Postprint version. Original publication in:
Molec. gen. Genet. 162, 59–67 (1978)

Recombinant Plasmids Capable of Replication in *B. subtilis* and *E. coli*

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Summary. The plasmid *pBC16* (4.25 kbases), originally isolated from *Bacillus cereus*, determines tetracycline resistance and can be transformed into competent cells of *B. subtilis*. A miniplasmid of *pBC*16 (*pBC*16-1), 2,7 kb) which has lost an *Eco*RI fragment of *pBC*16 retains the replication functions and the tetracycline resistance. This plasmid which carries only one *Eco*RI site has been joined in vitro to *pBS*1, a cryptic plasmid previously isolated from *B. subtilis* and shown to carry also a single *Eco*RI site (Bernhard et al., 1978). The recombinant plasmid is unstable and dissociates into the plasmid *pBS*161 (8.2 kb) and the smaller plasmid *pBS*162 (2.1 kb). Plasmid *pBS*161 retains the tetracycline resistance. It possesses a single *Eco*RI site and 6 *Hind*III sites. The largest *Hind*III fragment of *pBS*161 carries the tetracycline resistance gene and the replication function. After circularization *in vitro* of this fragment a new plasmid, *pBS*161-1 is generated, which can be used as a *Hind*III and *Eco*RI cloning vector in *Bacillus subtilis*.

Hybrid plasmids consisting of the *E. coli* plasmids *pBR*322, *pWL*7 or *pAC*184 and different *Hind*III fragments of *pBS*161 were constructed in vitro. Hybrids containing together with the *E. coli* plasmid the largest *Hind*III fragment of *pBS*161 can replicate in *E. coli* and *B. subtilis*. In *E. coli* only the replicon of the *E. coli* plasmid part is functioning whereas in *B. subtilis* replication of the hybrid plasmid is under the control of the *Bacillus* replicon. The tetracycline resistance of the *B. subtilis* plasmid is expressed in *E. coli*, but several antibiotic resistances of the *E. coli* plasmids (ampicillin, kanamycin and chloramphenicol) are not expressed in *B. subtilis*. The hybrid plasmids seem to be more unstable in *B. subtilis* than in *E. coli*.

Introduction

Recently, we have reported on the isolation and characterization of a large number of plasmids from *B. subtilis* and *B. cereus* (Bernhard et al., 1978). Among those a rather small plasmid, *pBC*16, originally isolated from a tetracycline resistant strain of *B. cereus* could be transformed into *B. subtilis*, where it is stably maintained rendering the transformants resistant to tetracycline. The molecular properties of this plasmid (size, copy number, sites for restriction enzymes, genetic marker) made it appear a suitable vector for gene cloning in *B. subtilis*. We have therefore started experiments to insert fragments from other *B. subtilis* and *E. coli* DNA molecules into this plasmid and to analyze the resultant recombinant DNAs.

Other vector plasmids suitable for the application of this new genetic method in *B. subtilis* have been recently reported (S. Ehrlich, 1977). These plasmids derive from *Staphylococcus aureus*.

Like *pBC*16 they seem to be suitable vectors for gene cloning in *B. subtilis* (S. Ehrlich, personal communication; P. Lovett, personal communication). We have made the experience, however, that they seem to be considerably more unstable in the new host than *pBC*16. This report describes experiments, which demonstrate that *pBC*16 and some of its in vitro constructed derivatives can be used for cloning of homologous and heterologous DNA in *Bacillus subtilis*.

Materials and Methods

Bacterial Strains. E. coli 5K, r^-, m^+ was obtained from S. Glover. JC 1569 *rec*A$^-$, *thy*$^+$, *arg*$^-$, *his*$^-$, *met*$^-$, *leu*$^-$, *Sm*^{R} harbouring plasmid pAC184 was a gift from Cohen and Chang. *E. coli* DP9, *pol*A$^-$, r^-, m^- was provided by Ph. Kourilsky. *B. subtilis* 168 *trp*$^-$ (DSM 402) was from the Deutsche Sammlung von Mikroorganismen, *B. subtilis* BR151-CM1 *trp*$^-$, *lys*$^-$, *met*$^-$, *spo*$^-$ was kindly provided by P. Lovett. The other strains used here, were described before (Bernhard et al., 1978; Kollek et al., 1978).

Source of Reagents. All reagents if not otherwise stated were from Merck, Germany. The antibiotics used were a gift from Bayer, Germany. Sodium dodecyl sulfate (SDS), ethidium bromide, polyethylenglycol type 6000, lysozyme, pancreatic RNase and pronase P from *S. griseus* were obtained from Serva, Germany.

Enzymes. The restriction enzyme *Eco*RI was provided by H. Mayer (Braunschweig), *Hind*III and T4 DNA ligase was purchased from Bio Labs (USA).

Growth of Strains, Cell Lysis and Purification of Plasmid DNA were performed as previously described (Mayer et al., 1977; Bernhard et al., 1978).

Cleavage of DNA and Electrophoresis in Agarose Gels. Digestion with *Eco*RI was carried out in 100 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$ at 37° C for 30 min, with *Hind*III in 10 mM Tris-HCl pH 7.5, 25 mM NaCl, 10 mM $MgCl_2$ at 37° C for 60 min. The reactions were stopped by heating to 68° C for 8 min or by addition of 100 mM EDTA. Electrophoresis was performed on 1% agarose slab gels in 36 mM Tris-HCl, 30 mM NaH_2 PO_4 10 mM EDTA, pH 7.5 (Meyers et al., 1975)

Construction of Hybrid Plasmids and Transformation. The in vitro construction of hybrid plasmids (Cohen et al., 1973) using T4 DNA ligase, transformation of *E. coli* and of *B. subtilis* have been described (Goebel and Bonewald, 1975; Cahn and Fox, 1968). Transformants of *E. coli* were first selected on nutrient broth (ENB) plates containing 100 µg/ml ampicillin (in the case of *pBR*322 or *pWL*7 hybrids) or 100 µg/ml chloramphenicol (for *pAC*184 hybrids). Growing colonies were then tested for sensitivity to 5–50 µg/ml tetracycline (pBR322 and pAC184 hybrids), to 100 µg/ml kanamycin (for *pWL*7 hybrids). In addition *pWL*7 hybrids (Km^S) were tested for sensitivity against 5–50 µg/ml tetracycline.

Determination of the Intracellular β-Lactamase Activity. The enzyme was assayed with hydroxylamine/ammonium ferric sulphate (Dale and Smith, 1971). 1 unit corresponds to the amount of enzyme which catalyses the hydrolysis of one µmole ampicillin/min at 30° C in 0.025 M Tris-HCl, pH 7.4.

Results

In vitro Construction of Recombinant Plasmids from B. subtilis Plasmid pBS1 and B. cereus Plasmid pBC16

The tetracycline resistance plasmid *pBC*16 (4.2 kb) was previously isolated from *B. cereus* and transformed into *B. subtilis* 168 (Bernhard et al., 1978). Restriction endonuclease *Eco*RI cleaves this plasmid into two fragments of 2.7 kb and 1.5 kb. The cryptic plasmid *pBS*1 (7.8 kb) of *B. subtilis* carries a single *Eco*RI site. To construct in vitro recombinant plasmids carrying both replicons, *pBC*16 was either completely or partially digested by *Eco*RI and ligated, using T4 DNA ligase, with *Eco*RI cleaved *pBS*1. The ligated mixture was transformed into *B. subtilis* 168 made competent as described (Cahn and Fox, 1968). Tetracycline resistant (Tc^R) colonies were obtained with a frequency of about 10^{-6}. Covalently-closed circular (CCC) DNAs where isolated from several of the obtained clones. None of them represented the expected hybrid plasmid consisting of both replicons. Instead, the CCC-DNAs isolated from several Tc^R clones could be identified as:

a) pBC16-1: Some of tetracycline resistant cells harbour one small plasmid of 2.7 kb, which carries a single *Eco*RI site. This plasmid, which can be also obtained by transforming *B. subtilis* with *pBC*16, completely digested by *Eco*RI and then ligated *in vitro*, represents the circularized large *Eco*RI fragment of *pBC*16 (Fig. 1). This mini *pBC*16 plasmid, named *pBC*16-1, thus carries the tetracycline resistance gene and the replication functions of *pBC*16.

b) pBC16-2: Other Tc^R-transformants harbour two plasmids of 5.8 kb and 4.2 kb. The smaller one is identical to *pBC*16. Transformation of the isolated larger plasmid yields tetracycline resistant colonies which harbour again both plasmids. Cleavage of the mixture of both plasmids by *Eco*RI (Fig. 1) yields only the two fragments which are also obtained after cleavage of *pBC*16 DNA alone. Since the size difference between *pBC*16 and the larger plasmid, *pBC*16-2, is 1.5 kb and hence identical to the small *Eco*RI fragment of *pBC*16, we conclude that plasmid *pBC*16-2 carries a second small *Eco*RI fragment of *pBC*16 inserted into *pBC*16. This plasmid is, however, unstable and eliminates at a rather high rate one of the two identical fragments.

c) pBS161 and pBS162: A large number of the tetracycline resistant colonies harbour two plasmids of 8.2 kb (*pBS*161) and 2.1 kb (*pBS*162) (Fig. 1). Plasmid *pBS*161 alone but not *pBS*162 yields also tetracycline resistant colonies when transformed into competent *B. subtilis* cells. This plasmid being larger in size than *pBS*1 but smaller than the expected sum of *pBS*1 and *pBC*16 or one of its two *Eco*RI fragments, carries only one *Eco*RI site (Fig. 1) but 6 *Hind*III sites. Comparison of the *Hind*III patterns of *pBS*1 and *pBS*161 reveals that fragments B, C, E, F and G are identical with *pBS*1 (Fig. 2 and Fig. 4). Fragment D of *pBS*1 is lost, whereas fragment A' has obviously incorporated additional DNA from *pBC*16 including the gene determining tetracycline resistance. The smaller plasmid *pBS*162 being present in the original clones carries also one *Eco*RI and one *Hind*III site. Since it represents a replicating DNA element it must possess one of the replicon functions of the two parent plasmids *pBC*16 or *pBS*1. The sum of the sizes of *pBS*161 and *pBS*162 is 10.3 kb which is roughly identical to the sum of *pBS*1 and the larger *Eco*RI fragment of *pBC*16 (10.5 kb). It appears therefore rather likely that a hybrid plasmid consisting of the large *Eco*RI

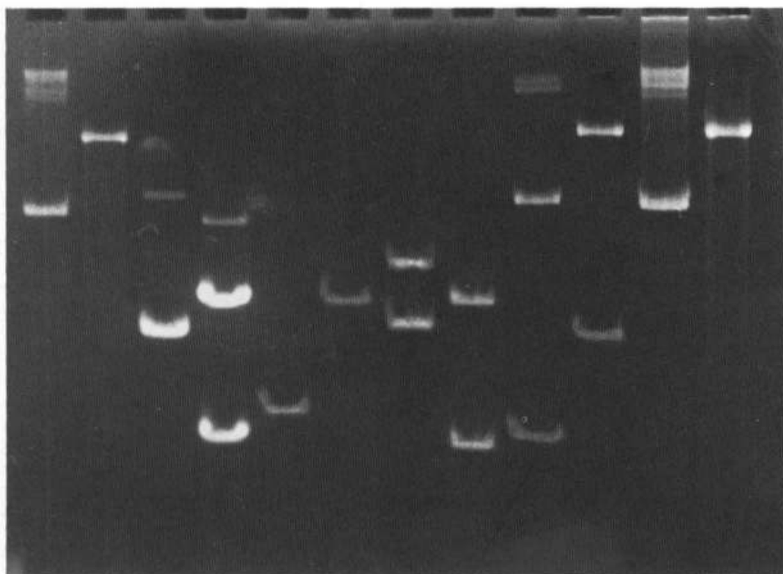

Fig. 1 A–M. Plasmids obtained after transformation of *B. subtilis* 168 with in vitro recombinant DNA consisting of *pBC*16 and *pBS*1. Plasmid *pBC*16 and *pBS*1 were cleaved by *Eco*RI and ligated with T4 DNA ligase as described (Cohen et al., 1973). Transformation into *B. subtilis* was performed by the procedure of Cahn and Fox, 1968. Tc^R-colonies were picked, their covalently-closed circular DNAs isolated and analysed on an 1% agarose gel (2 mA/cm, 17 h). **A** *pBS*1, **B** *pBS*1 cleaved with *Eco*RI, **C** *pBC*16, **D** *pBC*16 cleaved with *Eco*RI, **E** *pBC*16-1, **F** *pBC*16-1 cleaved with *Eco*RI, **G** *pBC*16 and *pBC*16-2, **H** *pBC*16 and *pBC*16-2 cleaved with *Eco*RI, **J** *pBS*161 and *pBS*162, **K** *pBS161* and *pBS*162 cleaved with *Eco*RI, **L** *pBS*161, **M** *pBS*161 cleaved with *Eco*RI

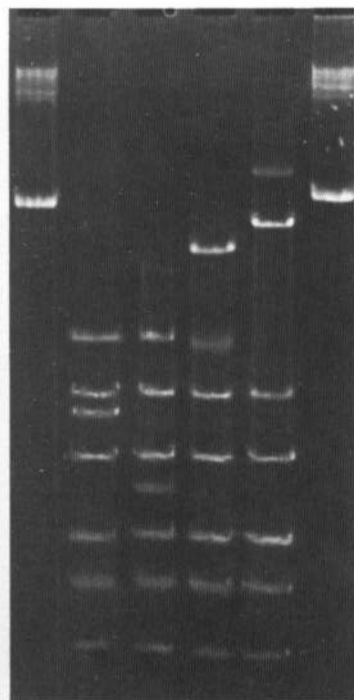

Fig. 2 A–F. Comparison of the *Hind*III and *Hind*III/*Eco*RI patterns of *pBS*1 and *pBS*161. Covalently closed DNA of *pBS*1 and *pBS*161 was cleaved with *Hind*III and *Hind*III + *Eco*RI, respectively. The fragments were separated by electrophoresis on an 1% agarose gel. **A** *pBS*1 CCC-DNA, **B** *pBS*1 cleaved with *Hind*III, **C** *pBS*1 cleaved with *Hind*III and *Eco* RI. **D** *pBS*161 cleaved with *Hind*III and *Eco*RI, **E** *pBS*161 cleaved with *Hind*III, **F** *pBS*161 CCC-DNA.

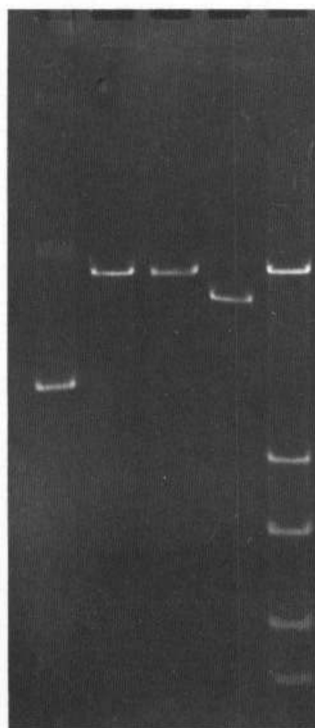

Fig. 3 A–E. Gel electrophoresis of plasmid *pBS*161-1, obtained by cleaving of *pBS*161 with *Hind*III and transformation of the religated DNA into *B. subtilis* 168. **A** Covalently closed *pBS*161-1 DNA, **B** *pBS*161-1 DNA cleaved with *Eco*RI, **C** with *Hind*III, **D** with *Eco*RI and *Hind*III (the small fragment of 300 bp can not be seen on this gel). **E** *pBS*161 cleaved with *Hind*III

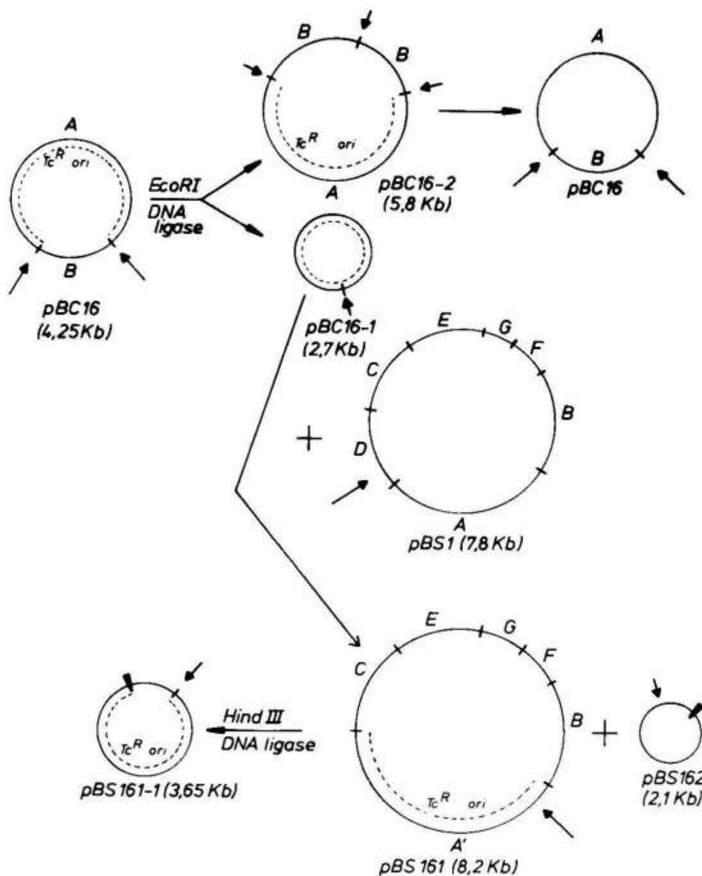

Fig. 4. Genealogy of the *B. subtilis* vector plasmids *pBC*16, *pBC*16-1, *pBS*161 and *pBS*161-1

fragment of *pBC*16, which carries the tetracycline resistance and the replication functions of *pBC*16, and *pBS*1 was formed in vitro. However, after transformation in *B. subtilis* the recombinant plasmid which consists now of two replicons is unstable and dissociates nonreciprocally into *pBS*161 and *pBS*162. *pBS*161 was cleaved with *Hind*III and the fragments were recircularized in vitro with DNA ligase. After transformation in *B. subtilis* Tc^R-colonies are obtained, that carry a new plasmid *pBS*161-1 which represents the circularized *Hind*III-A fragment of *pBS*161 (Fig. 3) which indicates that this fragment carries the tetracycline resistance determinant and the replication function of *pBS*161. This fragment has retained in addition one *Eco*RI site. Figure 4 summarizes the formation of the various derivatives of *pBC*16.

*Construction of Hybrid Plasmids Consisting of Hind*III *Fragments of pBS*161 *and E. coli Plasmids pBR*322, *pWL*7 *or pAC*184

Plasmid *pBS*161 was digested by restriction endonuclease *Hind*III and ligated, using T4 DNA ligase, with the *E. coli* plasmids *pBR*322 (Boyer et al., 1977), *pWL*7 (Kollek et al., 1978), or *pAC*184 (S. Cohen and A. Chang, unpublished), which were also linearized by *Hind*III. These *E. coli* plasmids were used as vectors since insertion of a *Hind*III fragment into their single *Hind*III sites inactivates the tetracycline resistance (in the case of *pBR*322 and *pAC*184) or the kanamycin resistance (in the case of *pWL*7), but leaves another antibiotic resistance determined by these plasmids intact which can then be used for selection of transformants (Ap^R for *pBR*322 and *pWL*7, and Cm^R for *pAC*184). The in vitro ligated mixtures were transformed into *E. coli* 5K (r^-, m^+) and hybrid clones were selected by inactivation of the corresponding marker. When clones, obtained after transformation with ligated *Hind*III cleaved *pBR*322 and *Hind*III fragments of *pBS*161 were tested for inactivation of tetracycline resistance, two different types of clones were obtained: Clones which were completely sensitive to tetracycline and clones which still retained a low level of tetracycline resistance. Whereas strains carrying the original plasmid *pBR*322 are resistant to 100 μg ml tetracycline, the upper limit of resistance of the latter clones is 15 μg/ml.

Both, tetracycline sensitive and low-level tetracycline resistant colonies contained hybrid plasmids, as

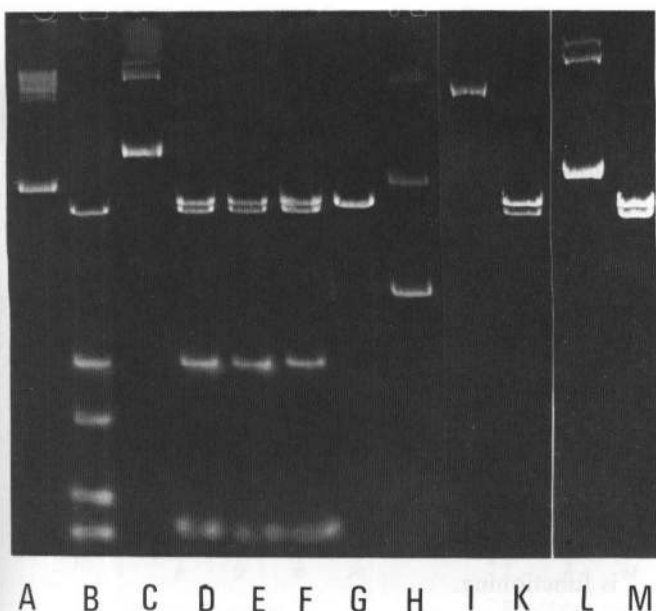

Fig. 5A–M. Agarose gel electrophoresis of *Hind*III-cleaved hybrid plasmids consisting of *E. coli* plasmid *pBR*322 and *Hind*III fragments of *pBS*161. Covalently closed hybrid DNA was isolated from the indicated strains and cleaved with *Hind*III. **A** *pBS*161-CCC-DNA, **B** *pBS*161 cleaved with *Hind*III, **C** CCC-*pJK*1 DNA from *E. coli* 5K, **D** cleaved with *Hind*III, **E** *pJK*1 DNA from *E. coli* MM383 (*polA*12) cleaved with *Hind*III, **F** *pJK*1 DNA from *B. subtilis* 168 cleaved with *Hind*III, **G** *pBR*322 cleaved with *Hind*III. **H** CCC-*pBR*322, **J** CCC-*pJK*2 DNA from *E. coli* 5K, **K** cleaved with *Hind*III. **L** CCC-*pJK*3 DNA from *E. coli* 5K. **M** cleaved with *Hind*III

Table 1. Characterization of hybrid plasmids consisting of *E. coli* vectors pBR322 or pWL7 and various *Hind*III fragments of *B. subtilis* plasmid pBS161

Plasmid	Molecular weight (× 1000 b.p.)	*E. coli* vector	HindIII fragments of pBS161 inserted into the *E. coli* vector	Transformation rate[b] in *B. subtilis*168
*p*JK1	9.8	*pBR*322	A, B, E	2.6×10^{-5}
*pJK*2	11.5[a]	*pBR*322	A	1.4×10^{-5}
*pJK*3	7.6	*pBR*322	A	5.3×10^{-5}
*pJK*4	5.6	*pBR*322	B	0
*pJK*101	15.75	*pWL*7	A	2.4×10^{-5}
*pBR*322	3.9	–	–	0
*pWL*7	12.1	–	–	0
*pBC*16	4.25	–	–	2.0×10^{-5}
*pBC*16-1	2.7	–	–	4.5×10^{-5}
*pBS*161	8.2	–	–	1.9×10^{-5}

[a] This plasmid contains two molecules of *pBR*322 linked to one *Hind*III-A fragment

[b] Transformation rate is defined as the ratio of Tc^R transformants per 10^8 cells and 3 µg of DNA used in the transformation assay

demonstrated by cleaving the isolated CCC-DNA with *Hind*III (Fig. 5). Most plasmids isolated from clones which retained the low tetracycline resistance had the largest *Hind*III fragment (*Hind*III-A) of *pBS*161 incorporated into *pBR*322 (Table 1). Low-level tetracycline resistant colonies were also obtained when *pWL*7 plasmid (Km^R, Ap^R) was used as vector for the cloning of the *Hind*III fragment of *pBS*1. The Tc^R colonies carried hybrid plasmids which had also *Hind*III fragment A incorporated into *pWL*7. Since plasmid *pWL*7 does not determine tetracycline resistance, this result indicates that the low tetracycline resistance is expressed by the cloned *Hind*III-A fragment of *pBS*161. One clone, also resistant to low concentrations of tetracycline, contained a hybrid plasmid, *pJK*4, which had fragment *Hind*III-B of *pBS*161 incorporated into *pBR*322 (Table 1). Since the *Hind*III site in *pBR*322 inactivates the promotor of the Tc^R gene, but not the structural gene it was assumed that the tetracycline resistance of *pJK*4 may be caused by the replacement of the inactive own promotor by a *B. subtilis* promotor located on fragment *Hind*III-B. Plasmid *pJK*4 was therefore cleaved by *Hind*III, religated with DNA ligase in vitro and transformed into *E. coli* 5K. Ampicillin resistant transformants were obtained, which were either Tc^R or Tc^S. The Tc^S transformants contained *pJK*4 with *Hind*III-B inserted in the opposite direction as in the original *pJK*4, whereas Tc^R transformants contained either *pJK*4 in the original orientation or *pBR*322 only (Fig. 6).

Purified hybrid plasmids obtained in the former cloning experiments in *E. coli* were used to transform competent *B. subtilis* cells. Selection for transformed colonies was performed on tetracycline containing plates. Tc^R transformants were obtained with all hybrid plasmids containing fragment *Hind*III-A from *pBS*161. Tetracycline resistance of these transformed *B. subtilis* strains is high and comparable to the tetracycline resistance (100 µg/ml) of the parent plasmid *pBS*161. CCC-DNA isolated from the *B. subtilis* clones is identical to the hybrid plasmid DNA isolated from *E. coli* and used for the transformation, i.e. the plasmid DNA in both hosts remains identical (Fig. 5). Similiar results were obtained with hybrid

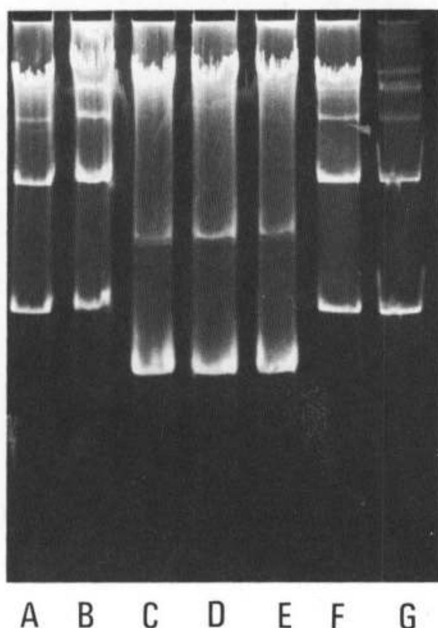

Fig. 6 A–G. Recloning of *pJK4* hybrid DNA. Hybrid *pJK*4 DNA from low-level Tc^R colonies of *E. coli* 5K was isolated. The DNA was cleaved with *Hind*III and religated with T4 DNA ligase. The DNA was transformed into *E. coli* 5K. Tc^R and Tc^S-colonies were obtained. Cleared lysates of Tc^R and Tc^S colonies were prepared and analysed by agarose gel electrophoresis. **A, B** cleared lysates from Tc^S-clones containing *pJK*4 DNA with *Hind*III-B fragment inserted into *pBR*322 in the opposite orientation as in the original *pJK*4 DNA. **C–E** cleared lysates from Tc^R-clones containing *pBR*322 DNA only, **F** cleared lysate from Tc^R-clone containing the original *pJK*4 DNA, **G** *pJK*4 DNA as control

plasmids of *pWL*7 having *Hind*III fragment A incorporated (data not shown).

Transformation of *B. subtilis* BR151-CM1 a nonsporulating derivative of *B. subtilis* 168 (kindly provided by P. Lovett) with *pBC*16-1 and the hybrid plasmid *pJK*101 leads also to tetracycline resistant colonies. The transformation rate is comparable to that of *B. subtilis* 168, indicating that this nonsporulating *B. subtilis* mutant can be also used as a host for recombinant DNA.

Replication Control of Hybrid Plasmids

The *E. coli* plasmids used as vectors for the described cloning experiments are *Col*E1 derivatives. Their replication is therefore dependent on high dosis of DNA polymerase I (Kingsbury and Helinski, 1970) and continues in the presence of chloramphenicol (Clewell, 1972). To test which replicon of the obtained hybrid plasmids capable of replication in both hosts is functional, the following experiments were performed:

a) Transformation of *pJK*1-3 (see Table 1) in *E. coli DP*9 *pol*A$^-$, r^-, m^- does not yield transformants. Transformants of MM383, *polA*12 (carrying a temperature sensitive DNA polymerase I) obtained with this plasmid are unable to grow at 43° C on tetracycline and/or ampicillin containing plates.

b) Addition of chloramphenicol to a culture of *E. coli* harbouring *pJK*1 leads to an amplification of this plasmid. However, the copy number of the hybrid plasmid increases only 3–5 fold after treatment with this antibiotic. Treatment of *B. subtilis* 168 harbouring *pJK*1 with chloramphenicol leads to a complete stop of plasmid synthesis (Fig. 7). The experiment was performed with chloramphenicol concentrations between 5 and 150 µg/ml. The results indicate that in *E. coli* the *Col*E1 replicon but not the *Bacillus subtilis* replicon is active whereas in *B. subtilis* only the *B. subtilis* but not the *Col*E1 replicon is functioning.

Differences in Gene Expression of the Hybrid Plasmids in E. coli and B. subtilis

As described in the preceeding section the gene determining resistance to tetracycline in *B. subtilis* is also expressed in *E. coli*, although the level of resistance in the new host is lower. Experiments to insert *in vitro* the *Eco*RI fragment of pML21 conferring resistance to kanamycin in *E. coli* (Hershfield et al., 1974) into *Eco*RI-cleaved plasmid *pBC*16 of *B. subtilis* were performed. The ligated mixture which contained according to the analysis on an agarose gel at least 80% ligated DNA was used to transform *B. subtilis*. Although a large number of Tc^R-transformants were obtained none of them were resistant to kanamycin.

The hybrid plasmids *pJK*1-3 and *pJK*101 which determine in *E. coli* resistance to ampicillin and tetracycline, showed resistance to tetracycline only, after transformation into *B. subtilis*. The lack of expression of the ampicillin resistance gene of *E. coli* in *B. subtilis* is not caused by a modification of the hybrid DNA in *B. subtilis*, since these plasmids isolated from *B. subtilis* and retransformed into *E. coli* lead again to Ap^R and Tc^R colonies.

To test whether β-lactamase of *pJK*2 is synthesized in *B. subtilis* but not excreted, *B. subtilis* harbouring *pJK*2 was analysed for intracellular β-lactamase activity by the method of Dale and Smith, 1971. No β-lactamase could be detected (Table 2).

Similiar experiments were performed with hybrid plasmids consisting of *pBS*161-1 (fragment *Hind*III-A of *pBS*1) and the *E. coli* vector plasmid *pAC*184 deter-

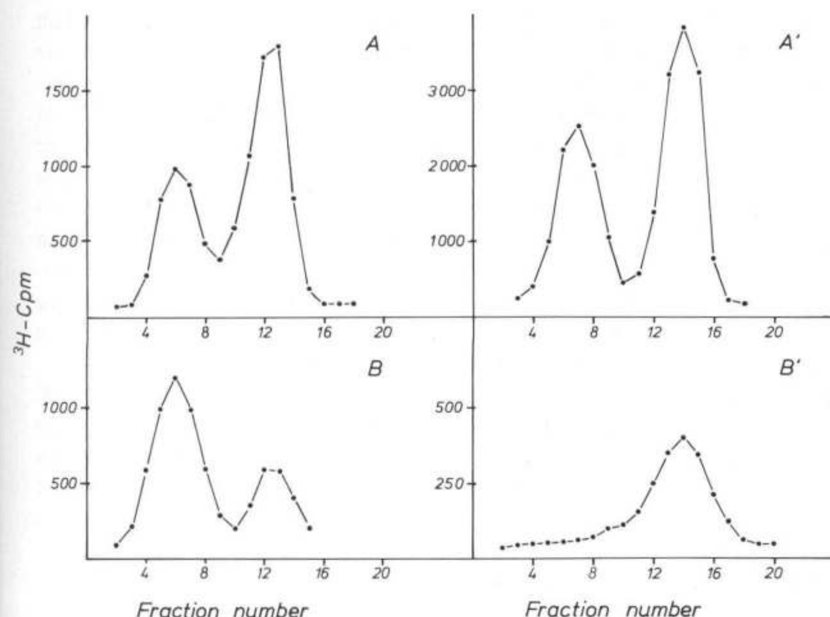

Fig. 7 A and B. Cesium chloride-ethidium bromide gradients of DNA from *E. coli* 5K, *pJK*3 and *B. subtilis* 168, *pJK*3 synthesized in the absence and presence of chloramphenicol (*Cm*). Cultures of *E. coli* 5K and *B. subtilis* 168, carrying the hybrid plasmid *pJK*3 were labeled at a cell density of 2×10^8/ml with 10 μCi/ml ^{3}H-thymidine (spec. act. 24.3 Ci/mol). **A** *E. coli* 5K, *pJK*3 without Cm, **B** *E. coli* 5K, *pJK*3 with 160 μg/ml Cm (**A'**) *B. subtilis*, *pJK*3 without Cm (**B'**) *B. subtilis*, *pJK*3 with 50 μg/ml Cm (other concentrations of Cm – 15 to 150 μg/ml – gave similiar results)

Table 2. Intracellular β-lactamase activity in *E. coli* 5K and *B. subtilis*168 harbouring hybrid plasmid *pJK*2

Bacterial strain	Protein concentration (mg/ml)	Specific activity (units/mg protein)[a]
E. coli 5K pJK2	32	7.3
*B. subtilis*168 pJK2	9	0
*B. subtilis*168	9	0

[a] One enzyme unit hydrolyses one μ/mole Ampicillin/min at 30° C in 25 mM Tris-HCl pH 7.4

mining resistance to chloramphenicol and tetracycline (S. Cohen and A. Chang, unpublished). Transformation of these plasmids into competent cells of *B. subtilis* 168 leads to tetracycline resistant but chloramphenicol sensitive clones, whereas the same DNA transformed in *E. coli* yields Tc^R and Cm^R colonies.

After cultivation of *B. subtilis*-clones, which harbour hybrid plasmids consisting of DNA from *E. coli* and *B. subtilis* (*pJK*1-3), over a longer period of time it was observed that the hybrid plasmids were unstable in this host. CCC-DNA from *B. subtilis* harbouring such hybrids was now isolated, which represents smaller plasmids (Fig. 8). They have lost various parts of the *E. coli* DNA. Some have retained essentially only the genetic information of the *B. subtilis* replicon, i.e. the capability of autonomous replication and the tetracycline resistance of *B. subtilis*. A similiar instability of these hybrids in *E. coli* has not been observed.

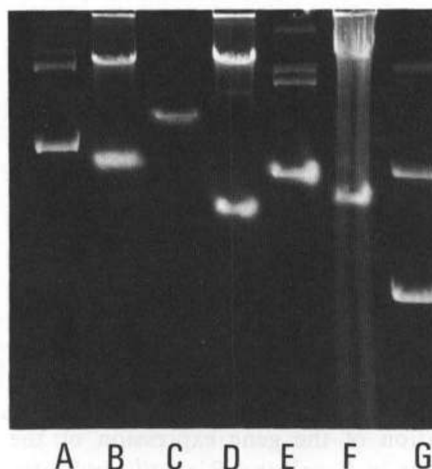

Fig. 8 A–G. *Covalently closed* DNA from *E. cli* and *B. subtilis* clones harbouring hybrid plasmids *pJK*1-3 after cultivation for a longer period of time. CCC-DNA was either purified by CsCl-ethidium bromid gradient centrifugation (**A, C, E**) or cleared lysates were directly applied to the gel (**B, D, F**). **A** *pJK*1 from *E. coli* 5K, **B** *pJK*1 from *B. subtilis* 168, **C** *pJK*2 from *E. coli* 5K, **D** *pJK*2 from *B. subtilis* 168, **E** *pJK*3 from *E. coli* 5K, **F** *pJK*3 from *B. subtilis* 168. **G** *pBR*322

Discussion

The plasmid *pBC*16, which was originally isolated from *Bacillus cereus* has been successfully used as a cloning vector in *B. subtilis*. The in vitro recombination experiments described in this report indicate, that the two important vector properties of this plasmid, namely the tetracycline resistance and the capability

of autonomous replication are located on an *Eco*RI fragment of 2.7 kb, which upon circularization results in a new autonomously replicating plasmid *pBC*16-1, which appears to be a suitable *Eco*RI vector for cloning of genes in *B. subtilis*.

Joining of this plasmid to another cryptic extrachromosomal element from *B. subtilis, pBS*1, does not lead to a stable bifunctional replicon as expected. Instead, the recombinant DNA dissociates in all clones studied into two new autonomously replicating elements *pBS*161 and *pBS*162. The reason for this apparent instability of a recombinant DNA consisting of two functional replicons in *B. subtilis* is unknown. In *E. coli* such bifunctional replicons have been constructed (Cabello et al., 1976; Kollek et al., 1978; Mayer et al., 1977) and shown to be stable.

The tetracycline resistance remains, after dissociation, on the larger plasmid, *pBS*161, which carries only one *Eco*RI site and can be also used as *Eco*RI vector for *B. subtilis*. The plasmid has in addition six HindIII sites from *pBS*1. The largest *Hind*III fragment carries the tetracycline resistance and the genetic information required for autonomous replication. Upon circularization of this fragment an autonomously replicating plasmid *pBS*161-1 is generated which represents a *Hind*III vector for *B. subtilis*.

Recombinant plasmids consisting of this *Hind*III fragment and an *E. coli* plasmid like *pBR*322, *pWL*7 or *pAC*184 are capable of stable replication in *B. subtilis* and *E. coli*. The dependency of replication of these recombinant plasmids on *pol*A in *E. coli* and their inability to replicate in *B. subtilis* in the presence of chloramphenicol indicate that only the homologous replicon is functioning in the corresponding host.

The investigation of the gene expression of the recombinant DNAs in *E. coli* and *B. subtilis* indicates a rather interesting difference between *E. coli* and *B. subtilis*. Whereas the tetracycline resistance of the *B. subtilis* plasmid can be expressed in *E. coli* – although at a lower level than in the natural host – several antibiotic resistances from *E. coli* (kanamycin-, ampicillin- and chloramphenicol resistance) are not expressed in *B. subtilis*. Trivial explanations for this lack in heterologous gene expression in *B. subtilis* could be ruled out: Modification of the recombinant plasmid in *E. coli* or *B. subtilis* does not occur. The orientation of insertion of the fragment is of no influence in any case. It could also be shown that no TEM-β-lactamase determined by the *E. coli* gene is synthesized inside the *B. subtilis* cell.

It remains to be seen whether this lack of heterologous gene expression is a general phenomenon in *B. subtilis*. Since gene expression in the opposite tion, i.e. *B. subtilis* genes in *E. coli*, is possible (Ehrlich et al., 1976; Mahler and Halvorson, 1977) and the expression of other heterologous genes from Gram-positive and Gram-negative bacteria in *E. coli* has been repeatedly demonstrated (for a summary, see Vosberg, 1978), one may speculate that Gram-positive bacteria may have a more limited potential for expression of genetic information from Gram-negative than vice versa. There is also evidence that the heterologous DNA from the recombinant plasmids is more unstable in *B. subtilis* than in *E. coli*. The reason for this difference is also unknown.

It should be emphasized that not only the wild-type strain of *B. subtilis*, but also a non-sporulating mutant of it yields comparable transformation rates with the *Bacillus subtilis* vector plasmid alone and recombinant DNA constructed with it. This makes *B. subtilis* a rather safe host system for gene cloning.

While this manuscript was in preparation, we learned that similiar results concerning the lack of expression of *E. coli* genes in *B. subtilis* were obtained by S.D. Ehrlich (PNAS, in press) using plasmids from *S. aureus* as cloning vehicle.

Acknowledgements. We thank S.D. Ehrlich for sending us his manuscript prior to publication. S. Cohen is thanked for providing the plasmid pAC184, Ph. Kourilsky for the *E. coli* strain DP9 polA$^-$, r$^-$, m$^-$ and P. Lovett for *B. subtilis* BR151-CM1.

We are thankful to Miss M. Vogel and Miss B. Schelle for skillful technical assistance.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 105-A1)

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Communicated by F. Kaudewitz