The Mechanism of Insertion of a Segment of Heterologous DNA into the Chromosome of Bacillus subtilis

By MICHAEL YOUNG

Department of Botany and Microbiology, University College of Wales, Aberystwyth SY23 3DA, U.K.

(Received 25 August 1982; revised 20 October 1982)

An Escherichia coli plasmid, p1949, that is derived from pMB9 and pC194, and unable to replicate in Bacillus subtilis, can give rise to stable Cm\(^{R}\) transformants of the latter species if it is inserted into the bacterial chromosome. A purified segment of the B. subtilis chromosome, with transforming activity against phe\(^{Al}\) and nic-38 recipients, was used to direct the insertion of p1949 into the B. subtilis chromosome. Insertion of the ligated DNA segments occurred in the region of the chromosome from which the purified phe–nic segment was derived. Many of the properties of the resulting Cm\(^{R}\) transformants of B. subtilis are consistent with the occurrence of a Campbell recombination mechanism leading to integration. However, certain of these properties are more easily explained if it is proposed that integration occurs by a reciprocal recombination event involving a linear ligation product. Evidence is presented suggesting that the inserted sequences may be tandemly duplicated. This may effectively vitiate the use of p1949 as a convenient means for complementation analysis of recessive mutations in B. subtilis.

INTRODUCTION

Several investigators have constructed plasmids that contain an antibiotic resistance determinant that is expressed in a particular host even though the plasmid is unable to replicate in that host (e.g. Haldenwang et al., 1980; Niaudet & Ehrlich, 1979). Plasmids of this type may be termed insertion vectors, since stable antibiotic-resistant progeny can be obtained in the non-permissive host if the plasmid integrates into a resident replicon. The ligation of host DNA sequences into the heterologous plasmid apparently directs insertion into the homologous region of the resident replicon. Insertion vectors have found three important uses to date. Firstly, they can be used to clone the replication origins of other replicons (e.g. see Messer et al., 1978; Niaudet & Ehrlich, 1979; Chan & Tye, 1980; Anderson et al., 1982). Secondly, they have been employed to determine the map position of cloned fragments of unknown origin (Haldenwang et al., 1980; Wilson et al., 1981). Thirdly, an insertion vector has been used to clone one DNA sequence repeatedly from different strains of the same organism (Méjean et al., 1981). This represents an important aid to the identification of gene products encoded by cloned DNA segments. If a family of cloned fragments can be obtained in this way, each one carrying a different allelic form of the gene of interest (ideally, suppressible alleles should be cloned), then the gene product can be identified quite readily, since high-efficiency temperature-sensitive amber suppressor strains of Escherichia coli have now been isolated (Oeschger et al., 1980).

There have been several reports of the integration of heterologous DNA into the Bacillus subtilis chromosome (Harris-Warwick & Lederberg, 1978; Duncan et al., 1978; Rapoport et al., 1979; Yoneda et al., 1979; Kawamura et al., 1979; Haldenwang et al., 1980; Galizzi et al., 1981). It is widely accepted that integration cannot occur in Rec\(^{-}\) (recE4) hosts (Haldenwang et al., 1980; Wilson et al., 1981, Niaudet et al., 1982) and that a region of sequence homology is usually necessary to direct insertion of the foreign DNA (however, see Rubin et al., 1980). However, comparatively little is known about the mechanism by which the heterologous DNA inserts into
the \textit{B. subtilis} chromosome. It has generally been assumed that integration is by a Campbell mechanism, by analogy with the mechanism of integration of bacteriophage \( \lambda \) into the chromosome of \textit{E. coli} (Campbell, 1962). In some cases evidence has been adduced, principally from Southern hybridization experiments, to support this contention (Haldenwang et al., 1980; Galizzi et al., 1981; Niaudet et al., 1982).

The mechanism by which foreign DNA inserts into the chromosome of \textit{B. subtilis} might be expected to depend on the precise physical nature of the transforming DNA. If it is provided as covalently closed plasmid DNA, then a Campbell mechanism would seem intuitively to be the most likely method of integration. However, if the source of DNA is simply a ligated mixture of vector and host fragments, then transformation by linear ligation products becomes a distinct possibility. Following on from this, if dissimilar host fragments are ligated to the ends of the vector DNA, then integration by a reciprocal recombination event will lead to deletion of the DNA segment that lies between the two host fragments. Indeed, Niaudet et al. (1982) have reported the isolation of an \textit{ilvA} auxotroph of \textit{B. subtilis} that apparently arose by this kind of recombination mechanism.

In this investigation further evidence has been obtained concerning the mechanism of insertion of foreign DNA into the \textit{B. subtilis} chromosome. The plasmid used was p1949 (Haldenwang et al., 1980) which encodes a chloramphenicol acetyltransferase that is expressed in \textit{B. subtilis}. This gene originated from pC194 (Iordânescu, 1975); the complete nucleotide sequence of pC194 has recently been published (Horinouchi & Weisblum, 1982). The plasmid p1949 is unable to replicate in \textit{B. subtilis}, whereas in \textit{E. coli} it replicates and confers resistance to both chloramphenicol and tetracycline (the latter resistance is derived from the \textit{E. coli} plasmid pMB9). A purified fragment of the \textit{B. subtilis} chromosome with transforming activity in \textit{pheA1} recipients (kindly donated by J. Szulmajster and C. Bonamy) was used to direct insertion of the plasmid into the \textit{B. subtilis} chromosome. This enabled direct examination of whether integration did indeed occur at the site where natural homology existed. Also, a suitably marked recipient strain was used so that genetic evidence concerning the mechanism of plasmid integration could be obtained. Many of the results are consistent with the occurrence of a Campbell integration mechanism. However, there are certain anomalies that can be explained if integration occurred via the formation of bridge structures involving linear ligation products.

\textbf{METHODS}

\textit{Strains.} The strains of \textit{E. coli} and \textit{B. subtilis} used in this investigation are listed in Table 1. Plasmid p1949 was harboured by \textit{E. coli} strain MM294, which was kindly supplied by A. L. Sonenshein. Plasmid pSC5 was constructed by cloning an \textit{EcoRI-BamHI} fragment of \textit{B. subtilis} DNA that had transforming activity against \textit{pheA1} recipients in the plasmid vector pBR322. Plasmid pSC5 was extracted from the \( r_{+} m_{+} \) \textit{recBC} derivative of \textit{E. coli} C600 listed in Table 1 and was a gift from C. Bonamy.

\textit{Transformation.} Strains of \textit{E. coli} were grown to mid-exponential phase in Luria broth and then permeabilized by calcium chloride treatment followed by heat shock (Mandel & Higa, 1970). Transformed bacteria were incubated in Luria broth at 37\(^\circ\)C for 90 min before plating out on selective medium.

The method of Anagnostopoulos & Spizizen (1961) was used for transformation of strains of \textit{B. subtilis}. Unless otherwise stated, DNA was added at a non-saturating concentration. In the crosses in Table 6 a saturating concentration of DNA was used and in one of them (cross 1) the spontaneous transformation technique of Ephrat-Eizur (1968) was employed. In this case the donor strain was grown overnight in Penassay broth (Difco) and 0·1 ml of the stationary phase culture was added to 1 ml of the competent recipient bacteria. This procedure resulted in a far higher frequency of congression of unselected donor markers than is normally found using a saturating concentration of purified donor DNA. It could only be used in one of the crosses in Table 6 since it depends on the application of counterselection against growth of the donor strain (this was achieved in cross 1 by the addition of 5 \( \mu \)g chloramphenicol ml\(^{-1}\) to the selective medium).

For the selection of \textit{Cm}\(^{R}\) transformants of \textit{E. coli} the selective medium used was Luria broth containing 15 \( \mu \)g chloramphenicol ml\(^{-1}\). \textit{Cm}\(^{R}\) transformants of \textit{B. subtilis} were selected on either nutrient agar supplemented with salts (the amounts added, per litre, were: \( \text{FeCl}_3 \), 6\( \text{H}_2\text{O} \), 0·98 mg; \( \text{MgCl}_2 \), 6\( \text{H}_2\text{O} \), 8·3 mg; \( \text{MnCl}_2 \), 4\( \text{H}_2\text{O} \), 19·8 mg; \( \text{NH}_4\text{Cl} \), 535 mg; \( \text{Na}_2\text{SO}_4 \), 106 mg; \( \text{KH}_2\text{PO}_4 \), 68 mg; \( \text{NH}_4\text{NO}_3 \), 96·5 mg) or an appropriately supplemented glucose glutamate minimal medium [this was based on the medium of Donellan \textit{et al.} (1964) as described by Pigott (1973), except that sodium lactate was replaced by glucose which was added to a final concentration of 0·2\% (w/v)]. In both cases chloramphenicol was added at a concentration of 5 \( \mu \)g ml\(^{-1}\).
Integration of heterologous DNA in B. subtilis

Table 1. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Origin/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli MM294</td>
<td>endoI thy hsdR (p1949 CmR TcR)</td>
<td>A. L. Sonenshein</td>
</tr>
<tr>
<td>C600 r- m*</td>
<td>thr-1 leu-6 thi-1 supE44 lacY1 tonA21 recBC tK- mK-</td>
<td>P. Kourilsky via J. Szulmajster</td>
</tr>
<tr>
<td>B. subtilis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>trpC2</td>
<td>Laboratory wild-type</td>
</tr>
<tr>
<td>MB21</td>
<td>metC3 leu-8 tal-1</td>
<td>Hranueli et al. (1974)</td>
</tr>
<tr>
<td>MB26</td>
<td>leu-8 pheA12 rif-2 tal-1</td>
<td>Grant (1974)</td>
</tr>
<tr>
<td>MB51</td>
<td>pheA12 nic-38 rif-2 tal-1</td>
<td>D. Hranueli; MB26 transformed to Leu+ Nic- with GSY860</td>
</tr>
<tr>
<td>JH648</td>
<td>trpC2 pheA1 spoOB136</td>
<td>J. Hoch</td>
</tr>
<tr>
<td>GSY225</td>
<td>pheA1 trpC2</td>
<td>C. Anagnostopoulos</td>
</tr>
<tr>
<td>GSY860</td>
<td>argA11 nic-38</td>
<td>C. Anagnostopoulos</td>
</tr>
<tr>
<td>SA101</td>
<td>trpC2 spoOB136</td>
<td>JH648 transformed to Phe+ with 168 DNA</td>
</tr>
<tr>
<td>SA102</td>
<td>nic-38 rif-2 tal-1</td>
<td>MB51 transformed to Phe+ with 168 DNA</td>
</tr>
<tr>
<td>SA103</td>
<td>trpC2 pheA1 nic-38 rif-2</td>
<td>GSY225 transformed to RifR Nic- with SA102 DNA</td>
</tr>
<tr>
<td>CL101–CL110</td>
<td>trpC2 spoOB136 cat</td>
<td>Ligation products of p1949 + pSC5 used to transform JH648 to CmR – all strains also Phe+ (see text)</td>
</tr>
<tr>
<td>CL104.2</td>
<td>trpC2 cat</td>
<td>Spo+ revertant of CL104</td>
</tr>
</tbody>
</table>

* This strain is a recBC tK- mK- derivative of strain C600.

Transduction. PBS1 transducing lysates were prepared essentially as described by Karamata & Gross (1970) and used for transduction as described by Takahashi (1963).

Preparation of DNA. Plasmid DNA was extracted from strains of E. coli by preparing cleared lysates as described by Clewell & Helinski (1969), and then subjecting them to cesium chloride density gradient centrifugation in the presence of ethidium bromide. Although the plasmid used here (p1949) encodes a chloramphenicol acetyltransferase, a chloramphenicol (250 μg ml⁻¹) amplification step was routinely employed (see Niaudet & Ehrlich, 1979). DNA was isolated from strains of B. subtilis using the method described by Marmur (1961). The CL104 DNA used in the Southern hybridization experiment (Fig. 2) was extracted and purified by caesium chloride density gradient centrifugation essentially as described by Harris-Warwick et al. (1975).

Restriction endonucleases. Restriction endonucleases were purchased from BRL and were used as specified by the manufacturer. For digestion of plasmid and chromosome DNA, the enzymes were used in two- and fivefold excess, respectively. Incubations were terminated by heating at 65 °C for 5 min.

Ligation of DNA fragments. DNA fragments generated by restriction endonucleases were ligated at a final concentration of 22 μg DNA ml⁻¹. This relatively low DNA concentration was chosen to promote the generation of circular products (Dugaiczyk et al., 1975). The ligation mixture contained 0.5 units T₄ DNA ligase (Boehringer) and 2.5 μg DNA in 114 μl ligation buffer (66 mM-Tris/HCl, pH 7.6; 6.6 mM-MgCl₂; 10 mM-DTT; 1 mM-ATP). Ligation was carried out for 20 h at 4°C.

Agarose gel electrophoresis. DNA restriction fragments were separated according to their molecular weights by electrophoresis through 0–6% (w/v) agarose (Sigma) in a horizontal gel electrophoresis apparatus. Electrophoresis was carried out at between 1.5 and 3 V cm⁻¹, using a buffer containing Tris (89 mM), EDTA (2.5 mM) and borate (89 mM), pH 8.3–8.5, and afterwards the gels were stained in electrophoresis buffer containing ethidium bromide (0.5 μg ml⁻¹). Stained gels were photographed using a polaroid camera mounted above a UV transilluminator (UV Products, San Gabriel, CA, U.S.A.).

Nick-translation. DNA samples were radioactively labelled to high specific activity [about 5 × 10⁷ d.p.m. (μg DNA)⁻¹] with ³²P-labelled ATP (Amersham) using the procedure described by Jeffries & Flavell (1977).

Southern hybridization. DNA–DNA hybridization experiments were carried out as follows. DNA restriction fragments (about 1 μg chromosome DNA and about 0.25 μg p1949 or λDNA) were separated by agarose gel electrophoresis and then depurinated in situ as described by Wahl et al. (1979). The DNA was then transferred to a nitrocellulose membrane (Schleicher & Schüll) using the method of Southern (1975). The filter was cut into suitable strips and the procedures used for hybridization and washing were essentially as described by Sager et al. (1981), except that dextran sulphate was omitted from the hybridization solution. The filter strips were then reassembled and autoradiographed at −20 °C by exposure to pre-fogged Kodak X-omat RP film using an intensifying screen.
Results

Characterization of plasmids p1949 and pSC5

Haldenwang et al. (1980) constructed the plasmid p1949 in three stages, the net result being the union of pMB9 (Tc^R, 3.55 MDal) that had been linearized with HpaI, and pC194 (Cm^R, 2.0 MDal) that had been linearized with HpaII. As expected, the resultant chimaera is unable to transform B. subtilis to Cm^R (see Table 4), since the HpaII (= MspI) recognition sequence lies in that region of pC194 that is thought to contain the origin of replication (Horinouchi & Weisblum, 1982). However, p1949 has two unexpected features: it is somewhat smaller than expected from the sizes of the two parental molecules; and it has an additional site for BamHI that is not present in either of the parent plasmids (Fig. 1).

A 3.2 MDal EcoRI–BamHI fragment of B. subtilis DNA was used to direct the insertion of p1949 sequences into the B. subtilis chromosome. This fragment was cloned into pBR322 and the resultant plasmid, pSC5, was shown to have transforming activity against a pheA1 recipient of B. subtilis (J. Szulmajster & C. Bonamy, personal communication). This finding is confirmed in Table 2, which also shows that pSC5 will generate Nic^+ transformants with a nic-38 recipient strain; however, no Phe^+ transformants were obtained with a pheA12 recipient strain. This suggests that the B. subtilis chromosomal fragment present in pSC5 does not carry an intact copy of the pheA gene. The plasmid failed to generate Spo^+ transformants with the spoOB136 recipient strain; this marker lies on the side of phe distal to nic (Hoch & Matthews, 1973).

Information concerning the disposition of the phe and nic genes on the purified B. subtilis DNA fragment was obtained by cleaving pSC5 with a variety of restriction endonucleases and then using the digested DNA to transform a pheA1 nic-38 strain of B. subtilis. As a precedent for the use of this approach, Lataste et al. (1981) have shown, using restriction endonuclease-cleaved DNA, that the residual transforming activity for mutations in the amiA locus of Streptococcus pneumoniae depends on the distance of the mutations from the restriction endonuclease cleavage site. The uncut plasmid has an equal transformation efficiency for both genetic markers (Table 3). When the B. subtilis DNA was released from the plasmid by double digestion with EcoRI and BamHI, the transformation efficiency for both markers was reduced about tenfold. Cleavage with EcoRI alone reduced the number of Phe^+ transformants to the same extent, which suggests that the pheA gene is at the end of the fragment that has the EcoRI...
Integration of heterologous DNA in B. subtilis

Table 2. Transforming activity of pSC5 for markers in the pheA region of the B. subtilis chromosome

<table>
<thead>
<tr>
<th>Donor DNA</th>
<th>Recipient strain</th>
<th>Genotype</th>
<th>Selected marker</th>
<th>10^{-5} \times \text{No. of transformants (\mu g DNA)}^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSC5</td>
<td>MB5</td>
<td>pheA12 nic-38</td>
<td>phe*</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>nic*</td>
<td>5.4</td>
</tr>
<tr>
<td>pSC5</td>
<td>JH648</td>
<td>pheA1 spoOB136 phe+</td>
<td>phe*</td>
<td>4.3 (0% Spo*)*</td>
</tr>
<tr>
<td>168</td>
<td>MB5</td>
<td>pheA12 nic-38</td>
<td>phe*</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>JH648</td>
<td>pheA1 spoOB136</td>
<td>phe*</td>
<td>2.5 (70% Spo*)*</td>
</tr>
</tbody>
</table>

* The spoOB136 marker is linked by transformation to pheA1; Spo* transformants were recognized by their brown colony pigmentation.

Table 3. Effect of cleavage with EcoRI, BamHI and SalGI on transforming activity of pSC5 against a pheA1 nic-38 recipient strain

Strain SA103 trpC2 pheA1 nic-38 was used as recipient.

<table>
<thead>
<tr>
<th>Selected marker</th>
<th>pSC5 (EcoRI)</th>
<th>pSC5 (BamHI)</th>
<th>pSC5 (EcoRI + BamHI)</th>
<th>pSC5 (SalGI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>phe*</td>
<td>3-3</td>
<td>0-2</td>
<td>2-2</td>
<td>0-3</td>
</tr>
<tr>
<td>nic*</td>
<td>3-3</td>
<td>1-9</td>
<td>1-2</td>
<td>0-5</td>
</tr>
</tbody>
</table>

Isolation of CmR transforms of B. subtilis

The two plasmids pSC5 and p1949 were digested with EcoRI and then with BamHI. This released the 3.2 MDal phe-nic fragment from pSC5 and a 3.4 MDal fragment from p1949 carrying the CmR gene. The digested plasmids were mixed, ligated and used to transform a pheA1 strain of B. subtilis. The numbers of Phe+ and CmR transformants obtained are given in Table 4. As observed previously, cleavage of pSC5 with EcoRI and BamHI reduced the number of Phe+ transformants obtained by about two orders of magnitude. After ligation in the presence of restriction fragments derived from p1949, the number of Phe+ transformants obtained increased about tenfold. This presumably reflects the generation of larger DNA fragments which may afford a degree of protection against nucleolytic degradation during DNA uptake and recombination. As expected from the reports that p1949 is unable to replicate autonomously in B. subtilis (Haldenwang et al., 1980; Wilson et al., 1981), no CmR transformants were obtained when p1949 DNA was used as donor (Table 4). However, after restriction and ligation with the fragments derived from pSC5, CmR transformants were obtained, albeit at a relatively low yield. Ten transformants were picked at random; they were purified by streaking for single colonies on nutrient agar containing chloramphenicol and tested for their phenylalanine requirement. In view of the data in Table 2 which shows that pSC5 apparently harbours an incomplete copy of the pheA gene, it was expected that a mixture of phenylalanine auxotrophs and phenylalanine
Table 4. Isolation of CmR transformants of B. subtilis using as donor a ligated mixture of EcoRI plus BamHI-generated restriction fragments of p1949 and pSC5

Strain JH648 trpC2 pheA1 spo0B136 was used as recipient. With E. coli strain C600 r- m- as recipient, p1949 gave 2.8 \times 10^5 CmR TeR transformants (\mu g DNA)^{-1}.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Phe+</th>
<th>CmR</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSC5</td>
<td>5.6 x 10^5</td>
<td>ND</td>
</tr>
<tr>
<td>p1949</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>Restricted pSC5 + restricted p1949*</td>
<td>juryl ligation</td>
<td>3.8 x 10^3</td>
</tr>
<tr>
<td></td>
<td>Before ligation</td>
<td>3.8 x 10^2</td>
</tr>
<tr>
<td></td>
<td>After ligation</td>
<td>2.7 x 10^4</td>
</tr>
</tbody>
</table>

ND. Not determined.
* Both plasmids were restricted with EcoRI and BamHI.

Table 5. Three factor transduction crosses to determine the position of cat relative to pheA12 and nic-38 in strain CL104

The donor was CL104.2 trpC2 cat and the recipient was MB26 leu-8 pheA12.

<table>
<thead>
<tr>
<th>Selected marker</th>
<th>Recombinant class</th>
<th>Frequency</th>
<th>Suggested order</th>
</tr>
</thead>
<tbody>
<tr>
<td>phe+</td>
<td>Leu- CmR</td>
<td>11</td>
<td>leu-phe-cat</td>
</tr>
<tr>
<td></td>
<td>Leu+ CmR</td>
<td>21</td>
<td>leu-phe-cat</td>
</tr>
<tr>
<td></td>
<td>Leu- CmR</td>
<td>20</td>
<td>leu-phe-cat</td>
</tr>
<tr>
<td></td>
<td>Leu+ CmR</td>
<td>68</td>
<td>leu-phe-cat</td>
</tr>
<tr>
<td>leu+</td>
<td>Phe+ CmR</td>
<td>23</td>
<td>leu-phe-cat</td>
</tr>
<tr>
<td></td>
<td>Phe+ CmR</td>
<td>19</td>
<td>leu-phe-cat</td>
</tr>
<tr>
<td></td>
<td>Phe- CmR</td>
<td>0</td>
<td>leu-phe-cai</td>
</tr>
<tr>
<td></td>
<td>Phe+ CmR</td>
<td>55</td>
<td>leu-phe-cat</td>
</tr>
<tr>
<td>cat</td>
<td>Leu- Phe+</td>
<td>76</td>
<td>leu-phe-cat</td>
</tr>
<tr>
<td></td>
<td>Leu+ Phe+</td>
<td>14</td>
<td>leu-phe-cat</td>
</tr>
<tr>
<td></td>
<td>Leu+ Phe-</td>
<td>1</td>
<td>leu-phe-cat</td>
</tr>
<tr>
<td></td>
<td>Leu- Phe-</td>
<td>29</td>
<td>leu-phe-cat</td>
</tr>
</tbody>
</table>

prototrophs would be obtained (see Fig. 4). However, all 10 strains (designated CL101–CL110) proved to be phenylalanine prototrophs. In accordance with this unexpected finding, when selection was made for Phe+ CmR double transformants using as donor the ligated mixture of double digested plasmids (see Table 4), the number of transformants obtained was very similar (data not shown) to that observed when the selection was for CmR transformants.

Chromosomal location of the CmR determinant in strains CL101–CL110

DNA prepared from strains CL101–CL110 was used to transform E. coli strain C600 r- m-. None of the 10 DNA preparations yielded any CmR transformants, whereas p1949 gave 2.8 \times 10^5 transformants (\mu g DNA)^{-1}. This suggests that strains CL101–CL110 do not carry the CmR gene on an autonomous plasmid.

Two further experiments were undertaken which establish the chromosomal location of the CmR determinant. In the first, the DNA preparations mentioned above were used at a non-saturating concentration (10 ng DNA ml^{-1}) to transform a pheA1 strain of B. subtilis. In a representative experiment with strain CL104 as donor, Phe+ transformants arose at a frequency of 2.0 \times 10^6 (\mu g DNA)^{-1} and only 4% of them were simultaneously transformed for the CmR marker. CmR transformants arose at a reduced frequency of 2.2 \times 10^5 (\mu g DNA)^{-1}, but 97% of them were simultaneously transformed to Phe+. This marked asymmetry has been observed previously (Wilson et al., 1981); presumably it reflects the presence in the donor strain of p1949 sequences for which no homology exists in the recipient strain. This asymmetry was not observed in the second experiment in which PBS1 transduction was used to effect the transfer of
larger DNA fragments than are transferred during transformation. By this means we were able to demonstrate the linkage of the Cm\textsuperscript{R} determinant to the leu-8 marker, which was not involved in construction of the CL strains. The data from a representative experiment, using a Spo\textsuperscript{+} derivative of strain CL104 as donor, are given in Table 5, and they establish that the Cm\textsuperscript{R} marker is inserted into the B. subtilis chromosome on the side of pheAI2 distal to leu-8. Tests for linkage by transduction to a range of other genetic markers scattered about the B. subtilis chromosome indicated that insertion had occurred only in the pheA region. Similar results were obtained with several other CL strains (data not given).

\begin{itemize}
  \item **Strains CL101–110 produce Cm\textsuperscript{S} segregants**
  \begin{itemize}
    \item As a working hypothesis it was postulated that the Cm\textsuperscript{R} determinant had been inserted into the bacterial chromosome by a Campbell mechanism. This would result in the generation of a duplication of the phe–nic fragment that was used to direct the insertion and hence it would be expected that the Cm\textsuperscript{R} strains would segregate Cm\textsuperscript{S} progeny at a measurable frequency. Moreover, since the incoming copy of the pheA gene was wild type, and the resident copy was mutant, it was expected that the Cm\textsuperscript{S} segregants would fall into two classes: those that were Phe\textsuperscript{+} and those that were Phe\textsuperscript{−}. These predictions were tested by growing six of the 10 CL strains overnight in nutrient broth, and then plating them out on nutrient agar to obtain single colonies. Between 140 and 320 colonies derived from each strain were picked and replica plated on to (1) nutrient agar with and without chloramphenicol and (2) tryptophan minimal agar with and without phenylalanine. As expected, all the strains tested segregated Cm\textsuperscript{S} progeny at frequencies varying from 1.6\% to 14.5\%. However, none of the 150 Cm\textsuperscript{S} segregants that were tested, nor indeed any of the Cm\textsuperscript{R} parental types, proved to be phenylalanine auxotrophs. This therefore raised the question as to whether the pheAI mutation in the parental strain (JH648) was still present in the CL strains.

  \item **Strain CL104 does not appear to contain the pheAI mutation**
  \begin{itemize}
    \item DNA from strain CL104 was used at a saturating concentration (0.1 \(\mu\)g ml\(^{-1}\)) to transform strain MB21 of B. subtilis. Transformants to Met\textsuperscript{+}, Leu\textsuperscript{+} and Cm\textsuperscript{R} were selected separately using media that contained tryptophan and phenylalanine. This permitted the detection of double transformants that also carried the trpC2 or pheAI mutations presumed to be present in the donor strain. Transformants to Leu\textsuperscript{+}(160), Met\textsuperscript{+}(160) and Cm\textsuperscript{R}(320) were screened for the inheritance of the unselected donor markers. A total of 10 transformants were Trp\textsuperscript{−}, but no Phe\textsuperscript{−} colonies were detected. This was particularly surprising in the case where selection had been made for Cm\textsuperscript{R} transformants since it had been established previously that when selection is made for this marker, approximately 97\% of the recombinants are simultaneously transformed for the donor phe marker. The implication of this experiment is that the donor strain no longer carries the pheAI mutation. The fact that the CL strains readily segregate Cm\textsuperscript{S} progeny suggests that they are merodiploid for the pheA–nic fragment and further evidence to confirm this was obtained using two approaches, namely genetic analysis and Southern hybridization.

  \item **Genetic analysis of strain CL104**
  \begin{itemize}
    \item Trp\textsuperscript{+} transformants of strain CL104 were obtained using as donor a nic-38 pheAI2 rif-2 strain of B. subtilis. The Ephrati-Elizur (1968) technique was used in this experiment since it generally results in higher frequencies of congression of unlinked donor markers than is observed with purified donor DNA (Nester et al., 1963) (see Table 6). Chloramphenicol was added to the selective medium to counterselect against growth of the donor strain. Further analysis of the Trp\textsuperscript{+} transformants showed that simultaneous transformation to Phe\textsuperscript{−}, Rif\textsuperscript{R} and Spo\textsuperscript{+} had occurred at frequencies of 6.5\%, 10.6\% and 12.5\%, respectively. In contrast, however, none of the Trp\textsuperscript{+} transformants had inherited the Nic\textsuperscript{−} phenotype of the donor strain. In the control experiment, using as recipient a strain of B. subtilis that did not harbour the integrated Cm\textsuperscript{R} determinant, Trp\textsuperscript{+} Nic\textsuperscript{−} double transformants arose at a frequency comparable to that observed for the Trp\textsuperscript{+} Phe\textsuperscript{−}, Trp\textsuperscript{+} Rif\textsuperscript{R} and Trp\textsuperscript{+} Spo\textsuperscript{+} classes (Table 6). In the control cross it was not possible to counterselect against growth of the donor strain since the recipient was Cm\textsuperscript{S}.
  \end{itemize}
\end{itemize}
\end{itemize}
Fig. 2. Southern hybridization experiment to identify sequences homologous to p1949 and the purified phe-nic fragment in restriction digests of DNA from strains CL104 and 168. (a) Restricted DNA samples before transfer to the nitrocellulose membrane. The restricted DNA samples were as follows: lane 1, 168 restricted with EcoRI; lane 2, p1949 with EcoRI plus BamHI; (digestion incomplete) lanes 3–6, CL104 with EcoRI plus BamHI, EcoRI, BamHI, and SalGI, respectively; lane 7, λ with EcoRI; lanes 8–10, CL104 with EcoRI, BamHI, and SalGI, respectively; lanes 11–13, 168 with SalGI, EcoRI, and BamHI, respectively; lane 14, 168 with EcoRI plus BamHI; lane 15, CL104 with EcoRI plus BamHI. (b) Autoradiogram of hybridized membrane filter. Lanes 1–6 were hybridized against p1949 DNA, lane 7 against λ DNA and lanes 8–15 against the purified phe-nic fragment.
Table 6. Congestion of rif-2, spoOB136, pheA12 and nic-38 markers into strains CL104 and SA101

<table>
<thead>
<tr>
<th>Cross</th>
<th>Recipient</th>
<th>Donor</th>
<th>Selected marker‡</th>
<th>Recombinant class</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CL104 trpC2 spoOB136</td>
<td>MB51 nic-38 pheA12</td>
<td>trp*</td>
<td>Trp+ Rif*</td>
<td>10-6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Trp+ Spo*</td>
<td>12-5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Trp+ Phe*</td>
<td>6-5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Trp+ Nic*</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>SA101 trpC2 spoOB136</td>
<td>MB51 nic-38 pheA12</td>
<td>trp*</td>
<td>Trp+ Rif*</td>
<td>1-7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Trp+ Spo*</td>
<td>1-0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Trp+ Phe*</td>
<td>1-0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Trp+ Nic*</td>
<td>1-0</td>
</tr>
</tbody>
</table>

* The spontaneous transformation technique of Ephrati-Eliiur (1968) was used.
† A saturating concentration of purified DNA was used as donor.
‡ A total of 480 Trp+ transformants from each cross were tested for inheritance of the non-selected spo, rif, phe and nic markers.

Table 7. Efficiency of transformation of pheA12, pheA1 and nic-38 recipients with strain CL104 as donor

<table>
<thead>
<tr>
<th>Cross</th>
<th>Recipient</th>
<th>Donor</th>
<th>Selected marker</th>
<th>No. of recombinants (µg DNA)⁻¹</th>
<th>Ratio of Nic*/Phe* transformants</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MB51 nic-38 pheA12</td>
<td>CL104 trpC2 spoOB136</td>
<td>nic*</td>
<td>1-6 × 10⁴</td>
<td>5-6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>phe*</td>
<td>2-8 × 10³</td>
<td>2-5</td>
</tr>
<tr>
<td>2</td>
<td>MB51 nic-38 pheA12</td>
<td>168 trpC2</td>
<td>nic*</td>
<td>1-2 × 10⁴</td>
<td>1-4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>phe*</td>
<td>4-7 × 10³</td>
<td>1-1</td>
</tr>
<tr>
<td>3</td>
<td>SA103 nic-38 pheA1</td>
<td>CL104 trpC2 spoOB136</td>
<td>nic*</td>
<td>7-0 × 10⁴</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>phe*</td>
<td>5-1 × 10³</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>SA103 nic-38 pheA1</td>
<td>168 trpC2</td>
<td>nic*</td>
<td>2-2 × 10⁴</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>phe*</td>
<td>2-0 × 10³</td>
<td></td>
</tr>
</tbody>
</table>

Therefore, purified DNA was used as donor, and this accounts for the fact that transformation of the unselected donor markers occurred at a lower frequency than in the first cross in Table 6. These results, showing that CL104 is not readily transformed to Nic-, would be expected if the strain harbours two (or more) copies of the wild-type nic gene.

The putative merodiploid strain CL104 was then used as donor in further crosses to measure the transformation efficiency against recipients carrying the pheA12, pheA1 and nic-38 markers. It should be noted that the putative merodiploid region in strain CL104 does not include the sequences complementary to the pheA12 mutation (see Table 2). A non-saturating concentration of DNA from strain CL104 was used to transform a pheA12 nic-38 recipient strain; DNA from strain 168 was used as control (Table 7, crosses 1 and 2). Transformants to Phe+ and to Nic+ were selected separately, and the frequencies obtained were used to calculate the ratio of Nic*/Phe* transformants with each donor. When the donor was strain 168 (cross 2), the ratio was approximately 2-5, which presumably reflects a difference in the transformation efficiencies for the nic-38 and pheA12 markers. When the donor was strain CL104, the ratio was increased about twofold to 5-6. This suggests that sequences complementary to the nic-38 mutation are present in strain CL104 in approximately twice as many copies as are to be found in normal 168 strains. This supports the contention that the strain is merodiploid for the nic region. Evidence that the merodiploid region also includes the sequences complementary to the pheA1 mutation was obtained in crosses 3 and 4 in Table 7. In these crosses the same donor strains were used to transform a pheA1 nic-38 recipient. As previously, Phe+ and Nic+ transformants were selected separately, and the frequencies observed were used to calculate the ratio of Nic*/Phe* transformants. This ratio was close to unity with strain 168 as donor and was only slightly increased with strain CL104 as donor. However, it is noteworthy that the transformation efficiencies for both markers are 2-5–3-fold greater with strain CL104 as donor than with strain
Integration of heterologous DNA in B. subtilis

**DISCUSSION**

The results obtained in this investigation confirm previous reports (Duncan et al., 1978; Haldenwang et al., 1980; Wilson et al., 1981; Niaudet et al., 1982) that heterologous DNA can be inserted into the B. subtilis chromosome provided that the incoming DNA has some sequence homology with the bacterial chromosome. Moreover, they demonstrate that insertion occurs into that region of the chromosome from which the exogenote was derived. In none of the 10 independent CmR transformants studied here was there any evidence that there were sites of insertion into the bacterial chromosome other than the site in the pheA region from which the DNA from strains CL104 and 168 was cleaved with a fivefold excess of EcoRI, BamHI and SalGI. The restriction fragments were electrophoretically separated in agarose according to their molecular weight and transferred to a nitrocellulose membrane. The membrane was then cut into strips and the appropriate samples hybridized against (1) nick-translated p1949, or (2) nick-translated 3.2 MDal phe-nic fragment of pSC5 (see Fig. 1). A molecular weight standard of λ DNA cut with EcoRI was also included. The hybridized and washed membrane filter strips were then reassembled and autoradiographed and the results are given in Fig. 2. When p1949 was used as probe against strain CL104 DNA cleaved with EcoRI plus BamHI (lane 3) and against p1949 DNA cut with the same enzymes (lane 2), hybridization occurred with a 3.4 MDal fragment corresponding to the EcoRI-BamHI fragment of p1949 that carries the CmR determinant (see Fig. 1). When CL104 DNA was restricted with either EcoRI, BamHI or SalGI the probe hybridized to a 6.6 MDal fragment (lanes 4–6). This corresponds to the sum of the 3.4 MDal EcoRI-BamHI fragment of p1949 and the 3.2 MDal phe-nic fragment of pSC5 (see Fig. 1). There was no hybridization of the probe to EcoRI-cleaved DNA from strain 168 (lane 1). In lanes 8–15 the 3.2 MDal phe-nic fragment of pSC5 was used as hybridization probe. Hybridization to CL104 DNA restricted with either EcoRI, BamHI or SalGI showed the same 6.6 MDal fragment as was detected with the p1949 probe, but in addition each enzyme also produced a second fragment of higher molecular weight to which much weaker hybridization occurred (lanes 8–10). When hybridized against strain 168 DNA that had been restricted with the same enzymes, only the higher molecular weight bands appeared (lanes 11–13). Finally, when either strain 168 or strain CL104 DNA was cut with both EcoRI and BamHI (lanes 14 and 15), hybridization occurred with a 3.2 MDal fragment corresponding to the 3.2 MDal phe-nic fragment of pSC5 (see Fig. 1).

These data were used to construct the restriction map of the phe-nic region of the CL104 chromosome shown in Fig. 3.

**Restriction map of the phe-nic region of strain CL104**

DNA from strains CL104 and 168 was cleaved with a fivefold excess of EcoRI, BamHI and SalGI. The restriction fragments were electrophoretically separated in agarose according to their molecular weight and transferred to a nitrocellulose membrane. The membrane was then cut into strips and the appropriate samples hybridized against (1) nick-translated p1949, or (2) nick-translated 3.2 MDal phe-nic fragment of pSC5 (see Fig. 1). A molecular weight standard of λ DNA cut with EcoRI was also included. The hybridized and washed membrane filter strips were then reassembled and autoradiographed and the results are given in Fig. 2. When p1949 was used as probe against strain CL104 DNA cleaved with EcoRI plus BamHI (lane 3) and against p1949 DNA cut with the same enzymes (lane 2), hybridization occurred with a 3.4 MDal fragment corresponding to the EcoRI-BamHI fragment of p1949 that carries the CmR determinant (see Fig. 1). When CL104 DNA was restricted with either EcoRI, BamHI or SalGI the probe hybridized to a 6.6 MDal fragment (lanes 4–6). This corresponds to the sum of the 3.4 MDal EcoRI-BamHI fragment of p1949 and the 3.2 MDal phe-nic fragment of pSC5 (see Fig. 1). There was no hybridization of the probe to EcoRI-cleaved DNA from strain 168 (lane 1). In lanes 8–15 the 3.2 MDal phe-nic fragment of pSC5 was used as hybridization probe. Hybridization to CL104 DNA restricted with either EcoRI, BamHI or SalGI showed the same 6.6 MDal fragment as was detected with the p1949 probe, but in addition each enzyme also produced a second fragment of higher molecular weight to which much weaker hybridization occurred (lanes 8–10). When hybridized against strain 168 DNA that had been restricted with the same enzymes, only the higher molecular weight bands appeared (lanes 11–13). Finally, when either strain 168 or strain CL104 DNA was cut with both EcoRI and BamHI (lanes 14 and 15), hybridization occurred with a 3.2 MDal fragment corresponding to the 3.2 MDal phe-nic fragment of pSC5 (see Fig. 1).

These data were used to construct the restriction map of the phe-nic region of the CL104 chromosome shown in Fig. 3.

**DISCUSSION**

The results obtained in this investigation confirm previous reports (Duncan et al., 1978; Haldenwang et al., 1980; Wilson et al., 1981; Niaudet et al., 1982) that heterologous DNA can be inserted into the B. subtilis chromosome provided that the incoming DNA has some sequence homology with the bacterial chromosome. Moreover, they demonstrate that insertion occurs into that region of the chromosome from which the exogenote was derived. In none of the 10 independent CmR transformants studied here was there any evidence that there were sites of insertion into the bacterial chromosome other than the site in the pheA region from which the DNA from strains CL104 and 168 was cleaved with a fivefold excess of EcoRI, BamHI and SalGI. The restriction fragments were electrophoretically separated in agarose according to their molecular weight and transferred to a nitrocellulose membrane. The membrane was then cut into strips and the appropriate samples hybridized against (1) nick-translated p1949, or (2) nick-translated 3.2 MDal phe-nic fragment of pSC5 (see Fig. 1). A molecular weight standard of λ DNA cut with EcoRI was also included. The hybridized and washed membrane filter strips were then reassembled and autoradiographed and the results are given in Fig. 2. When p1949 was used as probe against strain CL104 DNA cleaved with EcoRI plus BamHI (lane 3) and against p1949 DNA cut with the same enzymes (lane 2), hybridization occurred with a 3.4 MDal fragment corresponding to the EcoRI-BamHI fragment of p1949 that carries the CmR determinant (see Fig. 1). When CL104 DNA was restricted with either EcoRI, BamHI or SalGI the probe hybridized to a 6.6 MDal fragment (lanes 4–6). This corresponds to the sum of the 3.4 MDal EcoRI-BamHI fragment of p1949 and the 3.2 MDal phe-nic fragment of pSC5 (see Fig. 1). There was no hybridization of the probe to EcoRI-cleaved DNA from strain 168 (lane 1). In lanes 8–15 the 3.2 MDal phe-nic fragment of pSC5 was used as hybridization probe. Hybridization to CL104 DNA restricted with either EcoRI, BamHI or SalGI showed the same 6.6 MDal fragment as was detected with the p1949 probe, but in addition each enzyme also produced a second fragment of higher molecular weight to which much weaker hybridization occurred (lanes 8–10). When hybridized against strain 168 DNA that had been restricted with the same enzymes, only the higher molecular weight bands appeared (lanes 11–13). Finally, when either strain 168 or strain CL104 DNA was cut with both EcoRI and BamHI (lanes 14 and 15), hybridization occurred with a 3.2 MDal fragment corresponding to the 3.2 MDal phe-nic fragment of pSC5 (see Fig. 1).

These data were used to construct the restriction map of the phe-nic region of the CL104 chromosome shown in Fig. 3.
exogenote was derived. Also, none of the transformants harboured an autonomous plasmid.

Many of the properties of the Cm\textsuperscript{R} transformants are consistent with the occurrence of integration by a Campbell recombination mechanism. Three independent experiments indicated that the strains are merodiploid for the sequences used to promote insertion of the Cm\textsuperscript{R} determinant. The Cm\textsuperscript{R} transformants readily gave rise to Cm\textsuperscript{S} segregants. When using phe\textit{A1} and nic\textit{-38} recipients, DNA from the Cm\textsuperscript{R} transformants gave about twice as many Phe\textsuperscript{+} and Nic\textsuperscript{+} transformants as did DNA from the wild type strain (Table 7). Southern hybridization experiments indicated that the heterologous plasmid DNA was bounded on both sides by copies of the phe–nic fragment (Fig. 2).

Since the exogenotic phe–nic fragment of \textit{B. subtilis} DNA apparently carries an incomplete copy of the wild type \textit{pheA} gene (Table 2) it was anticipated that integration by a Campbell mechanism would generate a mixture of Phe\textsuperscript{+} and Phe\textsuperscript{−} transformants, depending on the precise location of the recombination event (see Fig. 4). Contrary to expectation, all of the Cm\textsuperscript{R} transformants tested were Phe\textsuperscript{+}. Assuming for the present that integration was by a Campbell mechanism, then in order to account for this finding it must be proposed that the likelihood of a crossover occurring within the \textit{pheA} gene, between the EcoRI end of the cloned fragment and the site of the phe\textit{A1} mutation, is much greater than the likelihood of a crossover occurring between the BamHI end of the cloned fragment and the site of the phe\textit{A1} mutation (see Fig. 4). This proposal seems unlikely in view of the fact that the phe\textit{A1} mutation seems to lie relatively close to the EcoRI end of the cloned fragment (Table 2). However, it could be accounted for if there was a recombination hot spot (chi sequence?) (Stahl, 1979; Smith \textit{et al.,} 1981) in this region.

There is, however, another serious difficulty with the proposal that integration occurred by a Campbell type of mechanism: namely, that the Cm\textsuperscript{R} transformants do not appear to harbour the phe\textit{A1} mutation. Experimentally it was found that the phe\textit{A1} mutation, supposedly present, could not be rescued from the strains by transformation. Also, when a large number of Cm\textsuperscript{S} segregants were obtained from the Cm\textsuperscript{R} transformants, all of them were Phe\textsuperscript{+}. Finally, it was demonstrated that one of the Cm\textsuperscript{R} transformants (CL104) carries at least two wild type copies of the sequences complementary to the phe\textit{A1} mutation (Table 7).

Bresler \textit{et al.} (1968) and Spatz & Trautner (1970) have shown that during the processes of transformation and transfection in \textit{B. subtilis}, allelic conversion can occur. DNA heteroduplexes, containing wild type and mutant copies of a gene may be converted to homoduplexes before replication leads to the partitioning of the two allelic forms of the gene in the resultant
Integration of heterologous DNA in *B. subtilis* 1509

![Diagram](image)

**Fig. 5.** Model depicting integration of a linear ligation product carrying the p1949 Cm\(^R\) determinant into the pheA region of the *B. subtilis* chromosome. A copy of the phe-nic fragment is ligated to each end of the heterologous p1949 DNA (single line). Steric hindrance favours the occurrence of recombination near the extremities of the phe-nic fragments. As a result the pheA\(^+\) mutation in the recipient strain (JH648) is lost on integration. B, BamHI and E, EcoRI.

daughter chromosomes. Although in the present investigation we are not dealing with DNA heteroduplexes, it might still be argued that a form of allelic conversion has occurred, since it has been shown that alterations introduced into the recipient-homologous DNA of hybrid plasmids can be corrected in transformation (Iglesias *et al.*, 1981). Very recently Chak *et al.* (1982), have extended this finding by their demonstration of the *in vivo* transfer of mutations between the *B. subtilis* chromosome and a plasmid harbouring homologous DNA. If gene conversion is responsible for the results reported here, then it must be explained why the pheA\(^+\) endogenote has apparently been converted by the phe\(^+\) exogenote in all 10 transformants that were examined. The selective medium used to obtain the Cm\(^R\) transformants was supplemented with phenylalanine and, as far as I am aware, pheA\(^1\) strains of *B. subtilis* grow and sporulate normally under the conditions employed. Hence, no selective pressure was applied to force conversion of the mutant pheA\(^1\) allele to the wild-type phe\(^+\) allele. It is noteworthy, in this context, that Spatz & Trautner (1970) did observe asymmetric gene conversion in their experiments on the transformation of *B. subtilis* with heteroduplex SPPl DNA. However, wild type and mutant alleles of a variety of different genes have been shown to coexist in merodiploid strains of *B. subtilis* (Trowsdale & Anagnostopoulos, 1975, 1976; Schneider & Anagnostopoulos, 1981; Fink & Zahler, 1982). On balance therefore, it is improbable that the systematic loss of the pheA\(^1\) mutation observed here is the result of allelic conversion.

An alternative trivial explanation for the failure to detect the pheA\(^1\) mutation in the merodiploid strains must also be considered. It seems very likely that multiple copies of the exogenote are present in the strains (this is discussed later). If the exogenote is present in very large excess, we may have failed to detect the single copy of the endogenote for the trivial reason that insufficient numbers of Cm\(^R\) segregants and transformants were analysed (see Results). This explanation might also account for the initial finding that all of the Cm\(^R\) transformants obtained with the ligated DNA were Phe\(^+\). In conclusion, the possibility that the exogenote integrated by a Campbell mechanism cannot be ruled out on the basis of the evidence currently available.

An alternative mechanism for integration of the Cm\(^R\) determinant can, however, be envisaged. A reciprocal recombination event might have occurred between the bacterial chromosome and a linear ligation product comprising the 3-4 MDal plasmid fragment carrying the Cm\(^R\) determinant with a copy of the 3-2 MDal phe-nic fragment of *B. subtilis* DNA ligated to each extremity. Integration could then proceed by the formation of a bridge structure, as shown diagrammatically in Fig. 5. Steric hindrance might favour the occurrence of recombination in
the region between the pheA1 mutation and the EcoRI end of the fragment, thus accounting for
the fact that the resultant merodiploid strains have apparently lost the pheA1 mutation.
Evidently, it dissimilar chromosomal fragments were ligated to the ends of the heterologous
DNA, insertion by this mechanism would result in deletion of the chromosomal sequences that
lie between them (Niaudet et al., 1982). A relatively simple method for the generation of deletion
mutations, for which positive selection could be made, could be developed by the use of p1949 as
an insertion vector, and refinements enabling the production of deletions of varying extent from
the ends of fragments already cloned in the insertion vector can also be envisaged. A
recombination mechanism similar to that described here has been proposed for the
transformation of a large deletion mutation in B. subtilis (Adams, 1972), and also for the
transformation of a heterologous intergenote comprising the ‘Bacillus globigii’ aromatic region in
B. subtilis (Harris-Warwick & Lederberg, 1978).

It would be of interest to determine whether the integration of a plasmid comprising the
3-4 MDal CmR fragment of p1949 and the 3-2 MDal phe–nic fragment does in fact proceed by a
Campbell mechanism. Repeated attempts to construct such a plasmid by ligating the two
purified fragments and transforming either a recBC derivative of E. coli strain C600 or a recA
strain (HB101) have failed. As an alternative approach, EcoRI or BamHI digests of CL104
DNA have been ligated and used to transform the strains of E. coli mentioned above, on several
different occasions. However, it has not proved possible to obtain the desired product. It seems
that a plasmid comprising the 3-4 MDal p1949 sequences plus the 3-2 MDal phe–nic fragment is
not stably maintained in E. coli. This is made all the more difficult to understand since the
3-2 MDal phe–nic fragment is stably maintained in E. coli when it is cloned into pBR322 (J.
Szulmajster & C. Bonamy, personal communication). In view of these unexpected difficulties, it
has not yet proved possible to demonstrate that p1949 sequences can integrate into the B. subtilis
chromosome by a Campbell mechanism when they are carried on a covalently closed plasmid.

Finally, it is of interest that in the Southern hybridization experiment (Fig. 2, lanes 8–10)
using the phe–nic fragment as probe, hybridization occurred to two restriction fragments of
CL104 DNA, but with very disparate intensities. The 6-6 MDal fragment comprising the vector
plus phe–nic segment showed much more intense hybridization than did the larger fragment, also
seen in digests of DNA from the wild type strain (lanes 11–13). This presumably reflects
differences in the numbers of copies of the two fragments present in the digested DNA, the
implication being that the vector plus phe–nic fragment is present in multiple copies (probably at
least 10) in strain CL104. (The transformation analysis discussed above suggested that only
about two copies were present, and the reason for the discrepancy is not known.) This
phenomenon has previously been noted by Wilson et al. (1981) who employed the same plasmid
as used here, and also by Tanaka (1979) who reported that the leu region of the B. subtilis
chromosome undergoes spontaneous recE-independent duplications when it is carried on an
autonomously replicating plasmid. Independent evidence confirming that multiple copies of the
inserted sequences are present was obtained by digesting DNA from the 10 CmR strains
(CL101–110) with EcoRI and BamHI and examining the restriction digests by agarose gel
electrophoresis. In each case a more or less prominent band was discernible, with an apparent
molecular weight of about 6-7 MDal against the background of restriction fragments generated
from the chromosomal DNA (M. Young, unpublished results). Moreover, this band was
converted into two bands of apparent molecular weights of about 3-5 and 3-2 MDal after double
digestion with EcoRI and BamHI. In some of the CmR transformants the band was more
prominent than in others, but no correlation was observed between band intensity and the
maximum chloramphenicol tolerance of the strains. It is pertinent to ask where these multiple
copies of the inserted sequences reside. Since we were unable to detect free plasmids in the
strains, and there was no evidence for insertion occurring anywhere else than in the pheA region,
it may be concluded that the inserted sequences are probably tandemly duplicated in the pheA
region. Evidence to confirm this hypothesis is currently being sought. It is possible that growth
in the presence of chloramphenicol provides sufficient selective pressure to force duplication of
the inserted sequences in situ. Alternatively, they may have arisen by multiple integration events
in the initial transformation.
Integration of heterologous DNA in B. subtilis

In any event, the finding that multiple copies of the cloned B. subtilis DNA are present in the strains makes it much less probable that this particular insertion vector will prove to be particularly useful for the analysis of dominance and complementation relationships of mutations in B. subtilis. Whether or not other insertion vectors will show the same behaviour remains to be seen.

Note added in proof: Ferrari et al. (1982) have recently published a restriction map of the pheA region of the B. subtilis chromosome which confirms the results reported here.

I thank Dr J. Szulmajster, in whose laboratory this work was started, for his hospitality during a three month period when I was the recipient of an EMBO Short Term Fellowship. I also thank Dr M. G. Sargent for his help in the preparation of nick-translated DNA, Mr G. Price for his technical assistance, and Miss S. W. Evans for typing the manuscript. The financial support of the SERC (GR/B75082) is gratefully acknowledged.

REFERENCES


IORDANESCU, S. (1975). Recombinant plasmid ob-


