Identification of Restriction Fragments from Two Cryptic *Clostridium butyricum* Plasmids That Promote the Establishment of a Replication-defective Plasmid in *Bacillus subtilis*

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Clostridium butyricum NCIB 7423 carries two cryptic plasmids, pCB101 (6.05 kbp) and pCB102 (7.8 kbp). Sites for the restriction enzymes *EcoRI*, *EcoRV*, *HindIII*, *ClaI* and *PstI* have been found in one or both of these plasmids and their relative positions determined. Restriction fragments from both plasmids have been inserted into a vector plasmid (pJAB1) that is able to replicate in *Escherichia coli* but not in *Bacillus subtilis* and the recombinant plasmids have been established in *E. coli*. A 3.3 kbp *Sau3A* fragment of pCB101 conferred upon the vector the ability to transform both Rec⁺ and Rec⁻ strains of *B. subtilis*. Plasmid pRB1, a representative chimera carrying only the 3.3 kbp *Sau3A* fragment of pCB101, was successfully transferred from *B. subtilis* back to *E. coli*. Plasmid pRB1 was readily lost from *B. subtilis* in the absence of selection. This evidence, together with the results of hybridization experiments, suggests that pRB1 is present as a weakly replicating autonomous element in *B. subtilis*. A recombinant plasmid carrying a 2.0 kbp *Sau3A* fragment of pCB102 underwent integration into the *B. subtilis* chromosome.

INTRODUCTION

The presence of two cryptic plasmids in *Clostridium butyricum* NCIB 7423 has previously been reported (Minton & Morris, 1981). The plasmids, pCB101 and pCB102, are relatively small (5.6 and 7.4 kbp, respectively) and are stably maintained apparently in the absence of any selective pressures. These features suggest that the plasmids could form the basis of suitable cloning vectors for use in clostridia once methods for DNA transfer to these organisms have been developed.

Here we report the results of a detailed restriction analysis of pCB101 and pCB102 and the cloning of restriction fragments from both of these plasmids in *Escherichia coli* and *Bacillus subtilis*. The results suggest that the sizes of the two plasmids are slightly greater than was previously thought (6.05 kbp and 7.8 kbp). It appears that the replication origin of pCB101 functions weakly in *B. subtilis*.

METHODS

Organisms. *Clostridium butyricum* NCIB 7423, which produces the bacteriocin butyricin 7423, was a laboratory stock strain. *Bacillus subtilis* strain 168 *trpC2* is a laboratory wild-type, and strain GSY908 *argC4 hisA1 recE4* was obtained from C. Anagnostopoulos (Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette, France). Strain SA120 *trpC2 spoIIE::cat* harbours the integrational plasmid pPAT83. Upon transformation of Spo⁺ strains of *B. subtilis* the plasmid integrates into and insertionally inactivates the *spoIIE* locus (M. Young, unpublished results). The strain CA160 of *Escherichia coli* that was used as recipient in transformations was a r⁻ m⁻ recBC derivative of *E. coli* C600 *thi-1 thr-1 leu-6 lacY ton azi supE44*. *E. coli* strain SL2034, a transformable Dam⁻ strain whose origin is obscure (Piggot *et al.*, 1984), was used for the propagation of plasmid pJAB1.

Plasmids. The two cryptic plasmids pCB101 and pCB102 were present in *C. butyricum* NCIB 7423 (Minton & Morris, 1981).

Plasmid pJAB1 was constructed by M. Sargent and J. Brannigan by ligating together fragments of the positive cloning vector pTR262 (Roberts *et al.*, 1980) and pJH101 (Ferrari *et al.*, 1983) after they had been digested with *Ava*I and *Pst*I (Sargent & Bennett, 1985). Plasmid pJAB1 comprises the segment of pTR262 that contains the λ *cI* gene together with the pBR322 tetracycline-resistance gene and the segment of pJH101 that contains the pC194-derived chloramphenicol-resistance gene (see Fig. 2). The chloramphenicol-resistance gene is expressed in both *E. coli* and *B. subtilis*, but the plasmid can only replicate in the former host. Insertion of foreign DNA at the unique *Bcl*I or *Hind*III sites inactivates the λ *cI* gene and allows expression of tetracycline-resistance. The enzyme *Bcl*I is insensitive to the *dam* methylation system of *E. coli* and therefore pJAB1 was maintained in and prepared from a *Dam*⁻ strain of *E. coli* (SL2034).

Plasmid pHV33 has been described by Primrose & Ehrlich (1981).

Growth of organisms. *C. butyricum* NCIB 7423 was maintained by growth on Reinforced Clostridial Medium (Oxoid or Lab M) which was solidified by the addition of 1.5% (w/v) agar. The cultures were incubated anaerobically at 37 °C and induced to form spores by exposure to air. Sporulated cultures were stored aerobically at room temperature. *E. coli* strains were grown aerobically at 37 °C in LB medium (containing, per litre: tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g) or on LB medium solidified with 1.5% agar. Antibiotics (chloramphenicol and tetracycline) were added to the media as required to a final concentration of 20 µg ml⁻¹. *B. subtilis* strains were grown with good aeration at 37 °C in liquid cultures of Penassay broth (Difco) or Brain Heart Infusion Broth (Difco). The solid medium used for growth of *B. subtilis* contained, per litre: nutrient broth, 13 g; agar, 15 g; 1 ml salts solution A (which contained, per litre: FeCl₃·6H₂O, 0.98 g; MgCl₂·6H₂O, 8.3 g; MnCl₂·4H₂O, 19.79 g) and 10 ml salts solution B (which contained, per litre: NH₄Cl, 53.5 g; Na₂SO₄, 10.6 g; KH₂PO₄, 6.8 g; NH₄NO₃, 9.7 g; pH 7.0). Chloramphenicol (5 µg ml⁻¹) was added as appropriate.

Extraction of DNA. Plasmids pCB101 and pCB102 were extracted from *C. butyricum* after the growth of organisms in CY medium containing, per litre: glucose, 20 g; NH₄Cl, 3 g; CaCl₂·6H₂O, 0.1 g; yeast extract, 3 g; L-cysteine·HCl, 0.5 g; *p*-aminobenzoic acid, 100 µg; biotin, 10 µg; plus 10 ml of a trace elements solution, which was essentially as described by Vishniac & Santer (1957) except that the concentration of ZnSO₄·7H₂O was reduced from 22 to 2.2 g l⁻¹. Casamino acids (10 ml of a 10%, w/v, solution) and potassium phosphate (50 ml of a 1 M solution, pH 7.0) were added as sterile solutions after autoclaving. The method described by Minton & Morris (1981) was used to lyse the bacteria, except that the lysing solution contained, in addition, 0.5% Brij-58. After lysis, RNA was removed by incubation at 37 °C for 1 h with RNAase (20 µg ml⁻¹, pre-heated to 80 °C for 10 min). The lysate was deproteinized by extraction with 1 vol. phenol/chloroform (1:1, v/v) followed by 2 vols chloroform/isoamyl alcohol (24:1, v/v) and dialysed overnight against 5 litres 10 mM-Tris/HCl, pH 8.0, containing 0.1 mM-Na₂EDTA. DNA was precipitated by the addition of 2 vols chilled 95% (v/v) ethanol and the mixture was held at -20 °C for several hours. The DNA was then collected by centrifugation and redissolved in 15–20 ml 10 mM-Tris/HCl, pH 8.0, containing 0.1 mM-EDTA, before CsCl density gradient centrifugation.

Large scale plasmid extraction from *E. coli* was done by established methods. A cleared lysate was prepared according to the method of Clewell & Helinski (1969) and plasmid DNA was purified by two cycles of CsCl density gradient centrifugation in the presence of ethidium bromide. Plasmids were routinely amplified by the addition of spectinomycin (300 µg ml⁻¹) to late exponential phase cultures.

The large scale extraction of both chromosomal and plasmid DNA from *B. subtilis* was carried out essentially as described by Gryczan *et al.* (1978) except that after low speed centrifugation, nucleic acid was precipitated by the addition of PEG 6000 to a final concentration of 10% (w/v). After holding the mixture at 4 °C for 1 h the nucleic acid was collected by centrifugation and redissolved in 10 mM-Tris/HCl, containing 1 mM-EDTA, pH 8.0, before CsCl density gradient centrifugation.

Two methods were used to extract plasmids on a small scale from 1 ml volumes of culture: the method of Holmes & Quigley (1981) was used for *E. coli* strains and the method of Birnboim & Doly (1979) for *B. subtilis* strains.

Restriction and ligation of DNA. Restriction enzymes were purchased from Bethesda Research Laboratories (*Bam*HI, *Cl*aI, *Eco*RI, *Hind*III, *Pst*I, *Sal*I, *Sau*3A and *Xho*I), Cambridge Biotechnology Laboratories (*Bcl*I and *Sma*I) and Boehringer (*Eco*RV, *Pvu*II and *Xba*I). They were used according to the manufacturer's instructions. To ensure complete digestion of chromosomal DNA samples a tenfold excess of enzyme was used; half of the enzyme was added initially, and the remainder was added 60 min later. T₄ DNA ligase was also supplied by Boehringer.

Construction of recombinant plasmids. A DNA sample (3 µg) containing a mixture of pCB101 and pCB102 was digested to completion with *Hind*III and a separate sample (3 µg) was partially digested with *Sau*3A. The restriction fragments thus obtained were mixed with an equal quantity of pJAB1 that had been cleaved with either *Hind*III or *Bcl*I. The DNA mixture was heated to 55 °C and allowed to cool slowly. After ligation overnight at 4 °C the samples were used to transform *E. coli* CA160; selection was made for tetracycline-resistant transformants.

Agarose gel electrophoresis. DNA samples were subjected to electrophoresis in horizontal 0.6–1.5% (w/v) agarose gels (Sigma) in TEB buffer (which contained, per litre: Tris base, 10.8 g; EDTA, 0.93 g; boric acid, 5.5 g). Before

applying samples to the gel they were mixed with 10% of their volume of tracking dye (glycerol, 50%, v/v; Na₂EDTA, 50 mM; bromophenol blue, 15 µg ml⁻¹).

Transformation. *E. coli* CA160 was transformed with plasmid DNA after the bacteria had been permeabilized by treatment with CaCl₂ and heat-shocked (Mandel & Higa, 1970). Transformed bacteria were incubated for 90 min in broth before selection of tetracycline-resistant transformants on LB agar containing 20 µg tetracycline ml⁻¹.

For transformation of *B. subtilis*, competence was induced by the method of Anagnostopoulos & Spizizen (1961). Plasmid DNA (about 0.5 µg) was incubated with 1 ml competent cells for 90 min and transformants were selected on nutrient agar containing 5 µg chloramphenicol ml⁻¹.

Nick translation and Southern hybridization procedures. A sample of plasmid pJAB1 (about 0.5 µg DNA) was labelled to high specific activity (about 10⁷ d.p.m. per µg DNA) by nick translation (Jeffreys & Flavell, 1977). DNA fragments for hybridizations were separated by agarose gel electrophoresis, depurinated *in situ* (Wahl *et al.*, 1979) and transferred to a nitrocellulose filter (Southern, 1979). The protocol used for hybridization and washing of the filters was as described by Sager *et al.* (1981) except that dextran sulphate was omitted from the solutions. Autoradiography was done at -70 °C using pre-fogged Kodak X-omat RP3 film and an intensifying screen.

RESULTS

Restriction analysis of pCB101 and pCB102

The presence of both pCB101 and pCB102 in the plasmid DNA prepared from *C. butyricum* 7423 complicated analysis of the restriction data. However, since pCB101 was present at a higher concentration in the DNA preparation than was pCB102, the provenance of the restriction fragments could be inferred from the relative intensities of the bands in agarose gels stained with ethidium bromide and visualized under UV light. In several instances bands from both plasmids co-migrated (e.g. both gave rise to a fragment of about 4.5 kbp after *Hind*III digestion) but these were resolved by double digestion analysis. Sites for *Cla*I, *Eco*RV, *Hind*III and *Sau*3A were found in both plasmids, while pCB102 had, in addition, sites for *Eco*RI and *Pst*I. Neither plasmid was cut by the following enzymes: *Bam*HI, *Bcl*I, *Bgl*II, *Sal*I, *Sma*I, *Sst*I, *Xba*I or *Xho*I. Restriction maps of pCB101 and pCB102 are shown in Fig. 1. They are based, in part, on data obtained from the analysis of recombinant plasmids containing fragments of pCB101 and pCB102, some of which are also shown in Fig. 1.

Cloning fragments of pCB101 and pCB102 in the vector pJAB1

After partial digestion with *Sau*3A or complete digestion with *Hind*III, fragments of plasmids pCB101 and pCB102 were cloned into the *Bcl*I or *Hind*III sites of pJAB1 as described in Methods. Twelve tetracycline-resistant transformants carrying *Sau*3A fragments and eighteen tetracycline-resistant transformants carrying *Hind*III fragments were picked for further analysis. After they had been purified by streaking for single colonies on LB agar + tetracycline, plasmid DNA was extracted from each isolate by the method of Holmes & Quigley (1981). Recombinant plasmids carrying *Sau*3A and *Hind*III fragments were denoted pRB1-12 and pRJ1-18, respectively. They were initially characterized using the same enzyme as had been used in their construction. This revealed that all of the *Hind*III and *Sau*3A fragments of pCB101 (the more abundant plasmid) had been cloned successfully. However, the only portion of pCB102 that had been cloned was a 2 kbp *Sau*3A fragment present in pRB7.

Transformation of B. subtilis by recombinant plasmids

Samples of all of the recombinant plasmids were incubated with competent cells of *B. subtilis* 168 and selection was made for chloramphenicol-resistant transformants. The vector plasmid pJAB1 cannot transform *B. subtilis* to chloramphenicol-resistance since it lacks a functional replication origin for this organism. None of the pRJ plasmids (carrying *Hind*III fragments of pCB101) gave transformants of *B. subtilis*, whereas several of the pRB plasmids did transform *B. subtilis* to chloramphenicol-resistance (Table 1). These plasmids included pRB7, which carried a 2 kbp *Sau*3A fragment from pCB102, and two other isolates (pRB1 and pRB5), which had in common a 3.3 kbp *Sau*3A fragment from pCB101 (Fig. 1). None of the pRB plasmids that failed to transform *B. subtilis* harboured either of these fragments.

Fig. 1. Restriction maps of plasmids pCB101 and pCB102 and three recombinant plasmids, pRB1, 5 and 7. The recombinant plasmids carry *Sau*3A fragments of pCB101 (pRB1 and 5) or pCB102 (pRB7) inserted at the *Bcl*I site of the vector pJAB1. Plasmid pCB102 has several *Sau*3A sites in addition to the two that are shown. The approximate extent of λ DNA sequences in pJAB1 is indicated by the bold line. The sizes of the various plasmids are pCB101, 6.05 kbp; pCB102, 7.8 kbp; pJAB1, 5.8 kbp; pRB1, 9.1 kbp; pRB5, 10.3 kbp; pRB7, 7.8 kbp. Sites for the following restriction endonucleases are shown: *Bam*HI, B; *Cla*I, C; *Eco*RI, E; *Hind*III, H; *Pst*I, P; *Sau*3A, S; *Eco*RV, V.

Table 1. Transformation of *B. subtilis* 168 and GSY908 with recombinant plasmids

Plasmid	Fragments of pCB101 present*	No. of transformants†	
		Recipient: 168 (Rec ⁺)	GSY908 (Rec ⁻)
pJAB1	None	0	ND
pHV33	None	1810	305
pRJ1 and 2	<i>Hind</i> III 0.6	0	ND
pRJ6, 9, 10 and 13	<i>Hind</i> III 4.95	0	ND
pRJ14	<i>Hind</i> III 0.5 + 0.6	0	ND
pRB1	<i>Sau</i> 3A 3.3	550	110
pRB2	<i>Sau</i> 3A 0.25	0	ND
pRB5	<i>Sau</i> 3A 3.3 + 1.1 + 0.25	1280	20
pRB7‡	<i>Sau</i> 3A 2.0‡	28	0
pRB8	<i>Sau</i> 3A 1.1	7	ND
pRB9, 10	<i>Sau</i> 3A 1.4 + 1.1	0, 2	ND
pRB12	<i>Sau</i> 3A 1.1	5	ND

* The fragments are identified by their sizes (kbp).

† Values given are the numbers of chloramphenicol-resistant transformants obtained per ml culture using as donor, plasmid DNA extracted from 1 ml cultures, according to the method of Holmes & Quigley (1981). The data for strains 168 and GSY908 are from separate experiments. Plasmid DNA purified by CsCl/ethidium bromide density gradient centrifugation gave the following transformation efficiencies using *B. subtilis* 168 as recipient: pRB1, 1.9×10^4 ; pRB5, 1.4×10^4 ; pRB7, 7.5×10^1 (numbers of transformants per μ g DNA). ND, Not determined.

‡ The 2.0 kbp *Sau*3A fragment present in pRB7 comes from pCB102 (see text and Fig. 1).

Table 2. Segregation of chloramphenicol-sensitive bacteria from strains of *B. subtilis* 168 harbouring recombinant plasmids

Strain	No. of generations*	No. of colonies tested	Percentage of Cm ^R colonies
168(pHV33)	21	150	73
SA120(pPAT83)	22	46	100
168(pRB1)	23	58	0
168(pRB7)	24	56	100

* Bacteria were grown in Penassay broth without chloramphenicol.

The recombinant plasmids that showed appreciable transforming activity with *B. subtilis* 168 (Rec⁺) as recipient were used on several different occasions to transform strain GSY908 (Rec⁻). Data from a representative experiment are given in Table 1. Chloramphenicol-resistant transformants were consistently obtained, albeit at a reduced frequency, with plasmids pRB1 and pRB5. In contrast, plasmid pRB7 never yielded any transformants. Plasmid pHV33, which was used as a control in these experiments, transformed both Rec⁺ and Rec⁻ recipients.

Stability of recombinant plasmids in *B. subtilis*

Strains of *B. subtilis* 168 that had been transformed with plasmids pRB1 and pRB7 were grown in the absence of chloramphenicol for more than 20 generations. Strains of *B. subtilis* harbouring pHV33 (autonomous) and pPAT83 (integrated into the *spoIIIE* region of the chromosome – see Methods) were used as controls. Colonies that were obtained after plating suitably diluted samples of each culture on nutrient agar were then tested for their ability to grow on nutrient agar supplemented with chloramphenicol. The results (Table 2) indicate that chloramphenicol-resistance was stably maintained in the absence of selective pressure in strains 168(pRB7) and SA120(pPAT83), whereas it was lost from strain 168(pRB1) even more rapidly than from strain 168(pHV33).

Analysis of strains of *B. subtilis* harbouring recombinant plasmids by Southern hybridization

DNA samples from *B. subtilis* strains transformed with pRB1 and pRB7 were digested with *Pst*I and either *Bam*HI or *Eco*RV. The fragments produced were separated electrophoretically according to their size and transferred to a nitrocellulose filter. A nick-translated sample of pJAB1 DNA was used as hybridization probe. Both of the pRB plasmids have a single site for these three enzymes within the vector (pJAB1) sequences (see Fig. 1). Any free plasmid DNA in the preparations would therefore give rise to a single band of hybridization corresponding to the linearized plasmid. Alternatively, if the cloned DNA fragments enabled the recombinant plasmids to integrate into the bacterial chromosome then this should result in the appearance of two bands of hybridization. The integration of more than one copy of the recombinant plasmids would generate three hybridizing fragments, one corresponding to the linear plasmid and the other two representing the border fragments (Young, 1984).

The results of the hybridization with strain 168(pRB7) are shown in Fig. 2(a). Hybridization occurred to high molecular weight DNA in the undigested sample (lane 3). After digestion with either *Bam*HI or *Pst*I hybridization occurred to three DNA fragments. The most intense hybridization occurred to a fragment corresponding in size to the linear plasmid (lanes 2 and 4). There was weaker hybridization to the other two fragments, which were most readily visible in the *Pst*I-digested sample, where their sizes were about 3.7 and 10.5 kbp (lane 4).

DNA samples from six independently isolated strains of *B. subtilis* 168 harbouring pRB1 [strains 168(pRB1)a–f] were analysed after digestion with *Pst*I and *Eco*RV; the results from two representative isolates are given in Fig. 2(b). Digestion with either enzyme produced a single band of hybridization corresponding in size to linearized pRB1 (compare lanes 2, 4, 10 and 12 with lane 5). The two bands seen in doubly digested samples also corresponded (compare lanes 3 and 11 with lane 6). A faint band of hybridization that might be due to monomeric plasmid

Fig. 2. Hybridization of pJAB1 to DNA from strains of *B. subtilis* 168 transformed with recombinant plasmids. (a) Analysis of strain harbouring pRB7. Total DNA from a *B. subtilis* strain harbouring pRB7 (1 µg) or pRB7 DNA (2.5 ng) was digested with *Bam*HI or *Pst*I. DNA fragments were separated by electrophoresis through 0.7% agarose and transferred to a nitrocellulose filter (see Methods). After hybridization for 44 h to nick-translated pJAB1 DNA, the filter was subjected to autoradiography at -70°C for 48 h. Lane 1, pRB7 *Bam*HI; lane 2, 168(pRB7) *Bam*HI; lane 3, 168(pRB7) undigested; lane 4, 168(pRB7) *Pst*I; lane 5, pRB7 *Pst*I. The minor bands of hybridization seen in lane 1 result from the fact that plasmid pRB7 shows slight instability in *E. coli*.

(b) Analysis of two independently isolated strains harbouring pRB1. Details as for (a) except that digestions were with *Eco*RV and/or *Pst*I and autoradiography was for 24 h. Lane 1, 168(pRB1)a undigested; lane 2, 168(pRB1)a *Pst*I; lane 3, 168(pRB1)a *Eco*RV; lane 4, 168(pRB1)a *Eco*RV; lane 5, pRB1 *Pst*I; lane 6, pRB1 *Pst*I and *Eco*RV; lane 7, λ *Hind*III; lane 8, 168 undigested; lane 9, 168(pRB1)b undigested; lane 10, 168(pRB1)b *Pst*I; lane 11, 168(pRB1)b *Pst*I and *Eco*RV; lane 12, 168(pRB1)b *Eco*RV. Note that pJAB1 hybridizes with the 6.7 kbp *Hind*III fragment of λ DNA in lane 7.

Fig. 3. Hybridization of pJAB1 to *Eco*RI-, *Bgl*II- and *Cla*I-digested DNA from a strain of *B. subtilis* transformed with pRB1. DNA fragments generated after digestion of 1 µg samples of total DNA were separated by electrophoresis through 1% agarose. Other details as for Fig. 2(b). (a) Ethidium-bromide stained electrophoretogram; (b) autoradiogram. Lane 1, λ *Hind*III; lane 2, pRB1 (25 ng) undigested; lane 3, 168(pRB1)d undigested; lane 4, 168(pRB1)d *Eco*RI; lane 5, 168(pRB1)d *Bgl*II; lane 6, 168(pRB1)d *Cla*I. The arrow in (a) indicates the position of the DNA in lanes 4, 5 and 6 to which intense hybridization occurred in (b).

DNA is evident in the undigested DNA from one strain (lane 1), but this band was not seen in undigested DNA samples from the other five strains; hybridization occurred exclusively to high molecular weight DNA in these samples. Some further hybridizations were undertaken using DNA from one of the strains that had been digested with enzymes that do not cut pRB1. The gel electrophoretogram and the corresponding autoradiogram are shown in Fig. 3(a, b). In each case hybridization occurred almost exclusively to a band that comigrated with one of the predominant forms of pRB1 (extracted from *E. coli* CA160). The DNA corresponding to this hybridizing band is visible in the ethidium bromide-stained electrophoretogram (Fig. 3a, lanes 4 and 6).

Transfer of pRB1 from B. subtilis to E. coli

Total DNA from a strain of *B. subtilis* that had been transformed with pRB1 was used to transform permeabilized cells of *E. coli* CA160. Transformants to either chloramphenicol- or tetracycline-resistance arose at a very low frequency (about 10 transformants per µg DNA). Ninety-three percent of the 69 tetracycline-resistant transformants that were obtained were also resistant to chloramphenicol, but most of the 30 chloramphenicol-resistant transformants that were obtained proved to be tetracycline-sensitive. Plasmid DNA was prepared from 11 of the doubly-resistant transformants. The DNA fragments produced after *Hind*III digestion were indistinguishable from those produced after digestion of pRB1 with the same enzyme.

DISCUSSION

The 2.0 kbp *Sau*3A fragment of pCB102 that was inserted into pJAB1 to form the recombinant plasmid pRB7 enabled this last plasmid to become established in *B. subtilis*. The hybridization data (Fig. 2) indicate that pRB7 was inserted into the bacterial chromosome and that under the conditions used here, more than one copy of the plasmid became integrated in tandem, i.e. amplification occurred (Young, 1984). The chromosomal location of the site of integration has not been ascertained, nor has it been determined whether integration always occurs at this particular site when the plasmid becomes established in *B. subtilis*.

In accordance with the integration of plasmid pRB7 into the *B. subtilis* chromosome, transformation was Rec-dependent (Table 1). Presumably the low transformation efficiency with the Rec⁺ recipient is a reflection of very limited homology between donor and recipient DNA – we have been unable to detect it by Southern hybridization. Once integrated into the *B. subtilis* chromosome, the plasmid was stably maintained during growth for more than 20 generations in the absence of selection (Table 2).

Plasmid pRB1, on the other hand, appears to replicate autonomously in *B. subtilis*. The data in Fig. 2 are somewhat ambiguous because hybridization to high molecular weight material in the undigested DNA samples would not be expected. However, the experiment shown in Fig. 3 provided an explanation for this. After cleavage with three different enzymes that do not cut pRB1 and electrophoresis through 1% agarose, the mobility of the plasmid DNA was retarded relative to that of the linear chromosomal DNA fragments. In two cases (*Eco*RI and *Cla*I) pRB1 was clearly separated from the chromosomal DNA. In the undigested DNA sample in Fig. 3 pRB1 still tended to co-migrate with the high molecular weight chromosomal DNA, perhaps because of the relative overloading of the gel in this size range. The pattern of hybridization of the pRB1 DNA isolated from *B. subtilis* is unusual in that it does not present the appearance that might be expected of an autonomous plasmid; the DNA is almost exclusively in a single molecular form (Fig. 3).

The autonomous mode of replication of pRB1 in *B. subtilis* tentatively deduced from the hybridization data is supported by the ability of this plasmid, and another similar plasmid, pRB5, carrying the 3.3 kbp *Sau*3A fragment of pCB101, to transform both Rec⁺ and Rec⁻ strains of *B. subtilis* (Table 1). It also serves to explain the results of the segregation experiments (Table 2) which showed that pRB1 is rapidly lost from *B. subtilis* in the absence of selection. This may reflect weak replication of the plasmid or, alternatively, inaccurate partitioning of plasmid molecules into daughter cells at division. Finally, the autonomous mode of replication of pRB1 in *B. subtilis* was confirmed by transfer of the plasmid back into *E. coli*.

The plasmids constructed in the course of this investigation should prove useful in our attempts to devise techniques for protoplast-mediated transformation in *C. butyricum* and other saccharolytic clostridia. They are also being developed as shuttle plasmids for future use in saccharolytic clostridia and *E. coli*.

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