Construction and Characterization of Recombinant Phage $\phi 105$ d(Cm^rmet) for Cloning in *Bacillus subtilis*

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(Received 14 December 1983; revised 9 March 1984)

A 1.6 kb fragment of DNA of plasmid pBD64, obtained after partial digestion with *Hpa*II, carrying a chloramphenicol-resistance determinant and a single site for the enzyme *Bgl*II, was inserted into the genome of defective phage $\phi 105$ dlys. Two types of phage were subsequently isolated and both transduced cells of *Bacillus subtilis* to chloramphenicol resistance. One type contained 26 kb and the other 32 kb of DNA. *Bacillus subtilis* chromosomal DNA fragments generated by cleavage with *Bgl*II were ligated into the unique *Bgl*II site within the smaller phage genome. A specialized transducing phage was isolated which carried the *metC* gene on a 6 kb *Bgl*II fragment. This phage, denoted $\phi 105$ d(Cm^rmet), transduced *B. subtilis* strain MB79 *pheA12 metC3* to Met⁺ and to chloramphenicol resistance, and the *metC3* mutation was complemented in transductants.

INTRODUCTION

Vegetative and sporulation-specific genes of *Bacillus subtilis* have been cloned in the temperature phages $\rho 11$ (Kawamura *et al.*, 1979; Hirochika *et al.*, 1981; Ikeuchi *et al.*, 1983) and $\phi 105$ (Iijima *et al.*, 1980; Jenkinson & Mandelstam, 1983) using prophage transformation (Kawamura *et al.*, 1979). This technique relies on the insertion, by recombination, of the gene(s) to be cloned into the prophage genome, and then the recovery of the cloned DNA in specialized transducing phages. However, a specialized transducing phage carrying the cloned gene can only be obtained if the transformation event has not inactivated those phage genes that are essential for induction, replication, packaging etc. The insertion of passenger DNA into the phage genome results in deletion of phage genes (Iijima *et al.*, 1980; Jenkinson & Mandelstam, 1983) and may also lead to rearrangement of the phage genome (Ikeuchi *et al.*, 1983). This process of insertion of non-homologous DNA and deletion within the region of non-homology also occurs when a non-replicative plasmid inserts into the *B. subtilis* chromosome at a site of limited homology (Niaudet *et al.*, 1982; Young, 1983).

The prophage transformation method has, therefore, a major disadvantage that arises because the passenger DNA is not cloned at any specific site. Consequently, a transformation event is very likely to delete essential phage genes, so that specialized transducing particles are not formed. To circumvent this problem we have constructed a defective recombinant phage $\phi 105$ which carries a chloramphenicol-resistance determinant, and a single restriction site, for the enzyme *Bgl*II, in a non-essential region. Into this phage we inserted a 6 kb *Bgl*II fragment from the *B. subtilis* chromosome which complements the *metC3* mutation.

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Table 1. *Bacterial strains*

Strain	Genotype	Source/Reference
MB79	<i>metC3 pheA12</i>	Laboratory stock
MY2016	<i>lys-1 hisH2 rpoB2 sul</i>	Laboratory stock
IL11	<i>trpC2</i> ($\phi 105$)	BGSC* original source of $\phi 105$
IE22	<i>trpC2 thr-5</i> (pBD64)	BGSC*
CU267	<i>ilvB2 leuB16 trpC2</i>	S. A. Zahler
SG18	<i>lys-1 hisH2 rpoB2 sul</i> ($\phi 105$ dlys)	Jenkinson & Mandelstam (1983)
SG33	<i>ilvB2 leuB16 trpC2</i> ($\phi 105$)	Lysogen of CU267
SG34	<i>metC3 pheA12</i> ($\phi 105$ dCm')	This paper
SG35	<i>metC3 pheA12</i> ($\phi 105$ d(Cm'met))	This paper

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METHODS

Bacteria. The strains of *B. subtilis* used are listed in Table 1.

Media. *Bacillus subtilis* was grown on Nutrient agar (Oxoid) at 37 °C. Selection for antibiotic resistance was made on Nutrient Agar containing chloramphenicol (Sigma; 7 $\mu\text{g ml}^{-1}$). Selection for prototrophy and antibiotic resistance together was made either on lactate/glutamate minimal agar (Coote, 1972) or on glucose/glutamate minimal agar (lactate replaced by 2 g glucose l^{-1}) containing chloramphenicol (5 $\mu\text{g ml}^{-1}$) and appropriate amino acid supplements (200 $\mu\text{g ml}^{-1}$ each).

Preparation of transducing phage lysates. A strain of *B. subtilis* lysogenic for phage $\phi 105$ was grown at 37 °C in Brain Heart Infusion Broth (10 ml; Oxoid) containing yeast extract (Oxoid; 0.5%, w/v) (BHY). When the culture had reached a density of about 1×10^8 cells ml^{-1} it was treated for 25 min at 37 °C with mitomycin C (Sigma; 0.5 $\mu\text{g ml}^{-1}$). The cells were collected by centrifugation (5000 g, 5 min), suspended to the same density in fresh warm BHY, and incubated with shaking at 37 °C until they had lysed (usually about 3 h). The suspension was then centrifuged (5000 g, 15 min), and the supernatant, after treatment with DNAase I (10 $\mu\text{g ml}^{-1}$), was filtered through a 0.45 μm membrane filter and stored at 4 °C over chloroform (0.1 ml).

Transduction. In this paper the derivatives of phage $\phi 105$ denoted as defective, lacked tails. To use these phages for transduction, tails from wild-type phage lysates were supplied. For transduction, stationary-phage cells of the recipient strain in BHY medium (0.5 ml) were mixed with defective phage $\phi 105$ lysate (0.1 ml) and wild-type helper lysate (0.05 ml, 1.0×10^7 p.f.u.), and incubated for 30 min at 37 °C. To select for chloramphenicol resistance, 0.1 ml volumes of appropriate dilutions were plated onto selective plates; to select for prototrophy, the cells were washed and plated on lactate/glutamate minimal agar as previously described (Jenkinson & Mandelstam, 1983).

Measurement of phage titre. Exponential culture (0.1 ml) of strain CU267 in BHY medium was mixed with phage (0.1 ml), suitably diluted in Brain Heart Infusion Broth. One-third strength Tryptose Blood Agar Base (3 ml) at 50 °C was added, and the mixture was poured onto Tryptose Blood Agar Base plates. Plaques were counted after incubation at 30 °C for 16 h.

Large-scale purification of phage. Cultures (200 ml) of lysogens carrying phage $\phi 105$ and phage PBSX were treated with mitomycin C and, after lysis had occurred the phages were precipitated with polyethylene glycol 6000 (9%, w/v) in the presence of NaCl (0.4 M). The phage particles were banded by centrifugation in a CsCl step gradient (11000 g, 3 h, 17 °C; Jenkinson & Mandelstam, 1983). Phage PBSX was prepared from strain CU267; it packages chromosomal DNA, apparently randomly, into phage heads (Okamoto *et al.*, 1968) and was used as a source of *B. subtilis* DNA for cloning.

Preparation of plasmid. A culture of strain IE22 grown for 16 h at 30 °C in Penassay broth (Difco; 100 ml) containing chloramphenicol (5 $\mu\text{g ml}^{-1}$) was added to 100 ml fresh, warmed Penassay broth and incubated with shaking at 37 °C for 4 h. Cultures were centrifuged (6000 g, 10 min, 4 °C), and the cells washed once with TES buffer (50 mM-Tris/HCl buffer, pH 7.6, containing 5 mM-EDTA and 50 mM-NaCl). Subsequent treatment with lysozyme, RNAase, Pronase and Sarkosyl, and isopycnic centrifugation of the lysate in CsCl containing ethidium bromide were as described by Lovett & Keggins (1979).

Preparation of phage DNA. This was prepared from phage $\phi 105$ derivatives and from phage PBSX as described by Jenkinson & Madelstam (1983).

Restriction enzymes. The restriction conditions employed were those recommended by the manufacturers (BRL). For double digestions, samples were treated first with *Bam*HI or *Bg*II and the buffer components then adjusted for digestion with *Eco*RI. Approximately 1 μg DNA was digested with 2 units of enzyme for 3–5 h at 37 °C. Digestions were terminated by heating at 65 °C for 20 min.

Ligation. Ligation with T4 DNA ligase (BRL) was as described by Jenkinson & Mandelstam (1983).

Agarose gel electrophoresis. DNA fragments were separated using 0.8% (w/v) agarose gels submerged in 40 mM-Tris/acetate buffer, pH 8.1, in a horizontal gel electrophoresis apparatus (Jenkinson & Mandelstam, 1983). Gels were stained with ethidium bromide ($1 \mu\text{g ml}^{-1}$, 15 min), de-stained in water (15 min) and visualized on a UV transilluminator at 304 nm.

Rapid preparation of chromosomal DNA from *B. subtilis*. This was done by the method of Ward & Zahler (1973) as modified by Jenkinson & Mandelstam (1983).

Transformation. Competent cells were prepared and transformed as described previously (Jenkinson, 1983; Jenkinson & Mandelstam, 1983).

Southern transfer and hybridization. DNA (100 ng) from plasmid pBD64 was labelled with [α - ^{32}P]dCTP (3000 Ci mmol $^{-1}$; 111 TBq mmol $^{-1}$; Amersham) to a specific activity of about 10^7 c.p.m. μg^{-1} by nick-translation (Rigby *et al.*, 1977) using a kit provided by Amersham. Southern transfer and hybridization were performed according to Southern (1975).

RESULTS

Insertion of a unique restriction site into phage $\phi 105$

Phage $\phi 105$ *dlys* is a derivative of wild-type phage $\phi 105$ which lacks tails because of a 3 kb deletion of DNA which contains genes for tail synthesis or assembly (Jenkinson & Mandelstam, 1983). We chose to further modify this phage rather than the wild-type phage because the heads of the latter would be less likely to have space for additional DNA.

Phage $\phi 105$ *dlys* DNA has no *Bam*HI or *Bgl*II restriction sites (Jenkinson & Mandelstam, 1983). To insert a unique site into this phage DNA by prophage transformation, it was necessary to obtain a fragment of DNA with the restriction site close to, or within, a gene conferring a selectable phenotype. This was provided in plasmid pBD64 which replicates in *B. subtilis* and which confers resistance to kanamycin, neomycin and chloramphenicol (Gryczan *et al.*, 1980*b*). Cleavage of the plasmid with *Hpa*II generates three DNA fragments of 2.9 kb, 0.9 kb and 0.7 kb (Fig. 1, lane 3). This agrees with the findings of Bingham *et al.* (1982), but differs from those of Gryczan *et al.* (1980*b*), who only observed two fragments after *Hpa*II digestion. The 0.7 kb fragment has a single *Bgl*II site (Gryczan *et al.*, 1980*b*) and carries at least part of the kanamycin resistance gene from plasmid pUB110 (Bingham *et al.*, 1982). The 1.6 kb fragment obtained after partial digestion with *Hpa*II and consisting of the 0.9 kb and 0.7 kb *Hpa*II fragments, carries a functional chloramphenicol resistance gene originally from plasmid pC194 (Grandi *et al.*, 1981).

Thus, to clone the chloramphenicol-resistance gene and the fragment of pBD64 carrying the *Bgl*II site, plasmid DNA (5 μg) was partially digested with *Hpa*II and ligated with a partial *Hpa*II digest of $\phi 105$ *dlys* DNA (5 μg). The ligated mixture (0.1 ml) was added to competent cells (1 ml; 2×10^8 cells) of strain SG18 *lys-1 hisH2* ($\phi 105$ *dlys*), and chloramphenicol-resistant (Cm^r) transformants were selected by plating the entire transformation mixture on to five selective plates. Sixty-seven Cm^r colonies were picked on to nutrient agar containing chloramphenicol and grown for 16 h at 37 °C. The total growth was then pooled and grown further in BHY medium (10 ml). A phage lysate was prepared from the culture by treatment with mitomycin C, a portion (0.3 ml) was mixed with wild-type helper phage lysate and used to transduce the non-lysogenic strain, CU267, to chloramphenicol resistance. Approximately 4.8×10^3 Cm^r transductants ml^{-1} were obtained, and several of these were purified by streaking them on to selective plates.

Since phage $\phi 105$ can transduce plasmid DNA into which fragments of phage $\phi 105$ DNA have been inserted (Marrero & Lovett, 1980), it was necessary to determine whether these transductants contained free plasmid. Cell lysates were prepared from 10 transductants and screened for plasmid DNA by the rapid-screening method of Marrero & Lovett (1980). None of the transductants contained free plasmid (results not shown). In addition, out of 100 Cm^r transductants tested, none was kanamycin-resistant, which showed that they did not contain pBD64. One transductant colony was grown in BHY medium (100 ml), treated with mitomycin C and the phage from the culture supernatant concentrated. When the preparation was subjected to CsCl gradient centrifugation, three phage bands were visible; the uppermost was PBSX (buoyant density 1.375 g ml^{-1}) which is always induced from derivatives of strain 168 (Okamoto *et al.*,

Fig. 1. (a) Restriction endonuclease digestion patterns of DNA from wild-type phage $\phi 105$, $\phi 105$ dCm' and plasmid pBD64. Lane 1, *Hpa*II digest of $\phi 105$ dCm' (upper phage band); lane 2, *Hpa*II, $\phi 105$ dCm' (lower phage band); lane 3, *Hpa*II, plasmid pBD64; lane 4, *Eco*RI, $\phi 105$ (wild-type). (b) Southern hybridization of lanes 1, 2 and 3 above using as probe plasmid pBD64 labelled with [α^{32} -P]dCTP.

1968) and the two others were close together and much lower in the gradient at buoyant densities of about 1.49 g ml^{-1} and 1.50 g ml^{-1} . These upper and lower bands were separately removed through the side of the centrifuge tube using a syringe and needle. A sample ($1 \mu\text{l}$) from each phage band was mixed with wild-type helper phage, incubated with stationary phase cells of strain CU267 and Cm' transductants selected. The phage in each band transduced the recipients to chloroamphenicol resistance, but only when provided with wild-type helper phage. Neither phage band contained plaque-forming activity. Subsequently, we induced phage from several transductants lysogenized with phage from either the upper or the lower phage bands. However, each transductant, after lysis, exhibited two phage bands in CsCl gradients, again with buoyant densities of 1.49 and 1.50 g ml^{-1} . The significance of this was not investigated further.

The *Eco*RI digestion pattern of DNA extracted from the upper band was similar to that of $\phi 105$ dlys DNA (Fig. 2, lane 2) except that the 11.7 kb and 3.8 kb fragments were replaced by a single fragment of 6.3 kb (Fig. 2, compare lanes 2 and 4). The *Eco*RI digestion pattern of DNA extracted from the lower phage band was identical with that from the upper, except that an

Fig. 2. Restriction endonuclease digestion patterns of DNA from wild-type phage $\phi 105$, $\phi 105$ *dlys* and $\phi 105$ *dCm'*. Lane 1, *EcoRI* digest of $\phi 105$ wt; lane 2, *EcoRI*, $\phi 105$ *dlys*; lane 3, *EcoRI*, $\phi 105$ *dCm'*, lower phage band; lane 4, *EcoRI*, $\phi 105$ *dCm'*, upper phage band; lane 5, *Bgl/II*, $\phi 105$ *dCm'*; lane 6, *Bgl/II* plus *EcoRI*, $\phi 105$ *dCm'*. The band at about 16 kb which appears in all *EcoRI*-digested samples to a variable degree is an artefact caused by the annealing of the end fragments C and D (Scher *et al.*, 1977).

additional *EcoRI* fragment of 6 kb was present (Fig. 2, lane 3). Each phage DNA had a single site for the enzyme *Bgl/II* (see later). When added together, the *EcoRI* fragments give a total of 26 kb for the 'upper' phage, and 32 kb for the 'lower' phage. The 'upper' phage thus contains 13.0 kb less DNA than the wild-type phage and 10 kb less than $\phi 105$ *dlys*. Since the 'upper' phage contained less DNA than the 'lower', it would be potentially more useful as a cloning vector because it should be able to accept more passenger DNA. This phage was designated $\phi 105$ *dCm'*.

When the DNA of phage $\phi 105$ *dCm'* was treated with *Bgl/II*, two fragments were generated of 9.3 kb and 16.7 kb (Fig. 2, lane 5). The *Bgl/II* site resided in the 6.3 kb *EcoRI* fragment, since in a double digestion with *EcoRI* and *Bgl/II* this *EcoRI* fragment was cut into two fragments of size 4.1 kb and 2.2 kb (Fig. 2, lane 6). A restriction map of the phage $\phi 105$ *dCm'* is shown in Fig. 4, and included on this map are sites for other enzymes, *KpnI*, *SstI* and *SalI*, deduced from single

and double digestions. When DNA from $\phi 105$ dCm^r was digested with *Hpa*II and compared with *Hpa*II-digested plasmid pBD64 DNA, it was clear that the phage carried a 0.9 kb fragment similar in size to the 0.9 kb fragment from the plasmid (Fig. 1), while the wild-type phage did not (results not shown). It was not possible on these gels to distinguish a 0.7 kb fragment in $\phi 105$ dCm^r equivalent to the pBD64 0.7 kb fragment, but its presence was assumed since this fragment contains the *Bgl*II site (see Fig. 1).

The above observations suggested that $\phi 105$ dCm^r carried the 1.6 kb *Hpa*II fragment from pBD64 and that this plasmid DNA was within the 6.3 kb *Eco*RI fragment. To confirm this, *Hpa*II-digested DNA from $\phi 105$ dCm^r and pBD64 were separated by agarose gel electrophoresis and transferred to nitrocellulose paper. A labelled pBD64 probe showed hybridization with two *Hpa*II fragments in $\phi 105$ dCm^r DNA, and these fragments co-migrated with the 0.9 kb and 0.7 kb *Hpa*II fragments from pBD64 (Fig. 1). The probe also hybridized with the 6.3 kb *Eco*RI fragment but, as expected, it did not hybridize with wild-type $\phi 105$ DNA digested with *Hpa*II or *Eco*RI (results not shown).

Cloning of the metC gene into phage $\phi 105$ dCm^r

To investigate whether the *Bgl*II site in $\phi 105$ dCm^r DNA was a suitable cloning site, we ligated into this site chromosomal fragments of *B. subtilis* DNA and attempted to isolate a transducing phage which would complement the *metC3* mutation in *B. subtilis*. Phage $\phi 105$ dCm^r DNA (5 μ g) was digested with *Bgl*II and ligated with a *Bgl*II-digested sample of phage PBSX DNA (20 μ g). The mixture was then used to transform strain SG34 *metC3 pheA12* ($\phi 105$ dCm^r) to Met⁺. Transformants (126) were picked on to lactate/glutamate minimal agar containing phenylalanine and chloramphenicol, grown at 37 °C for 48 h and then pooled and inoculated into BHY medium (10 ml). A lysate was prepared by mitomycin C treatment and 0.3 ml of this was mixed with helper phage (0.1 ml) and incubated with stationary phase cells of non-lysogenic strain MB79. Transductants were selected on minimal agar containing phenylalanine and chloramphenicol, and Met⁺ Cm^r colonies appeared at a frequency of about 10³ colonies per ml of transduction mixture. These colonies (about 200) were picked, pooled, and a lysate was prepared from them which was used to transduce strain MG79 to Met⁺ and Cm^r simultaneously. One transductant colony, denoted strain SG35, was purified and grown up for a large-scale phage preparation (200 ml culture). After CsCl gradient centrifugation a single phage band was visible at a buoyant density of about 1.5 g ml⁻¹. When a sample (1 μ l) of this was mixed with helper phage, it transduced strain MB79 to Met⁺, and all of 350 Met⁺ transductants tested were also Cm^r. If selection was made for chloramphenicol resistance, all Cm^r transductants were Met⁺. This phage was denoted $\phi 105$ d(Cm^r *met*).

Complementation of the metC3 mutation

To show that Met⁺ transductants of strain MB79 still carried the *metC3* mutation, DNA was prepared from strain SG35 and used at a concentration of 0.5 μ g ml⁻¹ to transform strain MY2016 *lys-1 hisH2* to Lys⁺. Selection was made on lactate/glutamate minimal agar containing histidine and methionine. If the *metC3* mutation was being complemented then up to 5% of the Lys⁺ transformants should be Met⁻, having acquired the *metC3* mutation by congression [Nester *et al.*, 1963]. Of 128 transformants tested, four were Met⁻, confirming that the original *metC3* mutation in the Met⁺ transductants had not been lost by recombination.

*Restriction endonuclease map of $\phi 105$ d(Cm^r *met*) DNA*

When DNA from $\phi 105$ d(Cm^r *met*) was digested with *Bgl*II, three fragments were generated, whereas only two fragments were obtained with $\phi 105$ dCm^r (Fig. 3, lanes 1 and 2). The additional piece of DNA was 6 kb in size and contained the *metC* gene (see below). The 6 kb *Bgl*II fragment contained two *Eco*RI sites (see Fig. 4), and digestion of the phage DNA with *Eco*RI showed three new fragments of 5.2 kb, 4.8 kb and 2.4 kb, replacing the 6.3 kb *Eco*RI fragment of $\phi 105$ dCm^r (compare Fig. 3, lane 3 with Fig. 2, lane 4). The DNA of $\phi 105$ d(Cm^r *met*) was digested with *Sst*I, *Kpn*I and *Sal*I (gel not shown) and a restriction map

Fig. 3. Restriction endonuclease digestion patterns of $\phi 105$ d(Cm^r met) DNA. Lane 1, *Bgl*II digest of $\phi 105$ dCm^r; lane 2, *Bgl*II, $\phi 105$ d(Cm^r met); lane 3, *Eco*RI, $\phi 105$ d(Cm^r met); lane 4, *Bam*HI plus *Eco*RI, $\phi 105$ d(Cm^r met); lane 5, *Bam*HI, $\phi 105$ d(Cm^r met); fragment M (lane 3) and fragment MC (lane 4) carry *metC3*⁺-transforming activity (see text). The size markers refer to the fragments in lane 3. The faint bands at the top of lanes 2, 3 and 4 are junction fragments.

was constructed (Fig. 4). The phage DNA was found to have a unique *Bam*HI site (Fig. 3, lane 5) located in the 4.8 kb *Eco*RI fragment (Fig. 3, lane 4 and Fig. 4).

To locate the *metC3*⁺-transforming activity on the restriction map, the phage DNA was digested with *Eco*RI, *Eco*RI and *Bam*HI, or with *Bam*HI alone, and the digested samples were subjected to agarose gel electrophoresis. The gel was stained briefly to visualize the bands which were cut from the gel, homogenized in transformation medium and mixed with competent cells of strain MB79. Met⁺ transformants were selected on glucose/glutamate minimal agar containing phenylalanine. The results in Table 2 show that only the 4.8 kb *Eco*RI fragment (fragment M in Fig. 3) transformed strain MB79 to Met⁺. When the phage DNA was treated with *Bam*HI as well as *Eco*RI, the 4.8 kb fragment was cleaved into two portions, a 3.0 kb and 1.8 kb fragment (Fig. 3, lane 4). Met⁺-transforming activity was confined to the 3.0 kb fragment (Table 2), and no activity was found in the 1.8 kb portion. When the larger (20.6 kb) and smaller (11.4 kb) *Bam*HI fragments were extracted from a gel and separately mixed with competent

Fig. 4. Restriction endonuclease cleavage maps of DNAs from wild-type phage $\phi 105$, $\phi 105$ *dlys*, $\phi 105$ *dCm^r* and $\phi 105$ *d(Cm^r met)*. The lettering of the fragments of $\phi 105$ wild-type phage DNA follows that originally devised by Perkins *et al.* (1978) whilst the positions of G and I are according to the new restriction map for phage $\phi 105$ (Bugaichuk *et al.*, 1984). Ba, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; K, *Kpn*I; Sa, *Sal*I; Ss, *Sst*I.

Table 2. Localization of *metC3*⁺ transforming activity on $\phi 105$ *d(Cm^r met)* DNA

Restriction fragments were excised from an agarose gel, homogenized in transformation medium by passage through a syringe needle, and mixed with competent cells of strain MB79 *pheA12 metC3*. Met⁺ transformants were selected as described in Methods. Approximately 0.2 µg DNA was digested in each case.

Fragment	No. of Met ⁺ transformants (0.1 ml) ⁻¹
<i>Bam</i> HI 20.4 kb	87
<i>Bam</i> HI 11.6 kb	2
<i>Eco</i> RI 9.0 kb	0
<i>Eco</i> RI 7.6 kb	2
<i>Eco</i> RI 5.3 kb	5
<i>Eco</i> RI 4.8 kb	126
<i>Eco</i> RI 3.2 kb	2
<i>Eco</i> RI + <i>Bam</i> HI 3.0 kb	76
<i>Eco</i> RI 2.4 kb	0
<i>Eco</i> RI + <i>Bam</i> HI 1.8 kb	2

cells, only the larger fragment had Met⁺-transforming activity (Table 2). Thus, that part of the *metC*⁺ gene defined by the mutation *metC3* is to the right of the *Bam*HI site in the phage map (Fig. 4) and on the 3 kb *Bam*HI–*Eco*RI fragment (fragment MC in Fig. 3, lane 4).

DISCUSSION

Phage $\phi 105$ is a useful vector for cloning homologous DNA in *B. subtilis* (Iijima *et al.*, 1980; Jenkinson & Mandelstam, 1983). The construction of $\phi 105$ *d(Cm^r met)* now allows cloning DNA directly into a unique restriction site in a non-essential region of the phage genome. This

prevents insertion of DNA at a site lethal for the phage which is what may happen when DNA is incorporated non-specifically.

When a chloramphenicol-resistant lysogen carrying $\phi 105$ dCm^r was induced with mitomycin C, two types of phage particles with genomes of different size were produced and these have different buoyant densities. The reason for this is not clear; it is possible that the additional 6 kb *Eco*RI fragment in the larger genome (32 kb) may be due to a duplication of some sort occurring during replication and packaging of the smaller 26 kb DNA. Alternatively, the smaller 26 kb phage genome may have arisen through some instability associated with the larger 32 kb DNA. Nevertheless, the insertion of the fragment carrying *metC* into the *Bgl*II site in the smaller genome removed any problems of instability because when $\phi 105$ d(Cm^r*met*) was induced from a lysogen, only one phage band was ever observed in CsCl gradients.

When DNA from $\phi 105$ d(Cm^r*met*) was used to transform the chloramphenicol resistance gene into strain MB79, lysogenic from $\phi 105$ wild-type phage, all the Cm^r transformants were also Met⁺. Since Cm^r and Met⁺ phenotypes appear to be 100% co-transformed into the lysogen, it suggests that there is little or no phage DNA between the Cm^r determinant and the *metC*⁺ fragment. Thus, the 0.9 kb and 0.7 kb *Hpa*II fragments from pBD64 would appear to have been incorporated into the genome of the phage as a complete 1.6 kb fragment.

The cloning of additional DNA into the *Bgl*II site by prophage transformation is analogous to helper plasmid transformation (Contente & Dubnau, 1979) where the cloned DNA is recovered on a resident plasmid (Gryczan *et al.*, 1980a).

We have tried, without success, to clone additional DNA into the *Bam*HI site in $\phi 105$ d(Cm^r*met*) DNA. We have not determined, therefore, whether this site is within the *metC*⁺ gene or if cloning additional DNA into it inactivates Met⁺-complementing activity. However, the *Bam*HI site is of limited use in a cloning experiment of the sort described because, in order to clone the largest pieces of passenger DNA possible, it is preferable to remove the *metC*-carrying *Bgl*II fragment which is not essential to the phage.

Phage $\phi 105$ lysogens are quite stable, and we have observed that the Cm^r Met⁺ phenotype of strains harbouring $\phi 105$ d(Cm^r*met*) are stable without selection. This is not true for strains of *B. subtilis* which carry plasmids conferring chloramphenicol resistance. For example, pHV14 (Ferrari & Hoch, 1983) and pCS194 (Grandi *et al.*, 1981), both of which carry the same chloramphenicol-resistance determinant from pC194 as $\phi 105$ d(Cm^r*met*), are not stably maintained without selection. The use of a phage for cloning DNA in *B. subtilis* has two main advantages over the use of a multicopy plasmid. Firstly, it is possible to do complementation analysis without a large excess of the one gene product which otherwise might interfere with the interpretation of results. Secondly, the product of a heterologous gene cloned as a single copy is less likely to damage the host cell.

Phage $\phi 105$ d(Cm^r*met*) provides a means of introducing into *B. subtilis* genes for which there is no selection. Thus, a previously cloned heterologous gene could be ligated into *Bgl*II-cleaved $\phi 105$ d(Cm^r*met*) DNA and this used to transform a wild-type phage lysogen to chloramphenicol resistance. The method could be refined to favour the formation of recombinants by first purifying the 9.3 kb and 16.7 kb *Bgl*II fragments free of the 6 kb *metC*⁺ fragment, and then treating them with alkaline phosphatase to prevent their self-ligation. Phage $\phi 105$ d(Cm^r*met*) can also be used as a vector for complementation analysis since homologous DNA may be ligated into the phage DNA as described above and introduced as a single copy into the *B. subtilis* chromosome.

We thank Professor J. Mandelstam for his advice and encouragement, Dr Jeff Errington for nick-translating pBD64 and performing the Southern hybridizations, and Professor D. J. Tipper for helpful discussions. This work was supported by the Science and Engineering Research Council. H. F. J. thanks Miss Marilyn Kan for technical assistance.

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