). Nucleotide sequence and complementation analysis of a polycistronic sporulation operon, *spoVA*, in *Bacillus subtilis*. *Journal of General Microbiology* **131**, 1091–1105.
- FORT, P. & PIGGOT, P. J. (1984). Nucleotide sequence of sporulation locus *spoIIA* in *Bacillus subtilis*. *Journal of General Microbiology* **130**, 2147–2153.
- GARRO, A. J. & LAW, M.-F. (1974). Relationship between lysogeny, spontaneous induction, and transformation efficiencies in *Bacillus subtilis*. *Journal of Bacteriology* **120**, 1256–1259.
- GRYCZAN, T. & DUBNAU, D. (1982). Direct selection of recombinant plasmids in *Bacillus subtilis*. *Gene* **20**, 459–469.
- GUTTERSON, N. I. & KOSHLAND, D. E., JR (1983). Replacement and amplification of bacterial genes with sequences altered *in vitro*. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 4894–4898.
- HAHN, J. & DUBNAU, D. (1985). Analysis of plasmid deletional instability in *Bacillus subtilis*. *Journal of Bacteriology* **162**, 1014–1023.
- HALDENWANG, W. G., BANNER, C. D., OLLINGTON, J. F., LOSICK, R., HOCH, J. A., O'CONNOR, M. B. & SONENSHEIN, A. L. (1980). Mapping a cloned gene under sporulation control by insertion of a drug resistance marker. *Journal of Bacteriology* **142**, 90–98.
- IJIMA, T., KAWAMURA, F., SAITO, H. & IKEDA, Y. (1980). A specialized transducing phage constructed from *Bacillus subtilis* phage $\phi 105$. *Gene* **9**, 115–126.
- IKEUCHI, T., KUDOH, J. & KURAHASHI, K. (1983). Cloning of sporulation genes *spo0A* and *spo0C* of *Bacillus subtilis* onto $\phi 11$ temperate bacteriophage. *Journal of Bacteriology* **154**, 988–991.
- JAMES, W. & MANDELSTAM, J. (1985). Protease production during sporulation of germination mutants of *Bacillus subtilis* and the cloning of a functional *gerE* gene. *Journal of General Microbiology* **131**, 2421–2430.
- 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. & MANDELSTAM, J. (1983). Cloning of the *Bacillus subtilis* *lys* and *spoIIIB* genes in phage $\phi 105$. *Journal of General Microbiology* **129**, 2229–2240.
- KAWAMURA, F., SAITO, H. & IKEDA, Y. (1979). A method for construction of specialized transducing phage $\phi 11$ of *Bacillus subtilis*. *Gene* **5**, 87–91.
- KAWAMURA, F., SAITO, H., HIROCHIKA, H. & KOBAYASHI, Y. (1980). Cloning of sporulation gene, *spo0F*, 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 *spo0* 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. J. Tipper. Washington, DC: American Society for Microbiology.
- LEVI-MEYRUEIS, C., FODOR, K. & SCHAEFFER, P. (1980). Polyethyleneglycol-induced transformation of *Bacillus subtilis* protoplasts by bacterial chromosomal DNA. *Molecular and General Genetics* **179**, 589–594.
- LOPEZ-DIAZ, I., CLARKE, S. & MANDELSTAM, J. (1986). *spoIID* operon of *Bacillus subtilis*: cloning and sequencing. *Journal of General Microbiology* **132**, 341–354.
- LOVETT, P. S. & KEGGINS, K. M. (1979). *Bacillus subtilis* as a host for molecular cloning. *Methods in Enzymology* **68**, 342–357.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory.
- NORRANDER, J., KEMPE, T. & MESSING, J. (1983). Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. *Gene* **26**, 101–106.
- OKAMOTO, J., MUDD, J. A. & MARMUR, J. (1968). Conversion of *Bacillus subtilis* DNA to phage DNA following mitomycin C induction. *Journal of Molecular Biology* **34**, 429–437.
- PETERSON, A. M. & RUTBERG, L. (1969). Linked transformation of bacterial and prophage markers in *Bacillus subtilis* 168 lysogenic for bacteriophage $\phi 105$. *Journal of Bacteriology* **98**, 874–877.
- PIGGOT, P. J., CURTIS, C. A. M. & LENCASTRE, H. DE (1984). Use of integrational plasmid vectors to demonstrate the polycistronic nature of a transcription unit (*spoIIA*) required for sporulation of *Bacillus subtilis*. *Journal of General Microbiology* **130**, 2123–2136.
- RUTBERG, L. (1969). Mapping of a temperate bacteriophage active on *Bacillus subtilis*. *Journal of Virology* **3**, 38–44.
- SAVVA, D. & MANDELSTAM, J. (1984). Cloning of the *Bacillus subtilis* *spoIIA* and *spoVA* genes in phage $\phi 105DI$:lt. *Journal of General Microbiology* **130**, 2137–2145.
- SEGALL, J. & LOSICK, R. (1977). Cloned *Bacillus subtilis* DNA containing a gene that is activated early during sporulation. *Cell* **11**, 751–761.
- TANAKA, T. (1979). *recE*-independent recombination between homologous deoxyribonucleic acid segments of *Bacillus subtilis* plasmids. *Journal of Bacteriology* **139**, 775–782.
- TURNER, S. M. & MANDELSTAM, J. (1986). Cloning and sequencing of a gene from *Bacillus amyloliquefaciens* that complements the sporulation gene *spoIID* in *Bacillus subtilis*. *Journal of General Microbiology* **132**, 3025–3035.
- UHLEN, M., FLOCK, J.-I. & PHILLIPSON, L. (1981). *RecE* independent deletions of recombinant plasmids in *Bacillus subtilis*. *Plasmid* **5**, 161–169.
- VENEMA, G. (1979). Bacterial transformation. *Advances in Microbial Physiology* **19**, 245–331.
- YASBIN, R. E., WILSON, G. A. & YOUNG, F. E. (1973). Transformation and transfection in lysogenic strains of *Bacillus subtilis* 168. *Journal of Bacteriology* **113**, 540–548.