Use of the *Escherichia coli* Transposon Tn1000 ($\gamma\delta$) to Generate Mutations in *Bacillus subtilis* DNA

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Plasmid pHM2 contains a *Bacillus subtilis* *spoIIA* gene and is able to replicate in both *Escherichia coli* and *B. subtilis*. The plasmid was mobilized at low frequency by the *E. coli* F plasmid. Nearly 30% of the mobilized plasmids contained an insert of Tn1000 ($\gamma\delta$). Fourteen of the inserts were in the *B. subtilis* DNA portion of pHM2. Of these, two adjacent inserts abolished expression of the plasmid *spoIIA* gene in *B. subtilis*. From the map positions of flanking inserts that do not abolish *spoIIA* gene expression, it is estimated that the gene is probably not more than 700 bp long.

INTRODUCTION

One of the most substantial technical advances in the last decade in the genetic analysis of enterobacteria has been the development of methods that use translocatable DNA elements, transposons (see Kleckner *et al.*, 1977). Transposons can insert into the chromosome or into plasmids. Generally such inserts are stable once formed. Many transposons carry a drug-resistance determinant, and this is particularly useful for chromosome mutations caused by insertion as it provides a positive selection for mutant phenotypes that are otherwise unselectable. Transposons have a defined size and this is particularly useful with plasmids as it allows accurate mapping of the site of insertion by restriction endonuclease analysis.

Bacillus subtilis is a spore-forming Gram-positive bacterium which has very good systems of genetic exchange. For this reason it has been widely used in studies of cellular differentiation (see Piggot *et al.*, 1981). In addition it is potentially an important cloning host for industrial use (see Hoch *et al.*, 1982) as it is also non-pathogenic and is already widely used industrially. However, the species has been poorly served by transposons. Relatively few transposons have been identified in Gram-positive bacteria, and so far only one has been shown to transpose in *B. subtilis* (Youngman *et al.*, 1983). In general, drug-resistance determinants from Gram-negative bacteria are not expressed in *B. subtilis* and there is no evidence of enterobacterial transposons transposing in *B. subtilis*. However, the availability of *B. subtilis* genes cloned on chimeric plasmids that can replicate in *Escherichia coli* and *B. subtilis* opens the possibility of using transposons in *E. coli* to analyse *B. subtilis* DNA. We have previously described the isolation of a plasmid, pHM2, that contains a 3.3 kb insert of *B. subtilis* DNA in pHV33 (Liu *et al.*, 1982); the latter is a chimera of the enterobacterial plasmid pBR322, and the staphylococcal plasmid pC194 (Primrose & Ehrlich, 1981). Plasmid pHM2 confers resistance to chloramphenicol and ampicillin on *E. coli* and to chloramphenicol on *B. subtilis*. We report here the use of the 5.7 kb

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Table 1. *Escherichia coli* strains

Strain	Relevant genotype	Origin/Reference
Km 4104	(F ⁻) <i>lysA argA</i> Δ 2134(gal-bio) Δ (<i>srl-recA</i>) <i>strA</i>	S. Sedgwick
RB 85	(F ⁻) <i>thr leu lacY</i> (λ^-) <i>str supE44 tonA</i>	R. S. Buxton
RB 308	(F ⁺) <i>thr leu thyA deoC lacY</i> (λ^-)	R. S. Buxton
SL 2022	(F ⁻) <i>hsdR supE recA56</i> (λ^-)	Chak <i>et al.</i> (1982)
SL 3045	As RB 308 (pHM2)	pHM2 transformed into RB 308
SL 3051-3078	As RB 85 (pHM2::Tn1000 Ω 3051- Ω 3078)	pHM2 mobilized from SL 3045, into RB 85
IGC 1003, 1007, 1026-1028	As RB 85 (pHM2::Tn1000 Ω 1003 etc.)	pHM2 mobilized from SL 3045 into RB 85
IGC 1029, 1030, 1035	As HM 4100, (pHM2::Tn1000 Ω 1029 etc.)	pHM2 mobilized from SL 3045 into KM 4104

E. coli transposon $\gamma\delta$ (Tn 1000) to study the *B. subtilis spoIIA* gene on pHM2. Tn1000 is derived from the *E. coli* F plasmid, and is inserted into poorly transmissible plasmids such as pBR322 when they are mobilized by F during conjugation (Guyer, 1978); all such mobilized plasmids were found to contain Tn1000, and insertion was at random (Guyer, 1978). These properties make it very useful for analysing plasmids even though no easily selectable marker is associated with Tn1000.

METHODS

Strains. The *B. subtilis* 168 strains SL1013 (*spoIIA69 lys-3 trpC2*) and SL1060 (*spoIIA69 trpC2 recE4*) have been described previously (Liu *et al.*, 1982). The *E. coli* strains used are listed in Table 1.

Plasmid preparation. Plasmids were prepared from *E. coli* by the alkaline-SDS method of Birnboim & Doly (1979) as modified by Ish-Horowicz & Burke (1981). This method was modified further for *B. subtilis* by incubating bacteria with lysozyme (200 μ g ml⁻¹) in solution I for 30 min at 37 °C.

Mating. Donor (SL3045) was grown up in L-broth containing ampicillin (Amp, 25 μ g ml⁻¹), and recipient (RB85 or KM4104) in L-broth. Growth was overnight at 35 °C without shaking. The cultures were separately diluted 1 to 50 in L-broth, and incubated with very gentle shaking at 37 °C; after 2 to 3 h, 0.1 ml donor culture was mixed with 1.0 ml recipient culture and incubated without shaking for 1 h at 37 °C. The culture was then diluted into 4 ml L-broth containing 200 μ g streptomycin (Sm) ml⁻¹ and incubated at 37 °C with vigorous aeration for 2 h. The culture was then diluted and plated on L-agar containing 200 μ g Sm and 100 μ g Amp ml⁻¹ to select for mobilization of pHM2. Resulting colonies were screened for resistance to 10 μ g chloramphenicol (Cm) ml⁻¹.

Sporulation. Cultures were induced to sporulate by the method of Sterlini & Mandelstam (1969) except that all cultures contained 3 μ g Cm ml⁻¹ and were incubated at 42 °C.

Gel electrophoresis. Electrophoresis of DNA was generally carried out with horizontal 0.8% agarose gels as described previously (Liu *et al.*, 1982). DNA was extracted from gels by electrophoresis on to DEAE paper as described by Dretzen *et al.* (1981).

Enzymes. Restriction enzymes were purchased from P & S Biochemicals, Liverpool, U.K. or from BRL. They were used according to the manufacturers' specifications, or according to the methods of Davis *et al.* (1980). T4 DNA ligase was purchased from Boehringer and used according to manufacturer's instructions. The structures of plasmids constructed by ligating restriction fragments were confirmed by restricting the plasmids and analysing the products by gel electrophoresis.

Transformation. Protoplast transformation of *B. subtilis* was by the method of Chang & Cohen (1979). Transformants were selected, and bacilli regenerated, on DP agar (Wyrick & Rogers, 1973) containing 3 μ g Cm ml⁻¹. Sporulation type was tested by streaking transformants to single colonies on nutrient agar (NA) containing 3 μ g Cm ml⁻¹. 'Classical' transformation of competent bacilli was by the method of Bott & Wilson (1968). All other methods have been described previously (Liu *et al.*, 1982).

RESULTS

Insertion of Tn1000 into pHM2

Strain SL3045 containing pHM2 and the F plasmid was mated with a Sm^R F⁻ strain, and recipient bacteria containing mobilized pHM2 were selected on solid media containing Sm and Amp. Such exconjugants were obtained at a frequency of about 10⁻⁴ to 10⁻⁵ of the recipient bacteria. Colonies were purified by single colony isolation on media containing Sm, Amp and

Fig. 1. Restriction map of pHM2 showing the sites of Tn1000 insertion. The plasmid is represented as a linear structure having as its ends the *Hind*III site that joins the pBR322 and pC194 portions of the molecule (Primrose & Ehrlich, 1981) and that is almost coincident with the *Eco*RI site of pBR322. The region of *B. subtilis* DNA is outlined in bold. The Amp^R and Cm^R determinants are indicated. The replication functions are denoted by Rep. The order of *Hind*III fragments along the map (kb) is 2.9, 0.51, 0.85, 0.42, 4.9; the 2.9 kb fragment corresponds exactly to pC194. The order of *Eco*RI fragments (kb) is 3.6, 0.20, 0.38, 1.5, 4.9. The restriction sites are identified below the map: H, *Hind*III; E, *Eco*RI; B, *Bgl*II; S, *Sal*I. The sites of Tn1000 insertion are shown by vertical lines above the map; the longer lines indicate insertion in the orientation $\gamma\delta$, and the shorter lines insertion in the orientation $\delta\gamma$ (Guyer, 1978). The names of inserts in the *B. subtilis* DNA are indicated above these inserts. A restriction map of Tn1000 based on that of Guyer (1978) is shown below that for pHM2. The scale (in kb) is at the bottom of the Figure.

Cm, and plasmid prepared from each isolate. Of 225 clones examined, 159 contained plasmids of the same electrophoretic mobility as pHM2, 64 contained plasmids of the mobility expected for pHM2::Tn1000, and two contained plasmids of both sizes.

The sites of Tn1000 insertion were determined by restriction-endonuclease mapping, making use of the known restriction maps of pHM2 and Tn1000 (Liu *et al.*, 1982; Guyer, 1978; Fig. 1); it should be noted that there are no known restriction sites at the ends of Tn1000. In general, identification of the sizes of fragments formed by separate digestions with two enzymes, *Hind*III and *Eco*RI, was sufficient to work out the site and orientation of insertion. Digestion with *Bgl*II or a *Hind*III–*Sal*I double digestion, was usually sufficient to resolve cases where interpretation was ambiguous. Thus *Hind*III digestions of 15 plasmids showed that eight had lost the 5.9 kb fragment of pHM2 (Fig. 2, tracks B, E, G, K, L, M, P and Q); four of these, with inserts 3059, 3071, 3072 and 3078 (tracks G, K, L and Q, respectively), gave *Hind*III fragments that were similar to, but distinguishable from, the 5.9 kb fragment of pHM2; the preparation of pHM2:: Ω 3054 (track B) also appeared to contain a small amount of pHM2 without any insert. Five of the plasmids had lost the 2.9 kb fragment (tracks C, D, H, I and O); one had lost the 0.85 kb fragment (track N); and one, SL3058 (track F), gave a complex pattern that was not analysed.

Taking pHM2:: Ω 3074 as an example, the insert was in the 0.85 kb *Hind*III fragment of pHM2, and two *Hind*III fragments of 2.5 and 1.68 kb were generated that were unique to Ω 3074 (the 2.5 kb fragment unique to Ω 3074 co-migrates with the 2.5 kb fragment that is derived exclusively from Tn1000 and is seen for all pHM2::Tn1000 derivatives; note the extra strength of the 2.5 kb band compared to the 2.9 kb band, Fig. 2). Each of these unique bands was

Fig. 2. Agarose gel (0.8%) electrophoresis of pHM2::Tn1000 plasmids digested with *Hind*III. Sizes (kb) of the pHM2 *Hind*III, and pHM2 *Hind*III-*Sal*I fragments (Liu *et al.*, 1982), and the 2.5 kb Tn1000 *Hind*III fragment (Guyer, 1978) are indicated. Lanes A and J, pHM2 digested with *Hind*III; lane R, pHM2 digested with *Hind*III and *Sal*I; lanes B to I and K to Q, pHM2 with different Tn1000 inserts, digested with *Hind*III: B, Ω 3054; C, Ω 3055; D, Ω 3056; E, Ω 3057; F, Ω 3058; G, Ω 3059; H, Ω 3060; I, Ω 3061; K, Ω 3071; L, Ω 3072; M, Ω 3073; N, Ω 3074; O, Ω 3075; P, Ω 3076; Q, Ω 3078.

presumed to consist of a part of the 0.85 kb pHM2 fragment and one of the ends of Tn1000, which are approximately 1.1 and 2.1 kb from the nearest *Hind*III sites within Tn1000 (Guyer, 1978; Fig. 1). Thus the unique 2.5 kb fragment consisted to 2.1 kb from the δ end of Tn1000 and 0.4 kb from the 0.85 kb pHM2 fragment; the 1.68 kb fragment consisted of 1.1 kb from the γ end of Tn1000 and 0.58 kb from the 0.85 kb fragment. Thus the δ end of Tn1000 is nearer (0.4 kb) to a *Hind*III site on pHM2 than is the γ end (0.58 kb); there is some inaccuracy in measuring sizes of DNA fragments from their mobility in gels, so it is not surprising that these numbers do not add up exactly to 0.85 kb. An unambiguous determination of the orientation and site of the Ω 3074 insert was made from the additional information that Ω 3074 had disrupted the 0.20 kb *Eco*RI fragment of pHM2. In this way, the orientation and sites of insertion of 58 independent Tn1000 transpositions were determined (Fig. 1); in three further cases Tn1000 was shown to have inserted outside the *B. subtilis* DNA in pHM2, although the exact location was not determined.

The mobilized plasmids were selected for replication in *E. coli* and for Amp^R and Cm^R, so that insertions were not obtained in the region of pHM2 determining these functions. Insertions were found in most other regions of the plasmid although none were obtained in the pC194 replication functions which were thought not to be used in *E. coli*. Inserts were found in both orientations, but a substantial majority, 42/58, were orientated $\delta\gamma$ in the sense of Fig. 2. Fourteen inserts were in the *B. subtilis* DNA.

Table 2. Characterization of transformants of *B. subtilis* SL1060 (*spoIIA69 recE4*) by pHM2::Tn1000 plasmids

Transformants of SL1060 protoplasts were selected on DP regeneration agar (Wyrick & Rogers, 1973) containing $3 \mu\text{g Cm ml}^{-1}$. Individual clones were streaked to single colonies on NA-Cm and scored for their sporulation phenotype. The inserts are arranged in their order in *B. subtilis* DNA (Fig. 1). Plasmids with inserts Ω 1007 and Ω 1026 were not tested.

Insert in plasmid	No. of transformants in 0.2 ml culture	Behaviour on NA-Cm	
		No. tested	Sporulation phenotype
3053	1480	18*	+
1029	94	22	+
1030	120	23	+
3072	234	18*	+
1035	91	23*	—
1027	33	23*	—
3051	1336	18*	+
3052	788	18*	+
1028	9	7	+
1003	18	17	+
3074	331	18*	+
3062	536	18*	+

* Plasmid DNA was prepared from clones in these crosses, see text.

Effect of Tn1000 insertions on *spoIIA* in *B. subtilis*

Plasmids containing homologous DNA interact with the chromosome when transforming Rec^+ *B. subtilis* strains (Canosi *et al.*, 1981), and this can alter the plasmid DNA (Iglesias *et al.*, 1981; Chak *et al.*, 1982). Consequently, pHM2::Tn1000 derivatives were transformed into a *spoIIA69* strain, SL1060, that contains the *recE4* mutation which largely prevents such interaction. The possibility of such interactions was reduced further by transforming protoplasts (Chang & Cohen, 1979; Mottes *et al.*, 1979).

Twelve of the 14 plasmids having inserts in *B. subtilis* DNA were tested, and all transformed SL1060 protoplasts (Table 2). Several transformant clones from each cross were re-isolated on NA-Cm medium and scored for spore formation. All transformants with pHM2:: Ω 1027 and pHM2:: Ω 1035 were asporogenous and exhibited the abortive disporic SpoII phenotype characteristic of *spoIIA* mutants (Piggot & Coote, 1976). All transformants with all other donors were Spo⁺ (Table 2).

To ensure that the plasmids had not been altered during transformation, plasmids were prepared from transformant clones from several of the crosses (Table 2). In all cases except Ω 1027 and Ω 1035 they were prepared from isolates that had been heated to 85 °C for 20 min. This ensured that the plasmids were obtained from heat-resistant spores. The plasmids had the same electrophoretic mobility in 0.8% agarose as the original pHM2::Tn1000. The plasmids were also transformed into *E. coli*; plasmids were prepared from these transformants, and the tests repeated, with the same results. In addition, the preparations for Ω 1027 and Ω 1035 were shown to transform SL1013 (*spoIIA69 rec*⁺) to Spo⁺ by recombination, confirming that the wild-type allele of the *spoIIA69* mutation was still present in these plasmids.

We also transformed SL1060 with pHM2::Tn1000 by the classical competence method. Few transformants were obtained, and many of these contained altered plasmids, presumably resulting from interaction of the plasmid with the chromosome through slight leakiness of the *recE4* mutation. Nonetheless, the results also indicated that Ω 1027 and Ω 1035 abolished the *spoIIA* complementing activity of pHM2, whereas all other inserts were ineffective.

In an attempt to quantify the sporulating ability of protoplast transformant clones, cultures were induced to sporulate in the replacement system of Sterlini & Mandelstam (1969). Cm ($3 \mu\text{g ml}^{-1}$) was included in all media because the plasmids were lost rapidly in its absence. The bacteria grew slowly in the growth medium, having mass doubling times of approximately

Table 3. *Sporulation of strain SL1060 (spoIIA69 recE4) containing pHM2 with various inserts*

Cultures were induced to sporulate in the medium of Sterlini & Mandelstam (1969) supplemented with 3 µg Cm ml⁻¹. After 18 h, the sporulation medium cultures, with or without heating to 85 °C for 20 min, were diluted, plated on NA with or without 3 µg Cm ml⁻¹ and incubated at 42 °C for 40 h. The colonies obtained are recorded as white (W), brown (B), or small white (SW).

Tn1000 insert	Heat treatment	No. of c.f.u.*				
		NA-Cm			NA	
		W	B	SW	W	B
3051	—	0	66	134	179	0
	+	0	168	13	353	0
3052	—	0	86	116	177	0
	+	0	101	0	255	1
3053	—	0	109	83	263	0
	+	0	128	6	225	0
3072	—	4	171	109	323	4
	+	1	170	2	382	0

* Numbers multiplied by 10⁵ (unheated cultures) or 10⁴ (heated cultures) give the number of c.f.u. in 1 ml culture.

60 min. All sporulated to some extent after 18 h in the sporulation medium, with 5–20% of the c.f.u. being heat-resistant (Table 3); there was no evidence of plasmids with inserts near the ends of the *spoIIA* gene (3051 and 3072) behaving differently from plasmids with inserts further away (3052 and 3072). There was considerable lysis in the sporulating cultures, and this precludes a more forceful statement about sporulating frequencies. However, several other observations can be made. (1) The small white colonies seen on NA-Cm plates grew as large Spo⁻ colonies upon subculture on NA, but not at all upon subculture on NA-Cm. Presumably they contained bacteria that had lost the plasmid, indicating that it can be lost even in the presence of Cm. In agreement with this the sum of the numbers of brown and small white colonies from untreated cultures on NA-Cm approximately equalled the number of white colonies on NA (Table 3). (2) In the absence of Cm there is rapid loss of plasmid as indicated by the preponderance of Cm^S white (Spo⁻) colonies on nutrient agar. (3) The number of heat-resistant survivors that could form colonies on NA was about double the number that could form colonies on NA-Cm. This may indicate that, after the plasmid-borne *spo*⁺ allele had functioned, the plasmid was lost during either sporulation or germination; the paucity of small white colonies from heated cultures could indicate that germination is particularly sensitive to Cm. (4) Occasionally large white colonies were observed on NA-Cm. These occurred more frequently (about 1% of brown colonies; unpublished data) in unheated samples than in heated samples (0.1%). All those examined contained a plasmid that was smaller than pHM2::Tn1000, indicating deletion formation.

Analysis of subclones of pHM2

To confirm the location of the *spoIIA* gene on pHM2, we constructed a set of subclones. The *Hind*III 5.9 kb and the *Eco*RI 4.9 kb fragments of pHM2 each contain the Amp^R determinant and the replication functions of pBR322. Each was isolated, ligated to itself, and transformed into *E. coli*. The resulting plasmids were maintained in *E. coli*; they could not replicate in *B. subtilis*. Both plasmids transformed SL1013 to Spo⁺ by recombination (Table 4), so that they contained at least part of the *spoIIA* gene. To obtain derivatives that could replicate in *B. subtilis*, the *Hind*III 5.9 kb fragment was ligated to the *Hind*III 2.9 kb fragment, and the *Eco*RI 4.9 kb fragment to the *Eco*RI 3.6 kb fragment. As before, both could transform SL1013 to Spo⁺ by recombination. In addition, both could transform SL1060 to Cm^R and were maintained as plasmids in SL1060. The *Hind*III-constructed plasmid, but not the *Eco*RI-constructed plasmid complemented the chromosomal *spoIIA69* mutation. It is concluded that *Hind*III 5.9 kb

Table 4. Transformation of *spoIIA69* strains with plasmid subclones of pHM2Numbers in parentheses indicate the number of Spo⁺ transformants among the Cm^R transformants.

Donor plasmid	Recipient . . .	No. of transformants from 1 ml recipient culture			
		SL1013 (<i>rec</i> ⁺)		SL1060 (<i>recE4</i>)	
		Selection . . .		Selection . . .	
		Spo ⁺	Cm ^R	Spo ⁺	Cm ^R
<i>Hind</i> III 5.9 kb		48 120	ND	5	ND
<i>Eco</i> RI 4.9 kb		16 510	ND	0	ND
<i>Hind</i> III 5.9, 2.9 kb		124 000	2 725(2 650)	1 070	1 665(1 665)
<i>Eco</i> RI 4.9, 3.6 kb		7 930	75 (0)	0	1 945 (0)
None		20	0	0	0
pHM2		35 080	2 120(2 070)	6 320	2 520(2 520)

ND, Not determined.

fragment carried the complete *spoIIA* gene, and that the gene spanned the *Eco*RI site between the *Eco*RI 4.9 kb and 1.5 kb fragments (Fig. 1). This is consistent with the gene location determined by insertional mutagenesis.

DISCUSSION

Plasmid pHM2 was mobilized at a low frequency by the F plasmid. Approximately 30% of the mobilized plasmids contained Tn1000, and this high proportion obviated the need for a selective character for transposition. We did not obtain the 100% insertion in mobilized plasmids reported by Guyer (1978); however, Buxton & Drury (1983) have also obtained less than 100% insertion. It may be that Tn1000 was not essential for mobilization of pHM2, or that it was essential, but was subsequently excised from 70% of the mobilized plasmids. We cannot, at present, assess the relative merits of these possibilities. Nor can we assess the significance of the observation that the majority of inserts were in one orientation.

Fourteen of the Tn1000 inserts (approximately 23%) were in *B. subtilis* DNA. It was possible to transfer the plasmids concerned to *B. subtilis*, and hence study the effect of Tn1000 on the expression of the *spoIIA* gene. Two adjacent inserts, 1027 and 1035, abolished *spoIIA* expression whereas the flanking inserts 3051 and 3072 did not. It seems unlikely that any part of Tn1000 could substitute for a *spoIIA* gene function, and so it is concluded that the gene (or at least the functional part of the gene) lies between the insertion sites 3051 and 3072, a distance of about 0.7 kb. This location was confirmed by analysis of subclones of pHM2. A gene of this size could code for a polypeptide of up to about 26 000 Dal.

A group of *spoIIA* mutations that map close to *spoIIA69* on the chromosome (Piggot, 1973; Yudkin & Turley, 1981) are not covered by the *B. subtilis* DNA in pHM2, and are presumed to lie in an adjacent gene (Liu *et al.*, 1982). The distance of the *spoIIA* gene on pHM2 from the nearest end of the *B. subtilis* insert in pHM2 is approximately 0.45 kb which opens the possibility that there may be a gap between this gene and the gene(s) within which the other mutations lie.

The major problem in studying expression in *B. subtilis* was plasmid stability. This manifested itself in three ways. (1) Plasmid rearrangements during transformation because of interaction with the chromosome; this necessitated checks for each new transformant clone. (2) Rapid loss of plasmid in the absence of Cm. This behaviour is exhibited by derivatives of pC194, such as pHV33 and pHM2, in which the *Hind*III site of pC194 has been disrupted (Ehrlich *et al.*, 1981). pHM2 : Tn1000 derivatives appeared less stable than pHM2 and this may be a consequence of the larger plasmid having a lower copy number. (3) Formation of deletions in plasmids maintained in *B. subtilis*. This has been observed for many other plasmids maintained in *B. subtilis*. We had no indication that the deletions related specifically to the presence of Tn1000, although size, in itself, may be a destabilizing factor.

Despite these reservations, Tn1000 can be used to analyse gene expression in *B. subtilis* by insertional mutagenesis. As only one transposon is known to function in *B. subtilis* (Youngman *et*

al., 1983), this is an important additional technique. It is likely that other *E. coli* transposons, such as Tn5, can also be used in this way. Canosi *et al.* (1981) have shown that a region of homology with the host chromosome facilitates transformation of *B. subtilis* Rec⁺ strains by plasmid monomers. Iglesias *et al.* (1981) showed that deletion or insertion in the plasmid within the region of homology could be corrected by interaction with the chromosome. The set of plasmids described here, with inserts of defined size at different positions in or near the region of homology, may offer a way to analyse this phenomenon further.

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