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

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A series of plasmids has been constructed that can be used to fuse the β -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 ϕ 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* (β -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 β -galactosidase to the coding region of a heterologous gene. The appearance of β -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 β -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 β -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.

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 $\phi 105$ 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, 1986*a*), *spoVA* (Errington & Mandelstam, 1986*b*), *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 $\phi 105$ DS1 (Savva & Mandelstam, 1984) is a derivative of bacteriophage $\phi 105$ DI:1t (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 (Norrander *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 $\phi 105$ have been described elsewhere (Jenkinson & Mandelstam, 1983; Errington, 1984). Transduction of the 'tail-less' defective phage $\phi 105$ DS1 and its derivatives was achieved in the presence of 'helper' phage as described by Jenkinson & Mandelstam (1983) except that phage $\phi 105$ DI:1t 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 μ l reaction volumes containing 1 μ g 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 μ l reaction volume containing 2 μ g 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 μ g ml⁻¹). *E. coli* cells containing pUC plasmids or their derivatives were selected on Oxoid nutrient agar containing ampicillin (Sigma; sodium salt, 50 μ g ml⁻¹) or chloramphenicol (12.5 μ g 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 μ g 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 μ g ml⁻¹) to detect β -galactosidase activity in *B. subtilis*.

Table 1. *Bacterial strains and plasmids*

Strain	Genotype	Origin
<i>B. subtilis</i>		
MB75	<i>lys-1 metC3 tal-1 spo⁺</i>	Laboratory stock
2000.A4	<i>hisH2 pyrD1 rpoB2 tal-1 spoIIA4</i>	M. D. Yudkin, Oxford Univ., UK
612.1	<i>hisH2 pyrD1 rpoB2 tal-1 [spoIIA4] (ϕ105DS1 spoIIA⁺ spoVA⁺)</i>	Spo ⁺ transductant of 2000.A4
613	<i>lys-1 metC3 tal-1 spoIIA::lac-cat</i>	This paper
614	<i>lys-1 metC3 tal-1 spoVAA::lac-cat</i>	
SG36	<i>lys-1 metC3 tal-1 spo⁺ (ϕ105J19 spoIIA::lac-cat)</i>	
SG37	<i>lys-1 metC3 tal-1 spo⁺ (ϕ105J20 spoVAA::lac-cat)</i>	
<i>E. coli</i>		
JM103	$\Delta(lac-pro)$ <i>thi-1 strA supE44 endA sbcB15 F' traD36 proAB lacI^{ts} ZΔM15</i>	Messing <i>et al.</i> (1981)
JM109	<i>recA1 Δ(lac-pro) endA1 gyrA96 thi-1 hsdR17 supE44 relA1 F' traD36 proAB lacI^{ts} ZΔM15</i>	Norrrander <i>et al.</i> (1983)
JM109F ⁻	As JM109, but F ⁻	This paper
Plasmids	Relevant characteristics/construction	Reference
pUC12,	<i>bla lacZ'</i> , 2.7 kbp	Messing (1983)
pUC13		Norrrander <i>et al.</i> (1983)
pUC18,		
pUC19		
pMC1871	<i>tet lacZ'</i> , 7.3 kbp	Casadaban <i>et al.</i> (1983)
pSGMU1*	<i>bla cat</i> , 4.7 kbp	Errington (1984)
pSGMU2	<i>bla cat</i> , 3.7 kbp	Fort & Errington (1985)
pSGMU9	<i>bla cat</i> , 4.7 kbp; 950 bp <i>PvuII</i> fragment from <i>spoIIA/spoVA</i> region inserted in pSGMU2	Fort & Errington (1985)
pSGMU17	<i>bla cat</i> , 2.95 kbp; 224 bp fragment from <i>spoIIA</i> promoter region inserted in pSGMU2	P. Fort & J. Errington (unpublished results)
pSGMU19	<i>bla cat</i> , 4.5 kbp	This paper
pSGMU20	<i>bla cat</i> , 4.3 kbp	
pSGMU23	<i>bla lacZ⁺ cat</i> , 7.4 kbp	
pSGMU28,	<i>bla lacZ⁺ cat</i> , 7.6 kbp	
pSGMU31,		
pSGMU37		
pSGMU32,	<i>bla lacZ⁺ cat</i> , 7.8 kbp	
pSGMU38		

* 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 M13 template. Single-stranded phage DNA (1 µg) and dideoxy-sequencing primer (dGTAAAACGACGGCCAGT, 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 (1 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.*, 1977), 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 periods to Whatman 3MM filter papers soaked in the following solutions in turn: 0.5 M-NaOH, 1.5 M-NaCl; 1.5 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 *spoIIA::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 (*Xba*I), ~200 ng plasmid pSGMU23 (*Xba*I and *Bgl*II), ~3 µg phage ϕ105DS1 (*Bgl*II). 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 μ l) was used, containing the following DNAs: $\sim$ 400 ng plasmid pSGMU9 (*EcoRI*), $\sim$ 300 ng plasmid pSGMU23 (*SmaI* and *BglII*), $\sim$ 3 μ g ϕ 105DS1 (*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 $^{\circ}$ C, competent cells of strain MB75 were transformed with 10 μ l 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 *Bam*HI and *Sal*I 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 *Bam*HI, treated with Klenow polymerase to render the ends blunt and then digested with *Sal*I. The 1.8 kbp fragment containing the *cat* gene was isolated and cloned into plasmid pUC12 between its unique *Nar*I (also repaired with Klenow polymerase) and *Sal*I sites; these manipulations yielded plasmid pSGMU20. Plasmid pSGMU20 differs from pUC12 in that the region of the *lacZ'* gene downstream from the *Sal*I 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 *lacZ* Δ M15 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 *Bam*HI sites, one in the remaining part of the polylinker region and one reconstituted by blunt-end ligation of the repaired *Bam*HI and *Nar*I ends.

The 3.0 kbp *Bam*HI 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 *Bam*HI cohesive ends, this fragment was cloned by blunt-end ligation into the unique *Sal*I site of plasmid pSGMU20, which had been repaired by Klenow

Fig. 1. Construction of plasmid pSGMU23 and its derivatives pSGMU31 and pSGMU37 that all contained *lac*-*cat* translational fusion cartridges. Plasmids are drawn approximately to scale, with regions 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, *Bam*HI; E, *Eco*RI; F, *Sph*I; G, *Bgl*II; H, *Hind*III; K, *Kpn*I; M, *Sma*I; N, *Nar*I; P, *Pst*I; S, *Sal*I; T, *Sst*I; V, *Pvu*II; X, *Xba*I. Not all of the *Pvu*II 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; Ap^R and Cm^R 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.

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 *Bam*HI 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 *Bam*HI, which cleaves at both ends of the insert, or with a combination of *Bgl*II, 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 *Bam*HI and sub-cloned into the unique *Bam*HI 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 *lacZ* gene and restriction sites at the 'downstream' end of the cartridge are given for each plasmid in Table 2. Three enzymes, *Eco*RI, *Sal*I and *Sst*I, 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 *Xba*I site of plasmid pSGMU23 in such a way that the same functional gene fusion was obtained. Since this *Xba*I site is 'downstream' from the *Bam*HI 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 *Bam*HI. 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 *spoIIAA* gene to an *Mbo*I 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 *spoIIAA*, 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 *Eco*RI 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

Plasmid	Nucleotide sequence, amino acid translation and useful restriction sites at the 5' end of cartridge																Useful restriction sites at the 3' end of cartridge	
pSGMU23	Thr ATG	Met ACC	Ile ATG	Asn AAT	Ser TCG	Ser AGC	Ser TCG	Pro CCC	Gly GGG	Asp GAT	Pro CCT	Leu CTA	Glu GAG	Ser TCG	Thr ACC	Val GTC		BglII, BamHI
	EcoRI*			SsrI*			XmaI SmaI			BamHI			XbaI			SalI*		
pSGMU31	Thr ATG	Met ACC	Ile ATG	Asn AAT	Ser TCG	Ser AGC	Ser TCG	Val GTA	Pro CCC	Gly GGG	Asp GAT	Pro CCT	Leu CTA	Glu GAG	Ser TCG	Thr ACC	Val GTC	BglII, BamHI, XbaI, SalI*, PstI, SphI, HindIII
	EcoRI*			SsrI*			KpnI			BamHI			XbaI			SalI*		
										XmaI SmaI			XbaI					
pSGMU37	Thr ATG	Met ACC	Ile ATG	Asn CCA	Ser AGC	Ser TTG	Leu CAT	His GCC	Ala TGC	Cys AGG	Arg TCG	Ser ACT	Thr CTA	Glu GAG	Ser TCG	Thr ACC	Val GTC	BglII, BamHI, XmaI/SmaI, KpnI, SsrI*, EcoRI*
	HindIII									PstI			XbaI			BamHI	SalI*

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 *spoIIA* 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 *lacZ* gene. A termination codon (*) in this reading frame occurs within the region (overlined) that represents the ribosome binding site at the beginning of *spoIIA* ORF1. The reading frame chosen for the remainder of the amino acid translation is that proposed for translation of the *spoIIA* gene (Fort & Piggot, 1984) and this runs on, in-frame, into the distal portion of the *lacZ* gene to encode a fusion peptide (*spoIIA::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),

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 *Bam*HI fragment from plasmid pSGMU28 was sub-cloned into the unique *Bam*HI 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 on to 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 *Bal*31, 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 μ g 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, 1986*a, 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 *Xba*I and *Bgl*II (as shown at the top of the figure), and was ligated to DNAs containing regions homologous to the *B. subtilis* chromosome. The *Xba*I end of the *lac-cat* cartridge could ligate to a segment of *spoIIA* DNA contained in plasmid pSGMU17 and the *Bgl*II end of the cartridge to a fragment of DNA from phage ϕ 105DS1. 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 ϕ 105DS1 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 *Pvu*II fragment of DNA from the *spoIIA/spoVA* region (Fort & Errington, 1985), was digested with *Eco*RI and then treated with S1 nuclease to obtain the correct reading frame when ligated to *Sma*I-digested pSGMU23.

Transfer of the spo::lac fusions of ϕ 105DS1

Phage ϕ 105DS1 (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 ϕ 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, 1986*a, b*; Clarke *et al.*, 1986; Turner *et al.*, 1986)].

The procedure for transferring the *spo::lac* fusions on to the ϕ 105DS1 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 *spoIIA4* 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 ϕ 105DS1 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 10⁴ per μ 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 lysate 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 ϕ 105J19 (*spoIIA::lacZ*) and ϕ 105J20 (*spoVA::lacZ*), showed the expected results (Fig. 5). The largest *Hind*III fragment (7.0 kbp) of ϕ 105DS1, which contains the *spoIIA/spoVA* region, was increased in size by insertion of the *lac-cat* cartridge to approximately 8.1 kbp in ϕ 105J19 and 9.1 kbp in ϕ 105J20. After secondary digestion with *Xba*I, 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' *Hind*III sites to the location of the *spo::lacZ* fusion.

Zuber & Losick (1983) have described the transfer of *spo::lacZ* fusions to specialized transducing derivatives of phage SP β that can be used in much the same way as the ϕ 105 derivatives described here. However, phage ϕ 105 is much smaller (39.2 kb; Bugaichuk *et al.*, 1984) than SP β (118 kb; 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 *lacZ* fusions from chromosome to prophage for any gene cloned in ϕ 105.

Random lacZ 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 $\phi 105J19$ and $\phi 105J20$. DNA from phages $\phi 105DS1$ (lanes 2 and 3), $\phi 105J19$ (lanes 4 and 5) and $\phi 105J20$ (lanes 6 and 7) were digested with *Hind*III (lanes 2, 4 and 6) or with a combination of *Hind*III and *Xba*I (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 (*Hind*III-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

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*.

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