Construction of a Novel Gene Bank of *Bacillus subtilis* Using a Low Copy Number Vector in *Escherichia coli*

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Low copy number vector plasmid pCT571 was constructed to clone *Bacillus subtilis* genomic fragments in *Escherichia coli*. pCT571 confers Km^R, Tc^R and Cm^R in *E. coli* and Cm^R in *B. subtilis*. It has unique restriction sites within the Km^R and Tc^R markers to allow screening for recombinant plasmids by insertional inactivation of these genes. It contains the pSC101 replicon and replicates normally at six to eight copies per chromosome equivalent in *E. coli*. It also contains *oriV_{RK2}*, which when supplied with the product of the *trfA* gene of RK2 in *trans*, allows pCT571 to replicate at 35–40 copies per chromosome equivalent. A *B. subtilis* gene bank was created by cloning partially *Sau3A*-digested and size-fractionated fragments of *B. subtilis* chromosomal DNA into the *Bam*HI site of pCT571. DNA from 1097 Km^R Tc^S transformants was extracted and analysed electrophoretically as supercoiled DNA and after digesting with *Eco*RI or *Eco*RI and *Sal*I. Approximately 1000 hybrid plasmids were found with reasonably sized *B. subtilis* fragments. The mean size of the inserts in pCT571 is 8 kb, ranging from 4 to 20 kb in different plasmids. The gene bank covers most of the *B. subtilis* chromosome, as demonstrated by the results of screening the gene bank for selectable nutritional markers in *E. coli* and *B. subtilis*. Hybrid plasmids which complement *E. coli* mutants for *arg*, *his*, *lys*, *met*, *pdx*, *pyr* and *thr* markers were identified from the gene bank. In *B. subtilis* the presence of *argC*, *cysA*, *dal*, *hisA*, *ilvA*, *leuA*, *lys*, *metB*, *metC*, *phe*, *purA*, *purB*, *thr* and *trpC* was established by transformation experiments. The effects of copy number on cloning and long-term maintenance in the bacterial strains were also investigated. At high copy number some hybrid plasmids cannot be maintained at all, while others show an increased rate of structural deletions and rearrangements.

INTRODUCTION

The use of high copy number plasmid cloning vectors has the advantages of allowing the isolation of large amounts of cloned DNA fragments and also aids the purification of proteins coded by the cloned genes. While partially successful attempts have been made to prepare, in *Escherichia coli*, plasmid gene banks of the *Bacillus subtilis* genome, using fragments produced by shearing or by digestion with restriction endonucleases (Hutchinson & Halvorson, 1980; Rapoport *et al.*, 1979; Ostroff & Pène, 1984*a, b*), a number of problems have been encountered. The final gene bank often has a less than random assortment of relatively small genomic fragments (Hutchinson & Halvorson, 1980; Gryczan & Dubnau, 1982). This may be due to the dosage of cloned genes joined to high copy number plasmids having deleterious effects on the bacterial host (Banner *et al.*, 1983), as well as promoting instability of the inserted segment. Ostroff & Pène (1984*a, b*) succeeded in cloning 8–9 kb fragments of *B. subtilis* DNA in a bifunctional plasmid vector in *E. coli* as an intermediate host, but a variety of their cloned fragments were unstable, and they observed deletions of both the insert and vector plasmid sequences when hybrid plasmids were returned to *B. subtilis*.

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In specific cases (for example, Mountain *et al.*, 1984) it has been observed that some of these problems can be avoided by using low copy number cloning vectors. We therefore set out to construct a gene bank representative of the *B. subtilis* chromosome in a reasonably small, low copy number plasmid vector. However, we thought it desirable to design the vector so that the copy number could be increased under specific conditions. This should not only facilitate the isolation of hybrid plasmid DNA and purification of the products of cloned genes but should also allow us to determine whether deleterious effects on the host or structural instability of the hybrid plasmid at high copy number are a common problem with clones selected randomly from such a gene bank.

Here we report the construction of the vector pCT571, its use in the production and screening of a gene bank of *B. subtilis* in *E. coli* and the effects of high copy number on hybrid plasmids from this gene bank.

METHODS

Bacterial strains and plasmids. Bacterial strains used in this study are shown in Table 1. Previously described plasmids used for the construction of the vector pCT571 were pLG339 (Stoker *et al.*, 1982), pCT461 (Thomas, 1983) and pHV60 (from Myra Jacobs, G. D. Searle Ltd, High Wycombe, UK) which is indistinguishable from pHV32 (Primrose & Ehrlich, 1981). Plasmid pUC99 (from R. Sammons, University of Birmingham, UK) was constructed by inserting an 8.2 kb *EcoRI* fragment carrying the *his* genes of *B. subtilis* into pUC9 (Vieira & Messing, 1982). Plasmids constructed during the course of this work are described in the text.

Bacterial growth. Growth medium for *E. coli* was L-broth (Kahn *et al.*, 1979), which was solidified with 1.5% (w/v) agar for L-agar. Incubation was at 37 °C. For the selection of antibiotic resistance markers (Km^R , Tc^R , Cm^R and Pn^R) carried by plasmids, medium was supplemented with kanamycin (50 µg ml⁻¹), tetracycline (25 µg ml⁻¹), chloramphenicol (5 µg ml⁻¹) and penicillin (300 µg ml⁻¹). For the selection of nutritional markers, M9 minimal agar (Kahn *et al.*, 1979) was supplemented with growth requirements (for amino acids, at 50 µg ml⁻¹), except those for which selection was made.

For *B. subtilis*, potato/glucose/yeast extract (PGYE) agar and Difco Penassay broth were used as rich media. Minimal medium (Anagnostopoulos & Spizizen, 1961) was supplemented with glucose (0.5%) and amino acids (50 µg ml⁻¹) where required. Growth was at 37 °C or 42 °C.

Transformation of bacteria. *E. coli* strains were made competent essentially as described by Thomas (1981). *B. subtilis* strains were transformed as described by Anagnostopoulos & Spizizen (1961).

Preparation of DNA. Plasmid DNA was extracted by the alkaline-SDS method of Birnboim & Doly (1979) as modified by Smith & Thomas (1983). *B. subtilis* chromosomal DNA was prepared according to Marmur (1961).

Manipulation of DNA. Restriction endonucleases were obtained from BRL, P & S Biochemicals Ltd or NBL. Enzymes and used under conditions similar to those recommended by the suppliers. Terminal 5' phosphates were removed from plasmid vector DNA using calf intestinal alkaline phosphatase (BCL). Partially *Sau3A*-digested chromosomal DNA of *B. subtilis* was fractionated by velocity centrifugation through a sucrose density gradient to isolate fragments in the range of 4–20 kb as described by Maniatis *et al.* (1978). Agarose gel electrophoresis was done under standard conditions.

RESULTS

Construction of the vector plasmid pCT571

The first step in the construction of this vector was the derivation of a new plasmid with reasonably small size and a copy number which, while normally low, can be increased under particular circumstances. This was achieved using plasmids pLG339 and pCT461. Plasmid pLG339, which is derived from pSC101, has a copy number of six to eight per chromosome in *E. coli* and confers resistance to tetracycline and kanamycin (Stoker *et al.*, 1982). Plasmid pCT461 is a mini RK2 (*E. coli* IncP) derivative. The two regions essential for replication of this plasmid are *oriV_{RK2}*, the vegetative replication origin, and the *trfA* gene, whose protein product acts positively in *trans* to allow initiation of plasmid replication (Thomas, 1981). It has a copy number of 35–40 per chromosome due to deletions in the *oriV_{RK2}* region (Thomas *et al.*, 1984), and was used as the source of a fragment carrying a mutant *oriV_{RK2}* which when supplied with

Table 1. *Bacterial strains*

Strain	Description	Reference or source*
<i>E. coli</i> K12		
MV10	C600 $\Delta trpE5$ (<i>thr-1 leu-6 thi-1 lacY1 supE44 tonA21 trpE5</i>)	D. R. Helinski
MV10Ch3/86	MV10 carrying hybrid λ prophage Ch3/86 with <i>trfA</i> and <i>trfB</i> regions of plasmid RK2	Thomas <i>et al.</i> (1981)
HB101	F ⁻ <i>hsdS20</i> [Res ⁻ Mod ⁻ (B)] <i>recA13 ara-14 lacY1 proA2 galK2 rpsL20</i> (Sm ^R) <i>xyl-5 mtl-1 supE44</i> λ^-	D. R. Helinski
ED8654	<i>supE supF hsdR⁻ M⁺ S⁺ met trpR</i>	J. Guest; Murray <i>et al.</i> (1977)
2235	<i>his argH thr leu trp cysA phe mtl lac gal rpsL</i> (Sm ^R) <i>malA</i> (Muc ⁺)	Departmental stock
2848	[R ₁₀₀₋₁ (Tc ^S)] λ^+ <i>his trp lys gal malB rpsL</i> (Sm ^R)	Departmental stock
2608	<i>pyrF30 ilv-277 met-65 his-53 purE41 proC24 pdxC3 xyl-14 lacY29 str-97 cys-2 cycA1 tonA32</i>	Departmental stock
<i>B. subtilis</i>		
PY79	Prototroph	Youngman <i>et al.</i> (1984)
1606	<i>leuA metA thyA thyB</i>	N. Sueoka
1612	<i>dal-1 aroI sacA metB</i>	Departmental stock
1A10	<i>trpC2 hisA1 thr-5</i>	R. Dedonder
1A3	<i>trpC2 cysA14 purA16</i>	R. Dedonder
1A5	<i>trpC2 glyB733 metC3</i>	R. Dedonder
1412	<i>purA16 lys-21 phe-12 metB5 arg-3</i>	Departmental stock
1458	<i>ilvA trpC2</i>	C. Anagnostopoulos
1411	<i>purB6 leu-8 metB5</i>	N. Sueoka
1417	<i>hisA1 argC4</i>	Departmental stock

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trfA in *trans* will allow the plasmid to replicate at an elevated copy number. The new plasmid, pCT570, was constructed by combining the *oriV_{RK2}* and Km^R segment of pCT461 with the replicon and Tc^R of pLG339 (Fig. 1). A fragment carrying a Cm^R determinant which can be expressed in *B. subtilis* was then introduced into pCT570 from pHV60 (Fig. 1). The resulting plasmid, pCT571, is 9.5 kb in size, confers Km^R, Tc^R and Cm^R in *E. coli* and Cm^R in *B. subtilis* and has at least seven unique restriction sites: *Bam*HI, *Sal*I and *Sph*I in the Tc^R gene and *Hind*III, *Sma*I and *Xho*I in the Km^R gene, allowing insertional inactivation of these markers to be used to screen for insertion of foreign fragments. While pCT571 has *oriV_{RK2}*, it lacks *trfA*, which is essential for *oriV_{RK2}* activity. Normally, therefore, only the pSC101 replicon functions and the copy number of pCT571 will be six to eight copies per chromosome. However, in a bacterial strain carrying *trfA* in *trans* the copy number of pCT571 will rise.

Plasmid pCT571 cannot replicate in *B. subtilis*; it can only be used to transform *B. subtilis* to Cm^R if it gains either a *B. subtilis* replicon or chromosomal DNA allowing it to integrate into the chromosome by homologous recombination. We checked that pCT571 can be used to transform *B. subtilis* in this way by inserting into it an approximately 400 bp *Bam*HI fragment (from pUC99) carrying part of the *B. subtilis* *his* genes. DNA of this plasmid, pCT572, was used to transform the prototrophic *B. subtilis* strain PY79 to Cm^R. As expected, Cm^R transformants were obtained with pCT572 but not pCT571. That integration had occurred by homologous recombination at the *his* locus was confirmed by checking the His phenotype. If the *his* fragment in pCT572 contains an internal segment of the *his* operon then a Campbell-type integration will result in inactivation of the *his* genes, leading to a His⁻ phenotype. This was indeed observed. Selection for His⁺ bacteria subsequently resulted in loss of Cm^R, as expected. Therefore pCT571 behaves as expected in transformation of *B. subtilis*, when it carries a segment of *B. subtilis* DNA (see previous work with integrational vectors, e.g. Piggot *et al.*, 1984).

Fig. 1. Construction and structure of pCT571. Genetic loci are as defined in the text. The *Eco*RI to *Xho*I segment of pLG339 containing the *Tc^R* determinant and *pSC101 rep* were joined to the *Eco*RI to *Sal*I segment of pCT461 containing *Km^R* and *oriV_{RRK2}*. The resultant plasmid, pCT570, was cut with *Eco*RI and *Bam*HI and then the *Eco*RI to *Bam*HI segment of pHV60 carrying *Cm^R* was inserted, giving pCT571, which is shown on an enlarged scale. Restriction sites are abbreviated as follows: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; Sa, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; Ss, *Sst*II; X, *Xho*I.

Gene bank construction and screening

The *B. subtilis* gene bank was constructed by ligating fragments ranging in size from 4 to 20 kb of partially *Sau*3A-digested *B. subtilis* chromosomal DNA (from PY79), fractionated by sucrose velocity gradient centrifugation, to dephosphorylated *Bam*HI-digested pCT571, and subsequently transforming *E. coli* strain ED8654, in which pCT571 has a low copy number. DNA of 1097 *Km^R Tc^S* colonies was extracted from separate 1.5 ml cultures. Insertion of foreign fragments into pCT571 was confirmed by checking the electrophoretic mobility of the plasmid DNA on 1% (w/v) agarose gels with pCT571 as a marker, and was further characterized by

digesting all plasmids with *EcoRI*. The restriction pattern of some plasmids was also determined after *EcoRI* and *SalI* digestion. Out of 1097 plasmids, 120 plasmids were not used for further analysis, because of either having a very small insert or no apparent insert at all. The mean size of the inserted *B. subtilis* DNA segments is approximately 8 kb (8.15 ± 3.21), as calculated from 75 randomly selected plasmids from the gene bank.

To demonstrate that the cloned DNA fragments represent *B. subtilis* DNA, the ability of a range of plasmids to transform *B. subtilis* to Cm^R was tested. DNA of 17 plasmid representatives of the gene bank was transformed into low (MV10) and high (MV10Ch3/86) copy number strains and reisolated. For both strains one tenth of the plasmid DNA extracted from 1.5 ml overnight cultures was used to transform *B. subtilis* strain PY79 to Cm^R . While only four plasmid preparations from the low copy number strain gave any transformants (mean = 1.5 transformants per transformation of these four), only two of the 17 plasmids failed to give any transformants using DNA from the high copy number strain (mean = 69 transformants per transformation over all 17). A number of factors may be responsible for this difference. In addition to the greater absolute amount of plasmid DNA from the high copy number strain (greater by a factor of approximately five), the increase may be due to the lower ratio of RNA and chromosomal DNA fragments to plasmid DNA available, or to the increased proportion of plasmid multimers in preparations from the high copy number strain (Canosi *et al.*, 1978). The results show that the hybrid plasmids produced have gained segments with homology to the *B. subtilis* chromosome allowing transformation to Cm^R by homologous recombination.

After constructing the gene bank, the next step was to determine whether it contained cloned segments representative of the whole *B. subtilis* genome. Genetic loci, generally nutritional markers, representing most regions of the *B. subtilis* chromosome were chosen; the presence of these specific *B. subtilis* genes in the gene bank was tested using pooled plasmid DNA to transform *E. coli* and *B. subtilis* mutant strains, direct selection being applied for the ability to grow in the absence of particular nutritional requirements. In *E. coli*, conversion to prototrophy for particular markers should occur by complementation since it has been shown that *B. subtilis* genes which are expressed during vegetative growth are generally expressed and can function in *E. coli* (for example, Mahler & Halvorson, 1977; Nagahari & Sakaguchi, 1978; Rabinovich *et al.*, 1978; Chi *et al.*, 1978; Moir, 1983). In *B. subtilis*, conversion to prototrophy should occur by integration of the wild-type sequence into the chromosome.

The bank of 977 hybrid plasmids was first pooled in 95 mini pools consisting of between eight and 11 plasmids per mini pool. For *E. coli*, 95 mini pools were further pooled into nine groups each containing approximately 100 plasmids, whereas for studies in *B. subtilis*, the mini pools were pooled to give 30 batches of approximately 30 plasmids.

The following nutritional markers were chosen to screen the gene bank in *E. coli*: *arg*, *his*, *lys*, *thr*, *cysA*, *met-65*, *pdxC* and *pyrF30*, since in *B. subtilis* these are distributed over the whole of the *B. subtilis* chromosome. Competent mutant strains were transformed separately with DNA from the nine plasmid pools. Strains with multiple requirements were then spread on supplemented minimal plates, selecting for independence from one different nutritional requirement at a time. Experiments were repeated three times. DNA of plasmids apparently complementing different nutritional markers was extracted from selected transformants, and in each case the corresponding plasmid in the gene bank was determined by comparing their *EcoRI* and *EcoRI* plus *SalI* digestion patterns with those of the individual plasmids comprising the pool. Competent *E. coli* strains with the specific markers were transformed again with both the complementing plasmid and the apparently identical plasmid from the gene bank, to confirm and demonstrate the specificity of the *B. subtilis* genomic segment in that particular plasmid. If possible, plasmids identified in this way in *E. coli* were also used for transformation of appropriate *B. subtilis* strains, to confirm that the results obtained in *E. coli* did indeed genuinely identify specific *B. subtilis* genes. The cumulative results of these experiments are shown in Table 2 and show that a variety of *B. subtilis* genes were identified in this way.

Pooled plasmid DNA was screened in *B. subtilis* by transformation and selection for the prototrophic phenotype of each particular marker. *E. coli* strain MV10Ch3/86, in which hybrids have a high copy number, was transformed with DNA from each pool to obtain a better yield of

Table 2. *Summary of genetic loci identified in the gene bank*

+, Positive result for complementation (*E. coli*) or transformation (*B. subtilis*); ++, more than one plasmid was identified.

Genetic marker screened	Complementation in <i>E. coli</i>	Transformation of <i>B. subtilis</i>
<i>argA</i>	+	
<i>argC</i>		+
<i>cysA</i>		+
<i>dal-1</i>		+
<i>hisA</i>	+ *	+
<i>ilvA</i>		+
<i>leuA</i>		+
<i>lys</i>	++	+
<i>metB</i>	+ *	+
<i>metC</i>		+
<i>pxd</i>	+	
<i>phe-12</i>		+
<i>purA</i>		+
<i>purB</i>		+
<i>pyr</i>	+ †	
<i>thr</i>	++	+
<i>trpC</i>		+

* Genotype of plasmid from *E. coli* verified in *B. subtilis*.

† Genotype of plasmid verified by hybridization with *pyr* probe (Chi *et al.*, 1978).

pooled DNA, since such DNA transformed *B. subtilis* more efficiently than plasmid DNA from the low copy number strain (see above). All transformants from one pool were resuspended together in L broth and used to extract DNA directly and samples of this DNA were used to transform *B. subtilis* strains. In this way the gene bank was screened for the following nutritional markers: *argC4*, *cysA14*, *dal-1*, *hisA1*, *ilvA*, *leuA*, *lys-21*, *metB*, *metC3*, *phe-12*, *purA16*, *purB6*, *thr-5*, *trpC*. In each case a significant number of transformants were obtained, indicating the presence of DNA corresponding to these loci in the gene bank. Plasmids identified in transformation experiments for nutritional markers *arg*, *his*, *lys* and *met* in *E. coli* were also used to transform phenotypically similar *B. subtilis* mutant strains. The plasmids isolated as complementing the Lys⁻ phenotype in *E. coli* failed to give transformants for *lys-21* in *B. subtilis*, indicating the probable presence of at least two different *lys* genes of *B. subtilis* in the gene bank. Plasmids pSHarg1 and pSH526, which were identified as complementing an Arg⁻ *E. coli* strain (*argH*), failed to transform a *B. subtilis argC* strain to Arg⁺. It seems likely that pSH526 has a *B. subtilis* genomic segment with *argA* on it (Mountain *et al.*, 1984). Plasmids pSH296 and pSH113, which successfully complemented the *his* and *met* mutations respectively in *E. coli*, were subsequently identified as having the genomic segments of *B. subtilis* carrying *hisA* and *metB* genes by transformation experiments in *B. subtilis*. Identical plasmids (pSH993, pSH1012, pSH1018, pSH1022 and pSH1026) which complemented *pyr* in *E. coli* hybridized strongly with a 6.7 kb *EcoRI* fragment carrying *pyrD*, *F*, *E* and part of *B*, cloned in λ by Chi *et al.* (1978).

While individual plasmids carrying specific markers were identified by complementation in *E. coli*, the screening of pooled plasmids for nutritional markers by transformation of *B. subtilis* was not generally taken as far as the identification of individual plasmids. However, that positive results with pooled DNA can be the starting point for successful isolation of individual hybrid plasmids from this gene bank is illustrated by the isolation of hybrid plasmids carrying the *sdh* operon and *gerE* gene (Hasnain *et al.*, 1985). The range of the *B. subtilis* chromosome (Henner & Hoch, 1980) covered by genes which we have evidence for in the gene bank gives us confidence that a large proportion of all genes can be easily isolated from this gene bank using appropriate screening procedures.

Table 3. Comparison of the ability of vector and hybrid plasmid DNA to transform low (MV10) and high (MV10Ch3/86) copy number strains

Plasmids	No. of transformants†	
	MV10	MV10Ch3/86
pLG339	162	~ 5000
pCT571	~ 5000	~ 25000
pSHpyr2	406	5
pSH550	276	13
pSH550*‡	1318	10
pSH1019	62	2250
pSH1019*‡	0	~ 5000
No plasmid DNA	0	0

† For pLG339 and pCT571, approximately 1 µg CsCl/ethidium bromide purified DNA was used. For the other plasmids, one tenth of the DNA yielded from a 1.5 ml overnight culture by the alkaline SDS method was used.

‡ Plasmid DNA designated pSH550* and pSH1019* was extracted from MV10Ch3/86 which had been transformed with pSH550 and pSH1019 respectively (see Results).

Stability of hybrid plasmids

As already described in the Introduction, difficulties encountered in cloning and stably maintaining hybrid plasmids at high copy number were the primary reasons for constructing the gene bank in the low copy number plasmid pCT571. In order to determine whether or not this strategy allows one to clone segments which cannot be cloned at high copy number, we exploited the fact that in a strain carrying the *trfA* gene of RK2 the mutant *oriV_{RK2}* in pCT571 is functional and the copy number of pCT571 increases to 35–40 copies per chromosome equivalent. Under these circumstances we found that plasmids from the gene bank behaved in one of a number of different ways. Described in some detail below are examples of the major categories of plasmid found.

The first category of plasmid are those which cannot be introduced into the high copy number strain MV10Ch3/86. Examples of such plasmids are pSH550 and pSHpyr2, which are probably identical and which contain an approximately 7.5 kb insert, apparently carrying at least part of the *B. subtilis* *Pyr* genes. While with both pLG339 and pCT571 the number of transformants was greater in MV10Ch3/86 than in MV10 (which probably reflects competence of the bacteria, since replication of pSC101 is not affected by *trfA*), it was observed that the number of transformants obtained using DNA of pSH550 and pSHpyr2 was substantially higher in MV10 than in MV10Ch3/86 (Table 3). Plasmid DNA extracted from transformants of the high copy number strain contained various kinds of deletions or insertions as compared with pSH550. Even between the colonies from a single transformation experiment different *Eco*RI restriction patterns were observed, while no change in plasmid DNA from the low copy number strain was observed. It seemed possible that the deletions or insertions observed may have inactivated a function on the *B. subtilis* genomic segment in pSH550 which was deleterious at high copy number. Therefore plasmid DNA designated pSH550*, extracted from MV10Ch3/86 (in which hybrids based on pCT571 have a high copy number) which had been transformed with pSH550, was tested for its ability to retransform MV10 and MV10Ch3/86 efficiently. While there was an apparent increase in transformation efficiency for MV10, the number of transformants in MV10Ch3/86 was again very low (Table 3), and plasmid DNA extracted from the transformants of MV10Ch3/86 showed further changes in plasmid restriction pattern compared to pSH550*, while no change was observed in MV10. Although we do not yet know the reason for our inability to establish pSH550 in MV10Ch3/86, it does appear that the defect is not entirely removed by the changes in plasmid structure which apparently allow the plasmid to become established in MV10Ch3/86. Nor have we established at what stage the plasmid variant derivatives arise. It is possible that they represent spontaneous events in the strain from which the plasmid DNA was isolated. Alternatively they may represent derivatives which arise after

Table 4. Transformation activity and stability of various hybrid plasmids in low (MV10) and high (MV10Ch3/86) copy number strains

Plasmid	Size of <i>B. subtilis</i> segment (kb)	No. of transformants*		Changes observed†	
		MV10	MV10Ch3/86	Low copy strain	High copy strain
pSH2	7	18	75	—	After 6 weeks
pSH4	9	266	720	—	—
pSH8	5	150	1120	—	—
pSH29	13	56	120	—	—
pSH225	12	93	850	—	—
pSH245	12.5	71	510	—	—
pSH359	9	288	1200	—	—
pSH408	10	54	432	—	—
pSH471	11	133	520	—	—
pSH546	3.5	560	1500	—	—
pSH658	9	94	1230	—	—
pSH679	12	245	530	After 19 weeks	After 13 weeks
pSH692	10	259	1100	—	After 26 weeks
pSH715	16	67	620	After 6 weeks	After 6 weeks
pSH749	4.5	448	915	—	After 26 weeks
pSH763	8	243	1320	—	—
pSH1024	12.5	230	510	—	—
pSH1041	8	500	1500	—	After 13 weeks

* Plasmid DNA was extracted by the alkaline/SDS method and one tenth of the yield from a 1.5 ml overnight culture was used for each transformation.

† In some cases (—) no change was observed after 32 weeks; where changes were observed, the time of their earliest appearance is recorded.

transformation into the high copy number recipient. However, it is clear that the parent plasmid itself, pSH550, is unable to become established in the high copy number strain.

The second category of plasmid are those plasmids which can be introduced efficiently into the high copy number host but which are apparently maintained poorly and rapidly give rise to deletion derivatives. An example of such a plasmid is pSH1019, which has an approximately 20 kb insert that from hybridization data appears also to carry at least part of the *B. subtilis* *pyr* genes. This plasmid gave many more transformants in MV10Ch3/86 than in MV10, but plasmid DNA extracted from cultures grown from three randomly selected transformants of MV10Ch3/86 showed only small amounts of plasmid DNA in contrast to the increase in copy number expected. The *Eco*RI restriction pattern obtained suggested that the plasmids present were identical to pSH1019 isolated from the low copy number strain. However, if transformants were not used to extract DNA immediately but were stored at room temperature, then within 24 h, deletion derivatives could be identified in the plasmid DNA extracted. When plasmid DNA extracted from such transformants, designated pSH1019*, was used to transform MV10 and MV10Ch3/86, the transformation frequency in MV10Ch3/86 was high (Table 3) but no transformants were obtained in MV10. A possible explanation for this is that the deletion derivatives obtained have not only lost part of the inserted *B. subtilis* DNA segment but also part of the pSC101 replicon that is essential for plasmid replication in MV10. Thus, while the hybrid plasmid pSH1019 can be introduced into the high copy number strain, it rapidly gives rise to deletion derivatives which are maintained in preference to the parent.

The examples of plasmids in these first two categories were encountered by chance when investigating plasmids carrying the *pyr* genes of *B. subtilis*. A third category of plasmid, those which in the short to medium term are stable at both low and high copy number were encountered when 18 plasmids from the gene bank were selected for study of hybrid plasmid stability. These plasmids were selected by virtue of their possession of reasonably large segments of *B. subtilis* DNA (between 5 and 15 kb) so as to optimize the likelihood of encountering *B. subtilis* loci which may be deleterious at high copy number. For each of these plasmids, the number of transformants obtained in MV10Ch3/86 was greater than in MV10 (Table 4),

although the absolute number of transformants varied considerably. Plasmid DNA isolated from both high and low copy number strains looked identical with respect to restriction pattern, except for the expected increase in quantity of DNA in the MV10Ch3/86 strains. To study the long-term stability of hybrid plasmids, a single transformant was streaked to single colonies on selective medium and after growth at 37 °C was stored at room temperature. Plasmid DNA in these cultures was examined after 6, 13, 19, 26 and 32 weeks by inoculating 1.5 ml L broth supplemented with kanamycin.

Observations from these studies on long term stability fall into three groups (Table 4). First, plasmids which showed no detectable change in their *EcoRI* restriction pattern after storage in either low or high copy number strain over 32 weeks. Second, plasmids (pSH2, pSH692, pSH749 and pSH1041) which exhibited insertions and deletions only in the high copy number strain. Third, plasmids (pSH715 and pSH679) which suffered deletions in both low and high copy number strains, although with pSH679, deletions were observed earlier in the high copy number strain. Generally, changes, if they occur, lead to loss of the parental plasmid restriction pattern and the production of what seems to be a mixture of derivatives. In most cases the composition of the mixture changed with time, eventually resulting in one predominant derivative. In the case of pSH1041 and pSH679, these final stable forms were small plasmids which had lost all *EcoRI* restriction sites, indicating that the deletions had extended well into the vector DNA. DNA extracts of all 18 plasmids from the low copy number strain kept at 4 °C showed no changes in *EcoRI* restriction pattern even after 6 months. It appeared that temperature also contributed to the instability observed in the DNA of the hybrid plasmids.

DISCUSSION

The aim of this work was to construct in *E. coli* as complete a gene bank of *B. subtilis* as possible, using a low copy number vector whose copy number can be increased when desired, and so to determine whether construction of a gene bank at low copy number allows one to clone DNA segments which it would be difficult or impossible to clone in a high copy number plasmid. The vector which we have constructed for this purpose, pCT571, appears to work well. It differs from dual replicon runaway vectors (Andreoli, 1985) in being maintained stably at high copy number with no apparent harm to the host cell. On the other hand it does seem to be maintained at sufficiently high copy number, when the RK2 replicon is activated, to unmask at least some of the consequences of trying to maintain some *B. subtilis* chromosomal segments at high copy number.

In the low copy number strain pCT571 seems to be very suitable for constructing a gene bank. The pSC101 replicon, while having a relatively low copy number, yields enough plasmid DNA by rapid isolation methods to allow easy analysis of clones. Screening of the hybrids produced indicates that many of the clones contain large *B. subtilis* chromosome segments. By a combination of complementation in *E. coli* and recombination in *B. subtilis* we have shown the presence of markers which come from all regions of the chromosome. The fact that all auxotrophic mutants of *B. subtilis* tested could apparently be transformed to prototrophy by one or other of the pools of hybrid plasmids encourages us to think that the gene bank covers most of the genome. Using the formula of Clarke & Carbon (1976) we calculate that that probability of any given sequence of the *B. subtilis* genome being present in our gene bank is about 90%. Where we have studied a hybrid plasmid from the gene bank in detail (carrying the *sdh* operon and *gerE*; Hasnain *et al.*, 1985), the evidence suggests that the hybrids obtained contain a single segment of the genome with the same structure as in the *B. subtilis* chromosome, and that this genomic segment is maintained reasonably stably in contrast to the instability observed in a similar segment recently reported as having been cloned on a high copy number plasmid (Magnusson *et al.*, 1985). In addition, experiments with a hybridization probe show that for a given region of the genome (in this case the *pyr* operon) there are a number of clones containing non-identical but overlapping segments of the genome (Hasnain, 1985). Thus, not only does this gene bank cover most of the chromosome but it should also provide an opportunity to 'walk' from known and easily selected genes into less well defined regions. It is also stored as DNA of

individual hybrids and should not therefore be subject to loss of physically unstable or poorly replicating hybrids, as found in previously constructed gene banks (Rapoport *et al.*, 1979; Ferrari *et al.*, 1981; Ostroff & Pène, 1984a, b).

The gene bank provided us with the opportunity to study the effects of vector plasmid copy number on the cloning and long term maintenance of *B. subtilis* chromosomal DNA. While a random sample of hybrids carrying large inserts showed no instability in the short term, during the course of this work we did come across a number of hybrids which could not exist stably at high copy number. Both of the plasmids of this sort reported in this paper carry *B. subtilis* DNA from the region coding for the *pyr* operon (Hasnain, 1985). We do, however, have another hybrid, pSH1012, which complements *E. coli pyr* mutants and hybridizes strongly with the λ *pyr* isolated by Chi *et al.* (1978) that carries at least part of the *B. subtilis pyr* operon, and this plasmid is stable at high copy number. It is possible that the unstable plasmids carry deleterious genes absent from pSH1012. Alternatively, the unstable plasmids may lack regulatory elements which normally stop the over-production of deleterious gene products. A phenomenon which may be explained in the latter way has been described by Mountain *et al.* (1984), who cloned *EcoRI* fragments of the *B. subtilis* genome in *E. coli* using pBR322 as a vector. They found that a 12 kb fragment of hybrid plasmid pUL720, which complemented *E. coli* mutant strains defective for *argA*, *B*, *C*, *D*, *E*, *F/I*, *cerA* and *cerB*, can only be maintained intact in the presence of a second *EcoRI* fragment, from elsewhere on the *B. subtilis* chromosome, that appears to code for the *arg* repressor gene (Mountain *et al.*, 1985). They suggest that one of the products of the *arg* operon is host lethal in *E. coli* if transcription is not regulated.

Deletion derivatives arise from unstable plasmids at various stages. For pSH550, which cannot be transformed intact into the high copy number strain, it seems likely that the few transformants obtained may arise from deletion derivatives present in trace quantities in the plasmid DNA of the low copy number strain. With plasmids which can be introduced efficiently into the high copy number strain it is likely that derivatives arise within this strain itself. However, it is unclear whether the unstable plasmids promote structural instability or simply have a sufficiently deleterious effect on their host that bacteria with deletion derivatives have a major advantage. This question is particularly intriguing with respect to bacteria carrying hybrid plasmids that are stored at room temperature, since we do not know whether the appearance of plasmid derivatives after many weeks of storage results from preferential survival of bacteria carrying them. Whatever the underlying mechanism giving rise to the deletion derivatives, it is clear from the studies on long term stability that storage of high copy number hybrid plasmids at room temperature can lead to loss of the plasmid. Use of low copy number vectors helps to overcome this problem and storage at 4 °C provides effectively stable maintenance. Nevertheless, storage of isolated plasmid DNA is probably even more reliable.

Thus, the use of low copy number vectors in gene bank construction has been shown to provide a way of avoiding the considerable problems of genetic instability created by the use of high copy number vectors. We hope that the gene bank constructed in this study will prove useful in the isolation and analysis of previously uncharacterized regions of the *B. subtilis* chromosome.

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