A General Method for Fusion of the Escherichia coli lacZ Gene to Chromosomal Genes in Bacillus subtilis

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(Received 11 March 1986; revised 5 June 1986)

A series of plasmids has been constructed that can be used to fuse the $\beta$-galactosidase gene (lacZ) of Escherichia coli to chromosomal genes of Bacillus subtilis. Insertion of the lacZ gene is facilitated by the use of a selectable chloramphenicol acetyl-transferase (cat) gene. The latter is included, along with the lacZ gene, in a single DNA fragment or 'cartridge' that can be removed from the plasmid with a variety of different restriction endonucleases. Methods applicable to any cloned B. subtilis gene are described that enable the lac-cat cartridge to be inserted at specific sites, or at random, directly into the B. subtilis chromosome in a single step. These single-copy chromosomal fusions can be readily transferred, by selection for chloramphenicol resistance, to a temperate phage such as $\phi$105, to permit a more extensive genetic analysis of the expression of the target gene. Alternatively, the lac-cat cartridge and flanking DNA sequences can be transferred into different genetic backgrounds by transformation. These techniques have been used to construct, in a single step, lac fusions to genes in the sporulation operons spoIIA and spoVA.

INTRODUCTION

A major limitation in studying the control of expression of sporulation genes in Bacillus subtilis has been the difficulty of assaying directly the product of any spo gene. In other systems where a similar problem has been encountered, a useful approach has been to fuse the gene to the coding region of the lacZ ($\beta$-galactosidase) gene from Escherichia coli (Casadaban et al., 1983), for which a very sensitive and convenient assay is available (Miller, 1972). This approach has also been applied recently with some success to the study of sporulation genes (Zuber & Losick, 1983; Stephens et al., 1984; Piggot et al., 1985).

Two main types of fusion can be obtained. (i) Translational (gene) fusions are constructed by joining, in frame, a truncated form of the lacZ gene that lacks the coding region for the first 20 to 30 amino acids of $\beta$-galactosidase to the coding region of a heterologous gene. The appearance of $\beta$-galactosidase activity is then an indication of translation of the target operon. This type of fusion is feasible because the first 30 amino acids encoded by the lacZ gene can be replaced by a variety of other protein sequences to produce a hybrid protein that often has substantial $\beta$-galactosidase activity (Brickman et al., 1979). The success of a translational fusion thus depends on two factors: first, maintenance of the correct reading frame through the junction of the fused genes; second, retention of $\beta$-galactosidase activity by the fusion peptide. Since certain in-frame gene fusions do not produce an active product it is often desirable to be able to insert a fragment of DNA containing the truncated lacZ gene at several different locations, or, preferably, at random. To facilitate this kind of approach, Casadaban et al. (1983) have constructed a plasmid, pMC1871, that contains a suitable truncated version of the lacZ gene on a 3·0 kbp segment of DNA which is bounded by a series of unique restriction sites. The gene can therefore be

Abbreviations: RBS, ribosome-binding site; ORF, open reading frame.

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removed as a single fragment of DNA or 'cartridge' by cleavage at any of the flanking restriction sites and re-cloned into target DNA that has cleavage sites for compatible restriction endonucleases. (ii) Transcriptional (operon) fusions are constructed by inserting the complete lacZ gene, including its translation initiation signals but not its promoter, into the transcription unit of another gene. The appearance of β-galactosidase activity is then used as an indication of transcription of the target operon. This type of fusion can be made at almost any point in an operon provided that the orientation of the lacZ gene is correct and that the ribosome-binding site (RBS) and initiation codon of the lacZ gene are suitable for the initiation of translation in the host strain. Unfortunately, the lacZ gene seems not to be efficiently translated in B. subtilis (Donnelly & Sonenshein, 1984), presumably because of low homology between the lacZ RBS and the 3'-end of the 16S ribosomal RNA of B. subtilis (Moran et al., 1982).

However, as described in this paper this problem can be circumvented by replacing the translation initiation signals of the lacZ gene with sequences that are functional in B. subtilis. The genes are contained in a segment of DNA, the lac–cat cartridge, that can be released by digestion with any of several different restriction endonucleases. Methods are described that enable the lac–cat cartridge to be inserted, in a single step, directly into any region of the B. subtilis chromosome that has been cloned. The resultant single copy chromosomal fusions can be analysed in situ, or can readily be transferred to the prophage of temperate bacteriophage φ105 for further genetic analysis.

The following papers describe the application of this system to the study of the regulation of sporulation operons spoIIA (Errington & Mandelstam, 1986a), spoVA (Errington & Mandelstam, 1986b), spoIID (Clarke et al., 1986) and spoIIC (Turner et al., 1986).

**METHODS**

_Bacterial strains, plasmids and bacteriophages._ Bacterial strains and plasmids and their sources are described in Table 1. Strain JM109F− was derived by treatment of strain JM109 with acridine orange (Hirota, 1960), followed by screening for a Pro− phenotype. Bacteriophage φ105DS1 (Savva & Mandelstam, 1984) is a derivative of bacteriophage φ105DI (Flock, 1977) that contains a 7 kbp fragment of _B. subtilis_ chromosomal DNA and can complement all known mutations in the spoIIA and spoVA sporulation loci. Bacteriophage M13mp18 (Norlander et al., 1983) was kindly provided by J. Messing, University of Minnesota, USA.

_B. subtilis techniques._ Methods for preparation of chromosomal DNA and transformation of competent cells were as described by Errington & Mandelstam (1983). Techniques using phage φ105 have been described elsewhere (Jenkinson & Mandelstam, 1983; Errington, 1984). Transduction of the 'tail-less' defective phage φ105DS1 and its derivatives was achieved in the presence of 'helper' phage as described by Jenkinson & Mandelstam (1983) except that phage φ105DI·1·11 was used as helper.

_E. coli techniques._ DNA-mediated transformation of cells, preparation of plasmid DNA, agarose gel-electrophoresis, preparation of plasmid DNA, restriction endonuclease digestions, isolation of DNA from low-melting-point agarose and ligations were done as described previously (Errington, 1984; Fort & Errington, 1985). DNA molecules with 5'-protruding ends were repaired using the Klenow fragment of DNA polymerase I (Klenow polymerase, New England BioLabs) in 30 μl reaction volumes containing 1 μg DNA, 0·5 unit of enzyme and all four deoxynucleoside triphosphates (0·5 mM each). The reaction was done in restriction endonuclease buffer (< 50 mM-NaCl) for 10 min at 22 °C and was terminated by extraction with phenol. The DNA was then recovered by ethanol precipitation. Nuclease-S1 (New England BioLabs) was used to remove single-stranded DNA protruding ends in a 30 μl reaction volume containing 2 μg DNA, 50 mM-sodium acetate (pH 4.0), 50 mM-NaCl, 6 mM-ZnSO₄ and 50 units of enzyme. After 10 min at 22 °C the reaction was terminated by the addition of 0·2 vol. of a solution containing 0·1 M-EDTA and 1·5 M-sodium acetate. After extraction with phenol the DNA was precipitated by the addition of 2 vols ethanol and recovered by centrifugation in the usual manner (Guo et al., 1983).

_Selection of transformants._ B. subtilis cells containing an integrated lac–cat cartridge (see Results) were selected on Oxoid nutrient agar containing chloramphenicol (Sigma; 5 μg ml⁻¹). E. coli cells containing pUC plasmids or their derivatives were selected on Oxoid nutrient agar containing ampicillin (Sigma; sodium salt, 50 μg ml⁻¹) or chloramphenicol (12·5 μg ml⁻¹).

_Detection and assay of β-galactosidase activity._ The chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal; Sigma) was added to solid medium (final concentration 10 μg ml⁻¹) to identify Lac+ colonies in E. coli. A more sensitive fluorogenic substrate, 4-methylumbelliferyl-β-D-galactopyranoside (MUG), was used (final concentration 10 μg ml⁻¹) to detect β-galactosidase activity in _B. subtilis_.

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**Table 1.** Strain JM109F− was derived by treatment of strain JM109 with acridine orange (Hirota, 1960), followed by screening for a Pro− phenotype. Bacteriophage φ105DS1 (Savva & Mandelstam, 1984) is a derivative of bacteriophage φ105DI (Flock, 1977) that contains a 7 kbp fragment of _B. subtilis_ chromosomal DNA and can complement all known mutations in the spoIIA and spoVA sporulation loci. Bacteriophage M13mp18 (Norlander et al., 1983) was kindly provided by J. Messing, University of Minnesota, USA.
lacZ gene fusions in Bacillus subtilis

Table 1. Bacterial strains and plasmids

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<th>Strain</th>
<th>Genotype</th>
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<td></td>
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<td>MB75</td>
<td>lys-1 metC3 tal-1 spo*</td>
<td>Laboratory stock</td>
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<td>recA1 (lac-pro) endA1 gryA96 thi-1 hsdR17 superE4 relA1 F' traD36 proAB lacP ZΔM15</td>
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Plasmids

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<td>pSGMU38</td>
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* Previously designated pSG1.

Nucleotide sequencing. This was done by the chain terminator method of Sanger et al. (1977) with a kit supplied by Amersham. Buffer gradient gels and 35S-labelled nucleotides were used, as described by Biggen et al. (1983). Single-stranded phage DNA was isolated by the method of Sanger et al. (1980).

Second-strand synthesis on a single-stranded MI3 template. Single-stranded phage DNA (1 μg) and dideoxy-sequencing primer (dGTAACCGACGGCCAGT, 0.2 pmol; Amersham) were annealed (2 h, 57 °C) in a 10 μl reaction volume in buffer containing Tris/HCl (10 mM, pH 8.0) and MgCl₂ (10 mM). To the annealed template/primer mixture was added 1 μl of a mixture of all four deoxynucleoside triphosphates (0.5 mM each) and 1 μl (l unit) of Klenow polymerase. After 10 min at 22 °C the reaction was terminated by heating at 70 °C for 10 min.

Southern hybridization. Southern transfers (Southern, 1975), preparation of 32P-labelled probe DNAs (Rigby et al., 1980), hybridization and autoradiography were done as described previously (Errington, 1984).

Colony hybridization. This was done essentially as described by Grunstein & Hogness (1975). Colonies from a selective plate were picked up on 0.45 μm nitrocellulose filters (Schleicher & Schüll), then transferred for 15 min to Whatman 3MM filter papers soaked in the following solutions in turn: 0.5 M-NaOH, 1.5 M-NaCl, 3 M-Tris/HCl, pH 7.5, 3 M-NaCl, 0.3 M-NaCl, 0.03 M-sodium citrate. The nitrocellulose filter was baked, hybridized to labelled probe-DNA and autoradiographed as described for Southern transfer/hybridization (Errington, 1984).

Construction of a spoIIAA : : lacZ fusion. The following DNAs, predigested to completion with the enzymes shown in parentheses, were ligated in a 25 μl reaction volume: ~ 300 ng plasmid pSGMU17 (XbaI), ~ 200 ng plasmid pSGMU23 (XbaI and BglII), ~ 3 μg phage φ105DS1 (BglII). After ligation for 7 h at 15 °C, competent cells of strain MB75 were transformed with a 10 μl sample of the ligation mixture.
Construction of a spoVAA : lacZ fusion. A ligation reaction similar to the one described above (total vol. 25 µl) was used, containing the following DNAs: ~400 ng plasmid pSGMU9 (EcoRI), ~300 ng plasmid pSGMU23 (SmaI and BglII), ~3 µg φ105DS1 (BglII). The EcoRI single-stranded ends of plasmid pSGMU9 were removed by treatment with S1 nuclease prior to ligation (see above). After ligation for 24 h at 15 °C, competent cells of strain MB75 were transformed with 10 µl ligation mixture.

RESULTS AND DISCUSSION
Translational fusion plasmids

A lac fusion cartridge incorporating a selectable chloramphenicol-resistance determinant (cat) and a series of flanking restriction endonuclease cleavage sites was constructed in a series of stages as shown in Fig. 1. The lacZ gene was obtained from plasmid pMC1871 (Casadaban et al., 1983) and the cat gene was originally from the Staphylococcus aureus plasmid pC194 (Iordanescu et al., 1978). A 1.8 kbp fragment of DNA from plasmid pSGMU1 (Errington, 1984) that contains the cat gene was sub-cloned to a location between the BamHI and SalI sites of plasmid pUC12 (Messing, 1983) in E. coli. (In these and subsequent manipulations that required the identification of chloramphenicol resistant clones, transformants were first selected for ampicillin-resistance, encoded by the pUC portion of the plasmid, and then replica-plated to identify chloramphenicol-resistant clones. Direct selection for chloramphenicol resistance was difficult because of the relatively low-level of expression of the cat gene in E. coli.) The resultant plasmid, pSGMU19, was digested with BamHI, treated with Klenow polymerase to render the ends blunt and then digested with SalI. The 1.8 kbp fragment containing the cat gene was isolated and cloned into plasmid pUC12 between its unique NarI (also repaired with Klenow polymerase) and SalI sites; these manipulations yielded plasmid pSGMU20. Plasmid pSGMU20 differs from pUC12 in that the region of the lacZ' gene downstream from the SalI site in the polylinker region is removed and replaced by the 1.8 kbp DNA fragment containing the cat gene. The lacZ' gene is a segment of DNA encoding only the amino-terminal region of β-galactosidase. In a suitable host, such as strain JM103, the product of the lacZ' gene restores, by intracistronic complementation, activity to the otherwise inactive product of the lacZΔM15 allele. The removal of the downstream part of the lacZ' gene was necessary to prevent it from being duplicated when the remainder of the full-length lacZ gene was subsequently introduced into the plasmid. The DNA segment encoding the cat gene in pSGMU20 is flanked by BamHI sites, one in the remaining part of the polylinker region and one reconstituted by blunt-end ligation of the repaired BamHI and NarI ends.

The 3.0 kbp BamHI fragment of plasmid pMC1871 contains a lacZ gene devoid only of the region encoding the amino-terminal part of the β-galactosidase protein. After treatment with S1 nuclease to remove the BamHI cohesive ends, this fragment was cloned by blunt-end ligation into the unique SalI site of plasmid pSGMU20, which had been repaired by Klenow polymerase. The region encoding chloramphenicol-resistance (cat), β-lactamase (bla) and β-galactosidase (lacZ) shown as boxes that indicate the direction of transcription and translation. ori indicates the approximate location of the origin of replication that enables the plasmid to replicate autonomously in E. coli. Z' denotes a segment of DNA encoding only the amino-terminal portion of β-galactosidase. Zig-zag lines denote break points in the coding regions of lacZ or Z'. Polylinker' regions are shown on an expanded scale with the restriction endonucleases abbreviated as follows: B, BamHI; E, EcoRI; F, SphI; G, BglII; H, HindIII; K, KpnI; M, SmaI; N, NarI; P, PstI; S, SalI; T, SstI; V, PvuII; X, XbaI. Not all of the PvuII sites are shown. Brackets indicate that a restriction site was destroyed following blunt-end ligation. Stages involved in the construction of each plasmid are indicated by arrows and, alongside, abbreviated descriptions of the manipulations are given: limit digestions with restriction endonucleases are indicated by the name of the enzyme; 'S1 nuclease' refers to the removal of single-stranded ends from digested DNA fragments with this enzyme; 'Klenow' denotes that 3'-recessed cohesive ends were filled in using the Klenow fragment of DNA polymerase I; ApR and CmR stand for resistances to ampicillin and chloramphenicol, respectively. In general, the enzymic reactions were done in the order shown, and each enzyme was inactivated before proceeding to the next stage. A full account of the plasmid constructions is given in Results and Discussion.
lacZ gene fusions in Bacillus subtilis

lac2

lacZ

gene

fusions

in

Bacillus

subtilis

pSGMU1

4.7 kbp

ori

XbaI

EcoRI

BamHI

Isolate 1.8 kbp fragment containing cat

Ligate

pSGMU19

4.5 kbp

ori

BamHI, Klenow, SspI

Isolate 1.8 kbp fragment containing cat

Transform JM103

Select Ap<sup>+</sup>

then Cm<sup>R</sup>

Ligate

pSGMU20

4.3 kbp

ori

SspI, Klenow

Isolate 3.1 kbp fragment containing lacZ

Ligate

pSGMU23

7.4 kbp

ori

BamHI

Isolate 4.9 kbp fragment

Ligate

pUC19

2.7 kbp

ori

BamHI

Transform JM109F<sup>-</sup>

Select Ap<sup>+</sup>

Screen for Lac<sup>+</sup>

Cm<sup>R</sup>
polymerase. This method of insertion was designed to give an in-frame fusion of the fragment encoding the lacZ gene to its natural promoter and translation initiation signals in the pUC-derived sequences of plasmid pSGMU20, without introducing any further BamHI sites. Ampicillin-resistant transformants were selected in strain JM109F-, from which the lacZ gene is completely deleted, and a Lac+ colony was isolated. The resultant plasmid, pSGMU23, was shown to have the expected constitution (see Fig. 1) by restriction endonuclease analysis (data not shown). The plasmid contains a 4.8 kbp segment of DNA incorporating both the lacZ cartridge from plasmid pMC1871 and the selectable cat gene from pSGMU20. This lac–cat translational fusion cartridge can be removed from plasmid pSGMU23 with BamHI, which cleaves at both ends of the insert, or with a combination of BgIII, which cleaves at the 3' end of the cartridge (relative to the direction of lacZ translation), and any one of the several enzymes that cleave within the 5' terminus of the lacZ gene.

In order to increase the versatility of the system, the cartridge was removed from plasmid pSGMU23 with BamHI and sub-cloned into the unique BamHI sites of plasmids pUC18 and pUC19. Insertion of the lac–cat translational fusion cartridge from plasmid pSGMU23 in the correct orientation generated plasmids pSGMU31 and pSGMU32, respectively. These were again selected by transforming JM109F– to ampicillin-resistance and screening for the production of β-galactosidase. Plasmids isolated from these strains gave the correct restriction cleavage pattern.

The predicted nucleotide sequences of the polylinker regions contained within the coding region of the IacZ gene and restriction sites at the 'downstream' end of the cartridge are given for each plasmid in Table 2. Three enzymes, EcoRI, SalI and SstI, have, in addition to any polylinker sites, a further restriction site within the cartridge. Use of these enzymes would therefore reduce the efficiency with which functional fusions can be obtained.

**Transcriptional fusion plasmids**

The *E. coli* lacZ gene does not carry suitable translation initiation signals for efficient expression in *B. subtilis* (Donnelly & Sonenshein, 1984). However, the translational fusion plasmids described above could be adapted to form transcriptional fusion plasmids by the insertion of a fragment of DNA containing not only the RBS but also the first few codons of a suitable *B. subtilis* gene behind the polylinker region in such a way that an in-frame fusion to the lacZ gene was obtained. It seemed likely that a suitable fragment for such a construction could be obtained from the well-characterized spoIIA region of *B. subtilis* (Fort & Piggot, 1984). This expectation was confirmed when a functional translational fusion was obtained by inserting the lac–cat cartridge from plasmid pSGMU23 into spoIIA (see below). The fused gene product is presumed to be translated from the RBS of the spoIIA gene encoded by the first open reading frame of the operon (ORF1) (Fort & Piggot, 1984), and to consist of the first 23 amino acids of the spo gene product fused in frame to the lacZ gene in the polylinker region (Fig. 3). A simple procedure for modifying plasmid pSGMU23 and its derivative plasmids so that they could be used for transcriptional fusions would therefore be to insert a small fragment of DNA from the spoIIA region at the unique XbaI site of plasmid pSGMU23 in such a way that the same functional gene fusion was obtained. Since this XbaI site is 'downstream' from the BamHI site at the 5' end of the lac–cat cartridge in plasmid pSGMU23 (Fig. 1), the fragment of DNA providing *B. subtilis* translation initiation signals for the lacZ gene would be included within the fragment of DNA released by digestion with BamHI. The new lac–cat transcriptional fusion cartridge could then be moved into plasmids pUC18 and pUC19 as described above for the translational fusion cartridge.

Plasmid pSGMU17 (P. Fort & J. Errington, unpublished results) is a derivative of plasmid pUC13 containing a fragment of *B. subtilis* chromosomal DNA that extends from a point 150 bp upstream from the start codon of the spoIIA gene to an MboI site 70 bp into the coding region of the same ORF (see Fort & Piggot, 1984). This DNA fragment contains not only the RBS and first few codons of spoIIA, but also all of the upstream sequences required for transcription of the spoIIA operon (data not shown). However, the promoter can be at least partially removed by cleaving at an EcoRI site within the 5' untranslated region of the fragment (unpublished results.
Table 2. Properties of plasmids constructed to facilitate lacZ gene and operon fusions in B. subtilis

(a) Translational fusion plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Nucleotide sequence, amino acid translation and useful restriction sites at the 5' end of cartridge</th>
<th>Useful restriction sites at the 3' end of cartridge</th>
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<td>pSGMU23</td>
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<td>ATG ACC ATG ATT ACG AGC TCG GGG GAT CCC GTC ACC ACC GTC ...</td>
<td>BglII, BamHI</td>
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<td>SstI*</td>
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<tr>
<td></td>
<td>ATG ACC ATG ATT ACG AAT TCG AGC TCG CAT GCG TGC AGG TCG ACT CTG GAG GAT CAG CCT CTG GTC ACC GTC ...</td>
<td>BglII, BamHI, XbaI, SalI*, PstI, SpI, HindIII</td>
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<tr>
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<td>Thr Met Ile Thr Asn Ser Ser Ser Ser Val Pro Gly Asp Pro Leu Glu Ser Thr Val ...</td>
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<td>EcoRI* KpnI BamHI SalI*</td>
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(b) Transcriptional fusion plasmids

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<th>Useful restriction sites 'downstream' from cartridge</th>
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<td>BglII, BamH1, XmaI/SmaI, KpnI, SstI*</td>
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</table>

* This restriction endonuclease has an additional recognition site within the cartridge.
Fig. 2. Structure and construction of plasmid pSGMU28. The diagram in the lower part of the figure shows the structure of plasmid pSGMU28, containing the lac-cat transcriptional fusion cartridge (for abbreviations see Fig. 1). The nucleotide sequence of the polylinker region of this plasmid is shown above, with restriction sites and amino acid translation (standard one-letter abbreviations) indicated.

The sequence is broken at an XbaI site into which an adapted EcoRI-MboI fragment of DNA from the spoZZA region of the B. subtilis chromosome has been inserted (see text). The nucleotide sequence of this insert is shown at the top of the figure. Above the sequence, to the left, the amino acid translation is given assuming read-through from the lucZ gene. A termination codon (*) in this reading frame occurs within the region (overlined) that represents the ribosome binding site at the beginning of spoZZA ORFl. The reading frame chosen for the remainder of the amino acid translation is that proposed for translation of the spoIIAA gene (Fort & Piggot, 1984) and this runs on, in-frame, into the distal portion of the lacZ gene to encode a fusion peptide (spoIIAA::lacZ).

and Piggot et al., 1984). The MboI end of the fragment in plasmid pSGMU17 had been ligated to a BamHI end from the polylinker of the vector plasmid pUC13. Beyond this hybrid BamHI/MboI site are consecutive unique restriction sites for the enzymes XbaI, SalI, PstI and HindIII. To sub-clone the EcoRI-MboI fragment, plasmid pSGMU17 was cleaved with EcoRI, treated with Klenow polymerase to fill in the single-stranded ends and then digested with PstI. The vector M13mp18 was cleaved with XbaI, treated with Klenow polymerase and digested with PstI. The target fragment was therefore cloned between the XbaI and PstI sites of M13mp18. The XbaI site in plasmid pSGMU17 was retained in the M13mp18 derivative. At the other end of the inserted fragment, blunt-end ligation of the XbaI and EcoRI ends resulted in the reconstitution of both sites (Fig. 2).

After verifying the nucleotide sequence of the insert, the insert was removed by digestion with XbaI, following primed second-strand synthesis on the single-stranded phage DNA template, and ligated into the unique XbaI site of plasmid pSGMU23. After transformation of strain JM109F- and selection for ampicillin-resistance, about 8% of the transformants were white on indicator plates, i.e. did not produce β-galactosidase activity. The remaining colonies were blue, and colony hybridization confirmed that approximately 5% of these contained the XbaI insert. Restriction enzyme analysis of plasmid DNA from one of the colonies that showed hybridization confirmed that the construction was correct. In the new plasmid (pSGMU28),
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translation initiated at the lacZ RBS would be expected to read through 19 amino acid residues to a stop codon within the RBS of spoIIA ORF1 (Fig. 2). Since the latter is translated in a different reading frame, it seems likely that the pSGMU28-encoded β-galactosidase activity is initiated, as expected, at the amino terminus of spoIIA ORF1.

The 5.2 kbp BamHI fragment from plasmid pSGMU28 was sub-cloned into the unique BamHI sites of plasmids pUC18 and pUC19, as described above for the translational fusion cartridge. The relevant restriction sites in the lac-cat transcriptional fusion plasmids, which were designated pSGMU32 and pSGMU38 respectively, are shown in Table 2.

**Fusion of the lac-cat cartridge from pSGMU23 to genes in the spoIIA and spoVA operons**

The method used for insertion of the lac-cat cartridges into the B. subtilis chromosome depends upon the observation that a DNA fragment carrying a selectable gene, such as cat, can be rescued very efficiently onto the B. subtilis chromosome, during transformation of competent cells, if it is flanked by fragments of chromosomal DNA (unpublished results). This type of transformation event has been exploited previously in B. subtilis cloning using both phage (Kawamura et al., 1979) and plasmid (Gryczan et al., 1980) vectors.

The lac-cat cartridges in the plasmids described above can be removed using a variety of pairwise combinations of restriction endonucleases. The same enzymes (or, in some cases, a range of different enzymes) can then be used to digest cloned chromosomal DNA into fragments that can be ligated in vitro to the ends of the lac-cat cartridge. After transformation of competent cells, the flanking fragments of chromosomal DNA provide regions of homology that enable the lac-cat cartridge to be rescued onto the chromosome. By using suitable combinations of enzymes to digest the chromosomal DNA target at unique sites, the lac-cat cartridge can be inserted at a specific point. Alternatively, by using enzymes that have multiple cleavage sites, or by using sequential degradative exonucleases such as Bal31, or a combination of exonuclease III and nuclease S1 (Guo et al., 1983), the lac-cat cartridge can be inserted more or less at random. This procedure is therefore flexible and provides a powerful tool for investigating the control of expression of newly cloned or physically well-characterized regions. Moreover, even in the absence of an active β-galactosidase fusion product, such chromosomal insertions are highly mutagenic. By correlating the location and orientation of a series of independent chromosomal insertions (using Southern hybridization, for example) with the phenotypic consequences and the ability to express β-galactosidase, a great deal of information could be rapidly obtained on the genetic and physical organization of the cloned region.

To illustrate the use of the system described to integrate the lac-cat cartridge at a precise chromosomal location, the plasmid pSGMU23 was used to generate functional translational fusions to genes in both the spoIIA and spoVA loci. The cloning and further characterization of these loci has been described in detail elsewhere (Liu et al., 1982; Piggot et al., 1984, 1985; Savva & Mandelstam, 1984, 1985; Fort & Piggot, 1984; Fort & Errington, 1985). Plasmids or phages containing suitable chromosomal DNA inserts and restriction endonuclease sites were chosen to provide fragments of homologous DNA with which to flank the lac-cat cartridge of plasmid pSGMU23. These flanking fragments enable the cartridge to be inserted by marker rescue recombination at specific sites in the B. subtilis chromosome, as shown in Figs 3 and 4. In a single experiment using 1 μg of ligated DNAs (see Methods), five chloramphenicol-resistant transformants of strain MB75 were obtained with the spoIIA system and 34 with the spoVA system. Individual transformants picked at random were completely stable, even when sub-cultured in the absence of chloramphenicol, and β-galactosidase activity was detected on nutrient agar plates containing the fluorescent indicator substrate MUG, after 24 h at 37 °C. The low numbers of transformants in the case of the spoIIA fusion were not surprising, considering the very small size of the 5'-flanking homologous DNA (about 200 bp). [The following two papers describe in detail analyses of the control of expression of both of these loci (Errington & Mandelstam, 1986a, b).] Southern hybridization was used to verify that a single copy of the lac-cat cartridge was present in the correct chromosomal location for each strain (data not shown).
Fig. 3. Translational fusion of the lac–cat cartridge from plasmid pSGMU23 to a gene in the spoIIA operon in B. subtilis. The lower part of the figure shows a physical map of the spoIIA/spoVA region of the B. subtilis chromosome. The spoIIA operon contains three genes (Fort & Piggot, 1984) and the spoVA operon five genes (Fort & Errington, 1985), as indicated by the letters in the boxes. The lac–cat cartridge was released from plasmid pSGMU23 by digestion with enzymes XbaI and BglII (as shown at the top of the figure), and was ligated to DNAs containing regions homologous to the B. subtilis chromosome. The XbaI end of the lac–cat cartridge could ligate to a segment of spoZIA DNA contained in plasmid pSGMU17 and the BglII end of the cartridge to a fragment of DNA from phage φ105DS1. The latter fragments of DNA are shown in the middle part of the figure and are aligned over their respective regions of homology to the chromosome. Broken vertical lines indicate the limits of these regions of homology. The coding portions of the lacZ gene and the cat gene are shown as hatched boxes and solid boxes respectively. Non-coding DNA of chromosomal (——) and plasmid (-----) origin are distinguished as shown. The end-point of the DNA insert in phage φ105DS1 downstream from the essential part of the spoVA operon has not been determined precisely (Fort & Errington, 1985). After ligation of the DNA molecules and transformation of strain MB75, only the arrangement shown for ligation of the participating molecules and a crossover event in both regions of homology could give rise to stable chloramphenicol-resistant colonies.

Fig. 4. Translational fusion of the lac–cat cartridge from plasmid pSGMU23 to a gene in the spoVA operon in B. subtilis. The arrangement of the figure is essentially similar to that in Fig. 3. Plasmid pSGMU9, containing an 0.96 kbp PvuII fragment of DNA from the spoIIA/spoVA region (Fort & Errington, 1985), was digested with EcoRI and then treated with S1 nuclease to obtain the correct reading frame when ligated to Smal-digested pSGMU23.
Transfer of the spo::lac fusions of \( \phi 105 \)

Phage \( \phi 105 \)DSI (Savva & Mandelstam, 1984) contains a 7 kbp fragment of chromosomal DNA that complements in trans all of the known \( spoIIA \) and \( spoVA \) mutations. The ends of the chromosomal DNA fragment cloned in this phage extend beyond the junctions of chromosomal DNA and \( lac-cat \) DNA in both the \( spoIIA \) and the \( spoVA \) fusion systems. Transfer of the chromosomal \( lac \) fusions to a mobile genetic element such as phage \( \phi 105 \) would be useful for two main reasons. It would facilitate the more detailed characterization of the genetic elements controlling the expression of the sporulation operon. It would also enable the fusion system to be transferred conveniently, by transduction and selection for chloramphenicol resistance, into different genetic backgrounds, where, for example, the effects of different \( spo \) mutations on its expression could be evaluated [see the following papers (Errington & Mandelstam, 1986a, b; Clarke et al., 1986)].

The procedure for transferring the spo::lac fusions on to the \( \phi 105 \)DSI genome was as follows. Chromosomal DNA from strains 613 (\( spoIIA::lac \)) and 614 (\( spoVA::lac \)) was used to transform strain 612.1, with selection for chloramphenicol resistance. The recipient strain carries the \( spoIIA \) mutation (mutation \( 4z \) of Ionesco & Schaeffer, 1968), which consists of an extensive deletion involving most of both the \( spoIIA \) and \( spoVA \) operons (P. J. Piggot, personal communication), but which is phenotypically \( Spo^+ \) as a result of complementation by the \( \phi 105 \)DSI prophage. Upon transformation, the \( lac-cat \) cartridge can be rescued (following selection for chloramphenicol resistance), by homologous recombination involving the flanking chromosomal DNA sequences, either on to the chromosome of the recipient or on to the prophage genome. In the former case the colony will remain phenotypically \( Spo^+ \), since it retains intact copies of \( spoIIA \) and \( spoVA \) sequences on the prophage. However, if rescue occurs on the prophage genome, the cell becomes phenotypically \( Spo^- \), since both copies of the \( spo \) operon in the cell are disrupted. Chloramphenicol-resistant transformants were obtained at a frequency of about \( 10^4 \) per \( \mu g \) DNA. The proportions of \( Spo^- \) colonies among the transformants were 6.4\% for the \( spoIIA::lac \) fusion and 16\% for the \( spoVA::lac \) fusion. (Presumably the difference in the proportions of \( Spo^+ \) transformants reflects the relatively small flanking homologous DNA at the 5’ end of the \( spoIIA::lac \) fusion.) Phage lysates were prepared from a single chloramphenicol-resistant \( Spo^- \) transformant of each type. In both cases the lyse was able to transduce sensitive cells of strain MB75 to chloramphenicol resistance (in the presence of wild-type helper phage) and the resulting transductants, designated strains SG36 and SG37, showed appropriate control of expression of the \( spo::lac \) fusion.

Restriction enzyme digestion of DNA from the two new phages, designated \( \phi 105 J19 \) (\( spoIIA::lac \)) and \( \phi 105 J20 \) (\( spoVA::lac \)), showed the expected results (Fig. 5). The largest \( HindIII \) fragment (7-0 kbp) of \( \phi 105 \)DSI, which contains the \( spoIIA/spoVA \) region, was increased in size by insertion of the \( lac-cat \) cartridge to approximately 8-1 kbp in \( \phi 105 J19 \) and 9-1 kbp in \( \phi 105 J20 \). After secondary digestion with \( XbaI \), the region of DNA upstream of the cartridge was released to give fragments of 1-2 kbp and 2-3 kbp respectively. (The latter fragment co-migrates with two other fragments on this gel.) These have the expected molecular masses for fragments that extend from the ‘upstream’ \( HindIII \) sites to the location of the \( spo::lac \) fusion.

Zuber & Losick (1983) have described the transfer of \( spo::lac \) fusions to specialized transducing derivatives of phage SP\( \beta \) that can be used in much the same way as the \( \phi 105 \) derivatives described here. However, phage \( \phi 105 \) is much smaller (39-2 kbp; Bugaichuk et al., 1984) than SP\( \beta \) (118 kbp; Zahler, 1982) and consequently its DNA is easier to characterize and to manipulate in vitro. Clearly the same approach could be used to transfer similar \( lac \) fusions from chromosome to prophage for any gene cloned in \( \phi 105 \).

Random \( lac\) fusions to chromosomal genes

In principle, the plasmids described here could also be used to generate random fusions to chromosomal genes in \( B. subtilis \). Plasmids such as these, which can replicate autonomously in \( E. coli \) but not in \( B. subtilis \), can sometimes be successfully transformed into the latter host if they carry a suitable selective marker (usually antibiotic resistance) and have a region of homology.
Fig. 5. Restriction enzyme analysis of bacteriophages φ105J19 and φ105J20. DNA from phages φ105DS1 (lanes 2 and 3), φ105J19 (lanes 4 and 5) and φ105J20 (lanes 6 and 7) were digested with HindIII (lanes 2, 4 and 6) or with a combination of HindIII and XbaI (lanes 3, 5 and 7). The completed digests were electrophoresed on a 0.7% (w/v) agarose gel. Lanes 1 and 8 contain molecular mass marker fragments (HindIII-digested λ).

with the *B. subtilis* chromosome. These transformations have been shown to occur by a single crossover event, involving the regions of homology in the plasmid and chromosome, that leads to a Campbell-like integration (Duncan *et al.*, 1978; Haldenwang *et al.*, 1980). Such integrational plasmids have been used for a variety of purposes; they have been used to map the chromosomal location of cloned DNAs (Haldenwang *et al.*, 1980), to study dominance and complementation relationships (Ferrari & Hoch, 1983), and to identify transcriptional units (Piggot *et al.*, 1984; Fort & Errington, 1985). Random chromosomal *lacZ* fusions could be generated by 'shotgun' cloning *B. subtilis* chromosomal DNA fragments into any of the upstream polylinker sites of the plasmids in *E. coli*, followed by transformation of *B. subtilis* with DNA from a pool of recombinant plasmids and selection for chloramphenicol-resistance. In the transformants, the lac-cat plasmid would be integrated at sites determined by the homology between its DNA insert and the *B. subtilis* chromosome (Niaudet *et al.*, 1982). Youngman *et al.* (1985) have described a system for generating essentially random chromosomal *lac* fusions by integration of derivatives of the transposon Tn917. A slight disadvantage with their method is the highly mutagenic nature of transposon insertions, which precludes its use for analysing the expression
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of essential genes. In contrast, the random plasmid integrations described above are often not mutagenic (Piggot et al., 1984).

In conclusion, the methods described here provide a general approach to study the regulation of gene expression in B. subtilis.

I thank Professor J. Mandelstam for advice and for many helpful discussions, Ms G. Roberts for excellent technical assistance, and the Royal Society for a 1983 Research Fellowship. This work was supported in part by the Science and Engineering Research Council.

REFERENCES


