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Gene Cloning in Organisms Other Than E. coli

Edited by P.H. Hofschneider and W. Goebel

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With 63 Figures

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Preface

Gene cloning for the production of recombinant DNA is usually performed with E. coli. There is, however, no doubt that gene cloning in organisms other than E. coli will assume a much more important role in the future: efficient cloning systems are needed for the analysis of gene expression and its regulation in eukaryotic cells, for the elucidation of the genetic mechanisms of transformation, and for the study of the genetic rearrangement during differentiation and embryogenesis. Furthermore, optimal cloning systems will be required for practical applications in the near future: for the optimal production of antibiotics, amino acids, vitamins, enzymes etc., for the expression of gene products under favorable energetic conditions for mass production, for the expression of glycolysated animal proteins, for the genetic manipulation of plants – and in the more distant future, for gene therapy in man.

The editors have therefore invited leading scientists to summarize the present status and the future applicability of gene cloning systems in their fields of research. To ensure a complete coverage of a genetic system, the editors have generally asked two authors to cover one system.

This volume is a first and unique account of our knowledge of cloning systems employing organisms other than E. coli such as B. subtilis, pseudomonas, neurospora, saccharomyces and streptomyces, as well as of vectors and selection procedures for animal and plant systems including the liposome technique.

October 1981

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Cloning Vectors Derived from Plasmids and Phage of Bacillus

JÜRGEN KREFT* AND COLIN HUGHES*

1 Introduction

Bacillus subtilis is a well-characterized, gram-positive, non-pathogenic, spore-forming soil bacterium which produces a wide array of extracellular enzymes (for reviews see *Young* 1980; *Priest* 1977; *Henner* and *Hoch* 1980). The development of molecular cloning systems within this organism will not only greatly assist biochemical and genetic studies but should play a fundamental role in the further development of biotechnological processes based on the *Bacilli.*

As described by *Ehrlich* (this volume), following the realization that antibiotic resistance plasmids from *Staphylococcus aureus* could be transformed into *B. subtilis* (*Ehrlich* 1977), much effort has been devoted to developing *S. aureus* plasmid cloning vectors for the *Bacilli.* In this review we will describe the current status of vectors constructed from plasmids and phage indigenous to the *Bacilli.*

In the wake of the development of recombinant DNA techniques in *E. coli,* studies of *Bacillus* plasmids gave way to searches aimed at isolating potential cloning vectors. Extrachromosomal DNA in *Bacillus* was first demonstrated in *B. megaterium* and since then many reports have been made of plasmids in this species (*Carlton* and *Smith* 1974; *Rostas* et al. 1980), *B. subtilis* (*Lovett* and *Bramucci* 1975; *Tanaka* and *Koshikawa* 1977; *Bernhard* et al. 1978; *Uozumi* et al. 1980), and *B. pumilus* (*Lovett* et al. 1976).

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Table 1. *Bacillus* plasmids developed as cloning vehicles

Plasmid	Source	Mol. wt. ($\times 10^{-6}$)	Copy number	Restriction sites	Marker	Reference
pLS 28	*B. subtilis (natto)*	4.1	5	*Eco*RI(2), *Bam*HI(1), *Hin*dIII(5)	–	*Tanaka* and *Koshikawa* 1977
pBS 1	*B. subtilis*	5.5	6	*Eco*RI(1), *Bam*HI(1), *Sal*I(1), *Pst*I(3), *Hin*dIII(6)	–	*Bernhard* et al. 1978
pBC 16	*B. cereus*	3.0	20	*Eco*RI(2), *Bam*HI(1)	Tc^R	*Bernhard* et al. 1978
pAB 124	*B. stearothermophilus*	2.9	?	*Eco*RI(3), *Hin*dIII(2), single sites for *Bst*EII, *Cau*I, *Hpa*I, *Xba*I	Tc^R	*Bingham* et al. 1979

Numbers in parentheses indicate the number of restriction sites for this enzyme

However, the majority of these plasmids, in most cases isolated from culture collection strains, lack readily identifiable markers and are thus not of immediate use as cloning vehicles. Nevertheless two such cryptic plasmids, *pBS*1 and *pLS*28, have been developed further. Examination of environmental isolates has proved more successful. *Bernhard* et al. (1978) isolated the tetracycline resistance (Tc^R) plasmid *pBC*16 from a strain of *B. cereus* found in soil, and the antibiotic-resistant thermophiles discovered in river sludge and silage yielded the two Tc^R plasmids *pAB*124 and *pAB*128 (*Bingham* et al. 1979).

The following section describes the development of some of these plasmids (Table 1) as cloning vehicles.

2 Development and Use of Vector Plasmids

2.1 Vectors Capable of Replication Only in B. subtilis

The tetracycline resistance plasmid *pBC*16 isolated from *B. cereus* can be transformed into *B. subtilis* (*Bernhard* et al. 1978) in which it replicates quite stably with no detectable segregation after more than 100 generations without selective pressure. It contains two *Eco*RI sites and in order to determine if these sites were within the tetracycline resistance gene(s) *pBC*16 was partially and completely digested with *Eco*RI and ligated with *Eco*RI linearized *pBS*1, a cryptic plasmid isolated from *B. subtilis*.

None of the Tc^R colonies obtained after transformation of competent cells of *B. subtilis* 168 with the ligated mixture contained a complete hybrid of the two parental plasmids but several derivatives were isolated (Fig. 1), *pBC*16-1, *pBS*161, and *pBS*161-1 being of particular interest (*Kreft* et al. 1978). The plasmid *pBC*16-1 is the circularized large *Eco*RI fragment (mol. wt. 1.8×10^6) of *pBC*16 which obviously carries both the replication functions and the tetracycline resistance determinant.

*pBS*161 and *pBS*162 have been found together in a large number of tetracycline-resistant colonies, the former plasmid alone carrying a Tc^R determinant.

Recircularization in vitro of the largest *Hin*dIII fragment of *pBS*161 yielded *pBS*161-1,

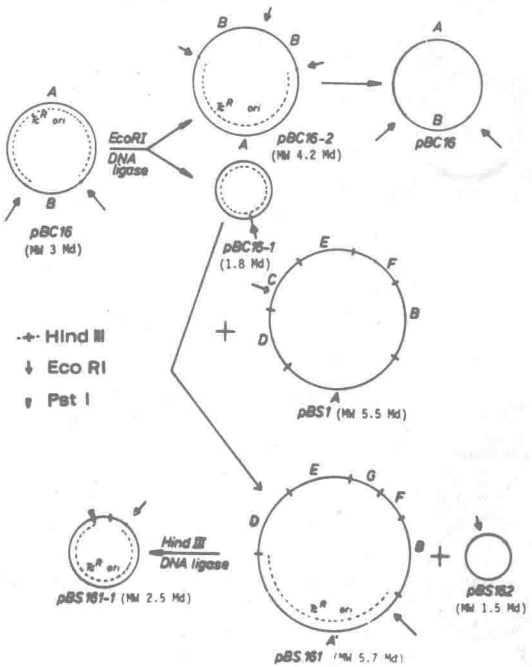

Fig. 1. Genealogy of *Bacillus* plasmids derived from *pBC*16 and *pBS*1. With the exception of *pBS*161-1 only the restriction sites for *Eco*RI and *Hin*dIII are shown

a small Tc^R plasmid (mol. wt. 2.5×10^6) with a high copy number (ca. 20), and single sites for *Hin*dIII, *Eco*RI, and *Pst*I, none of which lies within the replication region or the tetracycline resistance determinant.

The recombinant plasmid RSF2124-B. *leu* (*Nagahari* and *Sakaguchi* 1978), contains the leucine A, B, and C genes of *B. subtilis* and can transform not only *leu*$^-$ *E. coli* but also *B. subtilis* to prototrophy. This plasmid is able to replicate only in *E. coli*. After digestion with *Eco*RI, ligation to *Eco*RI-cleaved *B. subtilis* (*natto*) plasmid *pLS*28 (Table 1), and transformation of *B. subtilis* RM125 (leu$^-$, recE4), *leu*$^+$ transformants yielded two recombinant plasmids *pLS*101 (mol. wt. 6.5×10^6) and *pLS*102 (mol. wt. 10.7×10^6). After subcultivation of these clones slowly growing colonies have been observed containing only the plasmid *pLS*103, which is indistinguishable from *pLS*101 (Fig. 2).

Insertion of foreign DNA into the single *Bam*HI site inactivates *leu*A but not *leu*C, which can thus be used as a marker (*Tanaka* and *Sakaguchi* 1978). A derivative of *pLS*103 termed *pLL*10, has only one *Eco*RI site and complements *leu*A and *leu*B but not, in contrast to *pLS*103, *leu*C. In order to see whether DNA insertion into the remaining *Eco*RI site inactivated the *leu* function and also to introduce another marker, an *Eco*RI fragment carrying a *B. subtilis* 168 trimethoprim resistance determinant was recloned from *pBR*322-Tmp^R into *pLL*10 and transformed into *B. subtilis* ML112. *leu*$^+$ Tmp^R clones yielded *pTL*10 (Fig. 3), a plasmid of mol. wt. 9.4×10^6 giving two fragments of 5.7×10^6 and 3.7×10^6 after *Eco*RI digestion. The latter fragment could convert *B. subtilis* to Tmp^R when inserted in both orientations, indicating its retention of the promoter (*Tanaka* and *Kawano* 1980).

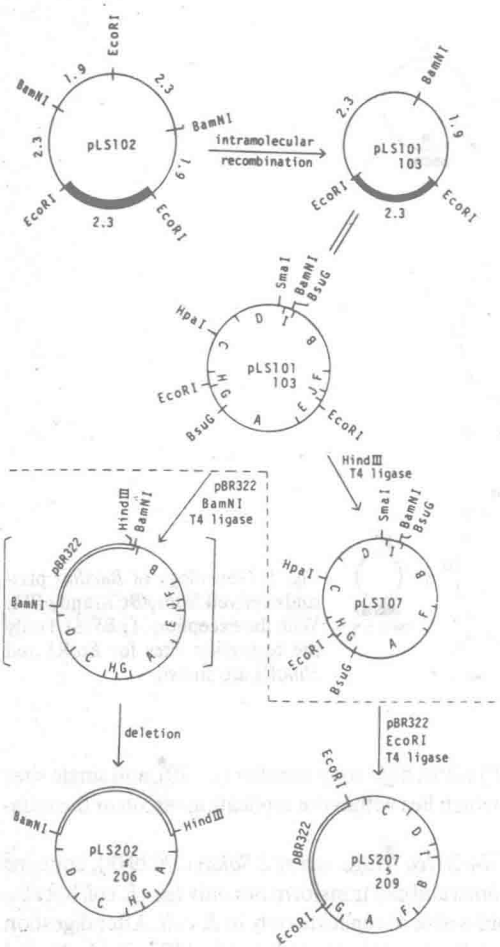

Fig. 2. Structure of constructed plasmids *pLS*101 etc. Numbers denote the molecular weights ($\times 10^{-6}$) of the DNA fragments. The *thick* and *thin lines* indicate the vector and the DNA segments containing the leucine gene respectively. Cleavage sites of *Eco*RI, *Bam*NI, *Sma*I, and *Bsu*G are shown *inside the circles* and those of *Hin*dIII outside. Courtesy of *T. Tanaka* and *K. Sakaguchi*

In order to reduce both the size and number of *Eco*RI and *Bgl*II sites on *pTL*10, the derivative *pTL*12 has been constructed (Fig. 3); a *leu*$^+$ Tmp^R plasmid of mol. wt. 6.4×10^6 carrying single sites for *Eco*RI, *Bgl*II, *Bam*HI, and *Xma*I. *leu* inactivation occurs following insertion at the *Bam*HI and *Xma*I sites, and *Bam*HI cleavage leaves a cohesive end (GATC), making possible the use of *Bgl*II, *Bcl*I, and *Mbo*I, which also leave this sequence. The presence of Tmp^R as a marker allows direct selection of transformants.

The Tc^R plasmid *pAB*124 isolated from *B. stearothermophilus* has three *Eco*RI sites (Table 1). The circularized *Eco*RI-A fragment (pAB224) (*Bingham* et al. 1980) is capable of autonomous replication and carries the tetracycline resistance determinant. It contains single sites for seven restriction enzymes, three of which produce cohesive termini. *pAB*524 has only one *Eco*RI fragment of *pAB*124 deleted (Fig. 4). Table 2 summarizes the properties of the plasmids described in this section. With the exception of *pTL*10 and *pTL*12, all these plasmids carry only one easily detectable genetic marker and do not allow

Table 2. Vectors derived from plasmids listed in Table 1

Vector	Source	Mol. wt. (x 10^{-6})	Single restriction sites	Markers
pBC 16-1	*pBC* 16	1.8	*Eco*RI	Tc^R
pBS 161-1	*pBC* 16/*pBS* 1	2.5	*Eco*RI, *Hin*dIII. *Pst*I	Tc^R
pLS 103	*pLS* 28/ RSF 2124-B. leu	6.5	*Bam*HI, *Sma*I, *Hpa*I, *Xma*I	leu
pLL 10	*pLS* 103	5.7	*Eco*RI, *Bam*HI, *Bgl*I, *Xma*I	leu
pTL 10	*pLL* 10	9.4	*Bam*HI, *Bgl*I, *Xma*I	leu, Tmp^R
pTL 12	*pTL* 10	6.4	*Bam*HI, *Eco*RI, *Bgl*II, *Xma*I	leu, Tmp^R
pAB 224	*pAB* 124	1.95	*Eco*RI, *Hpa*I, *Hpa*II, *Hha*I, *Tha*I, *Cau*II, *Bst*EII	Tc^R
pAB 524	*pAB* 124	2.3	*Hpa*I, *Hpa*II, *Cau*II, *Bst*EII	Tc^R

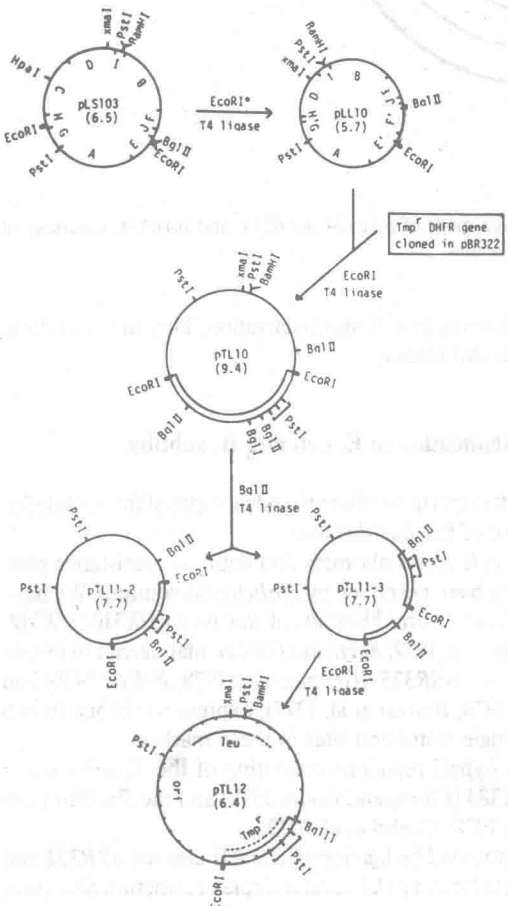

Fig. 3. Structure of plasmids *pLS*103 to *pTL*12. Numbers in parentheses are molecular weights. *Hin*dIII sites of *pLS*103/*pLL*10 are shown inside the circles and these were preserved in *pTL*10. Courtesy of *T. Tanaka* and *N. Kawano*

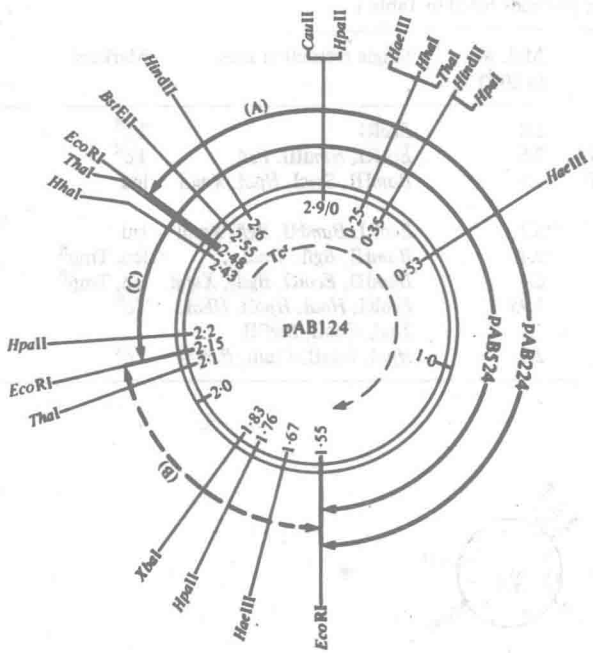

Fig. 4. Restriction endonuclease cleavage maps of *pAB*124, *pAB*224, and *pAB*524. Courtesy of *A.H.A. Bingham*, *C.J. Bruton*, and *T. Atkinson*

identification of recombinant molecules by insertional inactivation. They are, therefore, of limited value as vectors for molecular cloning.

2.2 Hybrid Vectors Capable of Replication in E. coli and B. subtilis

The construction of hybrid vectors attempts to combine the advantages of the well-defined *E. coli* cloning systems with those of the *Bacillus* host.

Hybrid replicons comprising *E. coli* vector plasmids and antibiotic resistance plasmids from *Staphylococcus aureus* have been described by *Ehrlich* (this volume). We have also constructed several *S. aureus/E. coli* hybrid plasmids of this type (*pJK*310, *pJK*312, *pJK*321, *pJK*521, and *pJK*523) (*Goebel* et al. 1979; *Kreft* and *Goebel*, manuscript in preparation). Two of them, *pJK*310 (*pUB*110 + *pBR*325) (*Gryczan* et al. 1978; *Bolivar* 1978) and *pJK*523 (*pC*221 + *pBR*322) (*Novick* 1976; *Bolivar* et al. 1977), express resistance to two antibiotics in *B. subtilis* and carry single restriction sites in these markers.

In addition we have developed hybrid replicons consisting of the *E. coli* vectors *pBR*322 (*Bolivar* et al. 1977), *pACYC*184 (*Chang* and *Cohen* 1978) and the *Bacillus* plasmids *pBS*161-1 and *pBS*1 (*Kreft* et al. 1978; *Goebel* et al. 1979).

*pJK*3 and *pJK*3-1 have been constructed by ligation of *Hin*dIII-cleaved *pBR*322 and *pBS*161-1. From the resulting complete hybrid *pJK*3 several duplex restriction sites have been removed by religation of *Pst*I cleaved *pJK*3, thus yielding *pJK*3-1 (Fig. 5). This plas-

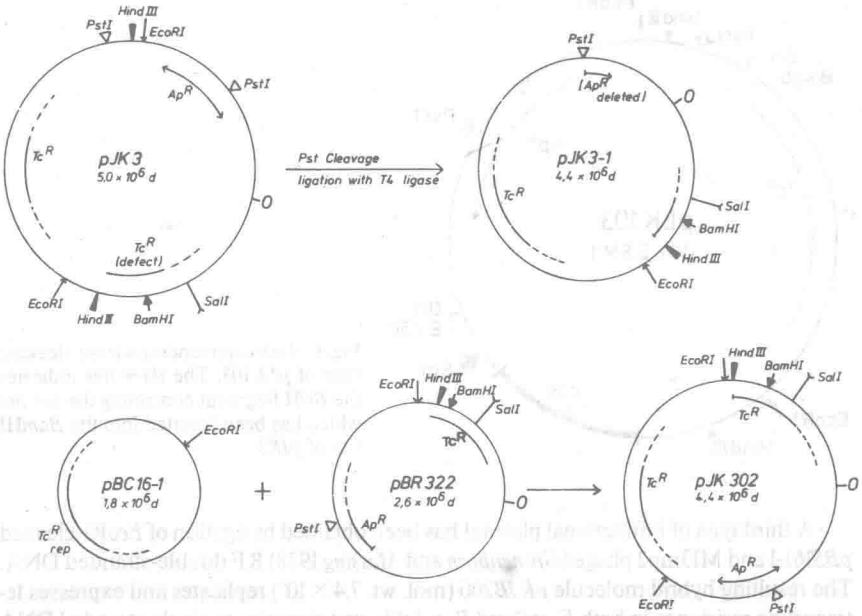

Fig. 5. Construction and restriction endonuclease cleavage maps of *pJK*3-1 and *pJK*302. Construction of *pJK*3 is described in the text

mid has retained only the tetracycline resistance determinant, but has single sites for five restriction enzymes and is capable of replication in both *E. coli* and *B. subtilis*. After transformation of *E. coli* with a ligation mixture from *Hin*dIII-cleaved *pACYC*184 and *pBS*161-1, the largely deleted hybrid plasmid *pJK*201, which carries Cm^R and Tc^R determinants, has been obtained (*Goebel* et al. 1979).

*pJK*302 is a hybrid consisting of *Eco*RI cleaved *pBR*322 and *pBC*16-1. It has single sites for four restriction enzymes, the *Pst*I site being situated in the Ap^R gene. Cleavage with *Eco*RI of both parental plasmids does not inactivate the Tc^R determinants on these; the hybrid *pJK*302 expresses a high level (more than 100 µg/ml) in both *E. coli* and *B. subtilis* (Fig. 5).

Ligation of the *Eco*RI-cleaved plasmids *pBR*322 and *pBS*1 yields in *E. coli* the expected complete hybrid *pJK*501. After transformation of competent cells or protoplasts of *B. subtilis* tetracycline-resistant colonies yield numerous derivatives of *pJK*501 which have deleted different parts of the original plasmid (*Kreft* and *Parrisius,* unpublished observations). One of those derivatives which do not undergo further rearrangements, *pJK*502, has single restriction sites for *Hin*dIII, *Bam*HI, *Sal*I (in the Tc^R determinant), and *Pst*I (in the Ap^R gene).

In order to convert such a bifunctional plasmid into a cosmid system, we have introduced the *cos* site from *pHC*79 (*Hohn* and *Collins* 1980) into *pJK*3, yielding *pLK*103 (Fig. 6). But for unknown reasons all attempts to package this plasmid in vitro into lambda heads have failed so far (*G. Luibrand,* unpublished observations).

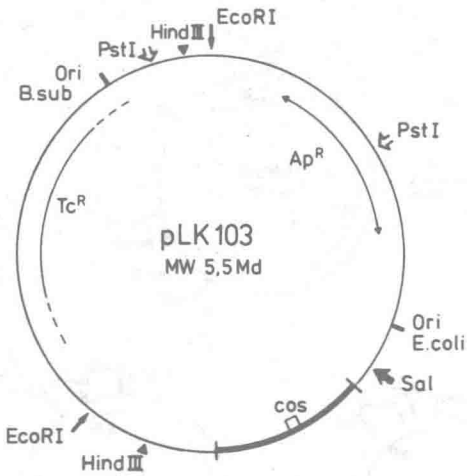

Fig. 6. Restriction endonuclease cleavage map of *pLK*103. The *thick line* indicates the *Bgl*II fragment containing the *cos* site which has been inserted into the *Bam*HI site of *pJK*3

A third type of bifunctional plasmid has been obtained by ligation of *Eco*RI-cleaved *pBS*161-1 and M13mp2 phage (*Gronenborn* and *Messing* 1978) RF double-stranded DNA. The resulting hybrid molecule *pKJB*200 (mol. wt. 7.4×10^6) replicates and expresses tetracycline resistance in both *E. coli* and *B. subtilis*, and gives rise to single-stranded DNA and phage in *E. coli* (*K.J. Burger*, unpublished observations). This plasmid has single restriction sites for *Hin*dIII and *Pst*I and should facilitate DNA sequencing of cloned fragments.

The plasmids *pJK*3, *pJK*302, *pKJ*502 and *pLK*103 carry two antibiotic resistance markers (Ap^R and Tc^R), both spanning single restriction sites, thus allowing the detection of recombinant plasmids by insertional inactivation. However, due to the nonexpression of the Ap^R gene from *pBR*322 in *B. subtilis* (see Sect. 4) its inactivation can only be detected in *E. coli*. Nevertheless recombinant plasmids can subsequently be transformed into *B. subtilis*, using Tc^R as a selective marker.

As will be discussed later, nonexpression and instability of cloned DNA fragments is an important problem in *B. subtilis*. It is, therefore, worthwhile to clone foreign DNA first in *E. coli* and to use the "bridge" character of the described hybrid replicons to introduce the cloned genes into *B. subtilis*.

2.3 Transformation

Bacillus subtilis cells can be transformed by DNA either at the stage of natural competence (*Spizizen* 1958; *Bott* and *Wilson* 1976) or after creation of protoplasts by lysozyme treatment (*Chang* and *Cohen* 1979).

In competent cells the transformability follows the same time course for chromosomal and plasmid DNA (*Contente* and *Dubnau* 1979). Competent cells are efficiently transformed only by oligomers of plasmid DNA (*Mottes* et al. 1979), whereas protoplasts can be transformed with equal efficiency by monomeric or oligomeric plasmid DNA (*Kreft*, unpublished observations).

It has been reported that a restriction-deficient mutant strain of *B. subtilis* can be more efficiently transformed by plasmid DNA than the restriction-proficient parental strain (*Tanaka* 1979). It has also been claimed that strains carrying the *rec*E mutation transform poorly (*Dubnau* et al. 1980). However, in our hands the protoplast system shows no difference between the transformation rates of *B. subtilis* BR151 and BR151 *rec*E4 or between *B. subtilis* MT120 r^-_M m^-_M *rec*E4 and MT128 r^+_M m^+_M *rec*E4 (*Tanaka* 1979). In all cases plasmids *pBS*161-1 (a *Bacillus* plasmid) and *pJK*3 (a *Bacillus/E. coli* hybrid plasmid) have been tested and, interestingly, no difference in transformation rate was seen regardless of whether the *pJK*3 plasmid DNA used for transformation had been isolated from *E. coli* or from *B. subtilis* (*Kreft,* unpublished observations). Clearly the influence of restriction and/or recombination systems on the transformation rate depends upon the particular plasmid used. The transformation efficiency with competent cells or protoplasts is highest with small plasmids, but nevertheless we have been able to transform *B. subtilis* protoplasts with a plasmid of mol. wt. 17×10^6.

2.4 Stability of Vector Plasmids

One prerequisite for the application of a host-vector system to the molecular cloning of DNA is the stable replication of vector plasmids and recombinant molecules in the host. It seems, however, that recombinant plasmids show a remarkable tendency to undergo alterations (mainly deletions) in *B. subtilis.*

Several different mechanisms may be responsible for the phenomena observed. Plasmids can of course recombine with each other or with the chromosome if they contain homologous segments (*Keggins* et al. 1978; *Tanaka* and *Sakaguchi* 1978), the *rec*E4 mutation (*Dubnau* and *Cirigliano* 1974) preventing this recombination process. Intramolecular recombination, on the other hand, can occur without involvement of the *rec*E4 function (*Tanaka* 1979b). In one case it has been shown that such an event can occur in a site-specific way, giving rise to two daughter plasmids containing the entire DNA sequence of the parental plasmid (*Fujii* and *Sakaguchi* 1980). The plasmids *pBS*161 and *pBS*162 (see. Sect. 2.1) may have been generated by the same process, as suggested by the distribution of restriction sites on these and the parental plasmids and the fact that the sum of the molecular weights of *pBS*161 and *pBS*162 is roughly the same as the sum of *pBS*1 and the large *Eco*RI fragment of *pBC*16.

All the hybrid plasmids described in Sect. 2.2 replicate stably in *E. coli* without detectable segregation. In *B. subtilis* the segregation rate after ten generations without selective pressure is 4%–65%, depending on the plasmid examined. They show no deletions or rearrangements while replicating in *E. coli,* but frequently display extensive deletions when isolated from *B. subtilis* transformants.

For example, from *B. subtilis* transformed by *pJK*501 a large variety of deleted plasmids may be obtained, the deletions affecting both parts (*pBS*1 and *pBR*322) of the hybrid plasmid and ranging from $3.6–6.1 \times 10^6$ in size. In some cases if plasmid DNA from *B. subtilis* cells transformed with this plasmid is isolated immediately after transformation, plasmids indistinguishable in size from *pJK*501 are found, while after subcultivation large deletions are again observed. This clearly demonstrates that in this case the deletion event occurs after the uptake of the plasmid into the cell, as has also been proposed by others (*Gryczan* and *Dubnau* 1978).

The occurrence of deletions, at least in the case of *pJK*501, is independent of the *rec*E function and the restriction/modification system of the recipient. Also the type of *E. coli* modification of *pJK*501 plasmid DNA isolated from *E. coli* and used for transformation of *B. subtilis* had no influence on the deletion phenomenon (*Kreft* and *Parrisius*, unpublished observations).

Insertion of different foreign DNA segments into the same vector plasmid showed that not the vector itself but rather the particular combination of vector with another segment of DNA determines whether this new structure is stable in *B. subtilis* (see. Sect. 4).

2.5 Clone Analysis

The screening for recombinant plasmids is difficult in cases where no marker inactivation and no primary selection for a cloned fragment is possible. To screen for plasmids with inserts (or deletions) the use of a rapid lysis procedure is of great advantage, the method of *Birnboim* and *Doly* (1979) giving in our hands satisfactory results for *B. subtilis*.

It should be kept in mind, however, that due to the remarkable tendency for deletions to occur in *B. subtilis* the mere size of a plasmid isolated from transformants is not a reliable criterion in assessing its structure.

Immunologic (*Broome* and *Gilbert* 1978) and colony hybridization methods (*Grunstein* and *Hogness* 1975) can help to identify particular recombinant plasmids, but have yet to be adapted to *B. subtilis*.

For studies on the expression of cloned DNA fragments a minicell system is available for *B. subtilis* (*Reeve* et al. 1973).

3 Use of Bacteriophage Vectors

Several phage systems of *B. subtilis* have been well characterized (*Graham* et al. 1979; *Cregg* and *Ito* 1979; *Mizukami* et al. 1980). In particular, early work has involved the phages Φ3T and p11 which may be termed specialized transducing phages as they carry the thymidilate synthetase gene *thy*P3 (*Dean* et al. 1976). *Kawamura* et al. described in 1979 a method to construct specialized transducers of *B. subtilis* based on the phage p11. This involved cleavage by *Eco*RI of DNA from p11 and the defective phage PBSX induced from *B. subtilis* 168. The latter phage contains only host chromosomal DNA (*Okamoto* et al. 1968), thus limiting the method to the cloning of homologous DNA. It has been extended by *Yoneda* et al. (1979) to permit the cloning into *B. subtilis* of foreign DNA for which no primary selection exists. They chose to construct a specialized transducing phage containing the α-amylase gene(s) from *B. amyloliquefaciens*. Chromosomal DNA from this strain and Φ3T DNA were digested with *Bgl*II, mixed, and ligated. This ligated mixture was then added to a preparation of chromosomal DNA from *B. subtilis* RUB200, a strain prototrophic for threonine and defective in α-amylase synthesis. This mixture was in turn incubated with *B. subtilis* RUB201, a threonine auxotroph lysogenic for Φ3T. *thr*$^+$ transformants were selected and tested for α-amylase production. Competent cells may take up more than one fragment of DNA (congression) so that by selecting, in this case, *thr*$^+$ transformants one effectively enriches for cells carrying foreign DNA (such selection gave a 10^4-fold enrichment for *amy*$^+$ clones). Seven of 10^5 thr$^+$ transformants of RUB201

acquired α-amylase activity and from five of these Φ3T could be induced. Infection of the amy⁻ strain RUB200 with these phages showed a 100% correlation between establishment of lysogeny and the amy⁺ phenotype. Transformation and selection of amy⁺ clones showed cotransformation with the phage specific *thy*P3 gene.

The technique has been utilized for the cloning of *spo* 077 (quoted in *Kawamura* et al. 1980) and *amy*E (*Nomura* et al. 1979) into ρ11, but this phage, having a genome with mol. wt. 80 x 10^6, generates a large number of fragments after routine digestions. *Iijima* et al. (1980) have, therefore, adapted the procedure to the temperate phage Φ105, which has a genome size of 26 x 10^6. Chromosomal DNA of *B. subtilis* 168 (*trp*C2) was prepared from phage PBSX and after *Eco*RI digestion ligated with *Eco*RI digested DNA from Φ105C. Ligated DNA was used to transform *B. subtilis* (*trp*C2 *lys* 3 *met* B10) lysogenic for Φ105C. Selection for auxotrophic markers, subsequent mitomycin C induction, and transduction of the resulting lysate into *B. subtilis* (*trp*C2 *lys* 3 *met* B10) allowed isolation of *met* B⁺ transducing particles. While Φ105C DNA is insensitive to *Bam*HI, incorporation of the new *met* B fragment introduced a single site for this enzyme. This seems possible with other phages, e.g., the virulent Φ1 (*Kawamura* et al. 1980), which also have no *Bam*HI site. A deletion mutant of Φ1, Φ1E2Δ1, has been isolated with increased cloning capacity and this has been used to clone ρ11 fragments which introduced *Bam*HI and *Hae*III restriction sites.

To summarize, initial bacteriophage systems have been shown to be very efficient especially in shotgun cloning of heterologous DNA. Due to the selection marker thymidilate synthetase the phage Φ3T is particularly useful. Major limitations of the method are that by lysogenization normally only single gene copies can be introduced into recipient cells and that induction of lysogens leads to lysis, which might cause containment problems.

4 Molecular Cloning with Plasmid Vectors

In a strict sense the construction of vector plasmids like *pJK*3 (*Kreft* et al. 1978) or *pTL*12 (*Tanaka* and *Kawano* 1980) has already involved the cloning of either heterologous or homologous DNA. This section describes the cloning and expression of isolated genomic fragments and the expression of genetic markers on hybrid replicons.

It has been shown that, at least in certain cases, even DNA cloned in an *E. coli* vector plasmid can transform *B. subtilis* without replication of the recombinant plasmid in the *Bacillus* host (see also Sect. 2.1). The thymidilate synthetase gene from *B. subtilis* bacteriophage Φ3T, which has extensive homology to the chromosomal gene, can transform *thy*⁻ *B. subtilis* to *thy*⁺ when cloned in *pSC*101 or *pMB*9 (*Ehrlich et al.* 1976). On the other hand, the nonhomologous thymidilate synthetase gene from phage β22 can only transform *thy*⁻*B. subtilis* when cloned into an *E. coli* plasmid carrying a small fragment of DNA homologous to the *B. subtilis* chromosome (*Duncan* et al. 1978; *Young* 1980). In this case the whole recombinant plasmid becomes integrated into the chromosome. Recently it has been shown that the thymidilate synthetase gene from *E. coli* can also transform *B. subtilis* when cloned into *pBR*322 or *pMB*9 (*Rubin* et al. 1980). It is not yet clear, however, if there exists sequence homology between the cloned gene and the *B. subtilis* chromosome.

Most of the hybrid plasmids described in Sect. 2.2 carry more than one antibiotic