The Expression of the *Escherichia coli lacZ* Gene in *Streptomyces*

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The *Escherichia coli lacZ* gene was stably introduced into ϕ C31-based phage cloning vectors in *Streptomyces lividans*. However, *lacZ* could not be stably introduced into *S. lividans* on the plasmid vectors pIJ702 or pIJ41. Studies of the expression of *lacZ* in *S. lividans* and *S. coelicolor* were facilitated by the use of mutants and/or growth conditions in which endogenous β -galactosidase activity was low or absent. Plaques and lysogens of ϕ C31::*lacZ* constructs involving transcriptional fusions to the pBR322 *tet* gene contained β -galactosidase activity. Activities were markedly higher in *S. lividans* than in *S. coelicolor*. Insertion of the major transcriptional terminator of coliphage fd between the *tet* promoter and *lacZ* reduced *lacZ* expression more in lysogens than in lytically infected cultures, suggesting the existence of a phage-specified anti-termination function. Several ϕ C31 derivatives suitable for the construction of different kinds of transcriptional and translational fusions in *Streptomyces* were derived. The expression of *lacZ* in one transcriptional fusion vector (KC659) was increased both in plaques and in lysogens by the appropriately positioned insertion of a previously characterized promoter from the *aph* gene of *S. fradiae*. When ϕ C31 DNA fragments were inserted into KC659, at least one recombinant phage gave high β -galactosidase activity in lytic infections, but not in lysogens. The potential usefulness of *lacZ* fusions in *Streptomyces* is discussed.

INTRODUCTION

Among a large number of intriguing features of streptomycetes, the capacity for morphological and physiological differentiation (to give rise to sporulating hyphae and antibiotic production) towards the end of vegetative growth is perhaps the major reason for their intensive study (Chater, 1984). To this end, several plasmid and phage systems for DNA cloning in *Streptomyces* have been developed (Hopwood *et al.*, 1985a; Chater *et al.*, 1985), and among the numerous genes so far cloned are several related to morphological differentiation [*bldA*, *bldB* (Piret & Chater, 1985; J. M. Piret & K. F. Chater, unpublished), *whiG* (C. Mendez, personal communication)] and antibiotic production (Hopwood *et al.*, 1985b). Generally, the products of such genes are either unknown or are difficult to assay. Regulatory studies would, therefore, benefit from fusion of these genes to genes with easily detected products or phenotypic effects. Several probe genes of this kind have been successfully used in *Streptomyces*, viz. the *cat* gene of Tn9 (Bibb & Cohen, 1982); the *neo* gene of Tn5 (Janssen *et al.*, 1985); and the *vph* gene of *Streptomyces vinaceus* (Rodicio *et al.*, 1985). However, all these genes mediate antibiotic resistance, a property useful in vegetative growth but likely to be difficult to employ when controlled by promoters active mainly after growth is completed. This consideration has led us to investigate the expression in *Streptomyces* of the *lacZ* gene of *Escherichia coli*. The *lacZ* gene product, β -galactosidase, can be detected or measured by several methods including very

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Abbreviations: MUG, 4-methylumbelliferyl β -D-galactoside; MUG⁺ (MUG⁻), hydrolysis (non-hydrolysis) of MUG; NTG, N-methyl-N'-nitro-N-nitrosoguanidine; ONPG, o-nitrophenyl β -D-galactoside; X-gal, 5-bromo-4-chloro-3-indolyl β -D-galactoside; XG⁺ (XG⁻), hydrolysis (non-hydrolysis) of X-gal.

sensitive and localized colour reactions, and it has been the most widely used indicator gene in fusion studies (Silhavy *et al.*, 1984). Although some difficulties have been encountered, we have succeeded in stably maintaining *lacZ* in *Streptomyces* by using ϕ C31 phage vectors, and have constructed a variety of ϕ C31::*lacZ* derivatives for use in different kinds of gene fusions. The sources of the *lacZ* DNA in these experiments were *E. coli* plasmids developed for *lacZ* fusions by Casadaban *et al.* (1980), Shapira *et al.* (1983) and Guarente *et al.* (1980). Activation of *lacZ* expression in plaques and in lysogens by a previously characterized *Streptomyces* promoter was observed using a transcriptional fusion. A fragment of ϕ C31 DNA was cloned into the same transcriptional fusion vector and found to activate *lacZ* expression in plaques, but not in lysogens.

METHODS

Bacteria. The following streptomycetes were used: *S. lividans* 66 (Lomovskaya *et al.*, 1972; John Innes Institute stock no. 1326); *S. lividans* J1528, a β -galactosidase-deficient mutant of 1326 [strain 1326-9 of Eckhardt *et al.* (1982)]; *S. coelicolor* A3(2) strain M130 (Bibb *et al.*, 1977), which is a Pgl⁺ strain unable to support ϕ C31 plaque formation (but able to be lysogenized and to release phage; Chinenova *et al.*, 1982); *S. coelicolor* A3(2) strain J1501, a Pgl⁻ derivative of M130 which allows ϕ C31 plaque formation (Chater *et al.*, 1982); and *S. coelicolor* A3(2) strains J1510 and J1511, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG)-induced mutants of M130 and J1501, respectively, that remained white during growth in the presence of X-gal (5-bromo-4-chloro-3-indolyl β -D-galactoside). Other *Streptomyces* strains are described in the text. *E. coli* work was done with ED 8800 (Murray *et al.*, 1977).

Media. Minimal medium (MM), protoplast regeneration medium (R2YE) and methods for *Streptomyces* culture were as described by Hopwood *et al.* (1985c), unless stated otherwise. Soft Y agar was the *Rhizobium* minimal medium of Beringer (1974), but contained 0.5% agar.

Plasmids. *Streptomyces* vector plasmids pIJ41 (Thompson *et al.*, 1982a) and pIJ702 (Katz *et al.*, 1983) were isolated and used as described by Hopwood *et al.* (1985c). The *E. coli* plasmids used were pLG400 and pMC931 (*lacZ* translational fusion vectors; Guarente *et al.*, 1980; Casadaban *et al.*, 1980); pMC903 and pMC1871 (*lacZ* transcriptional fusion vectors; Casadaban *et al.*, 1980; Shapira *et al.*, 1983); pLBU-1 (containing the fd terminator; Gentz *et al.*, 1981); pIJ36 [pBR322 (Bolivar *et al.*, 1977) containing the *vph* (viomycin phosphotransferase) gene of *Streptomyces vinaceus*; Thompson *et al.*, 1982b]. The isolation of *E. coli* plasmids and their use in transformation were as described by Maniatis *et al.* (1982).

Phages. Derivatives of ϕ C31 are described in Fig. 1. Phage techniques were as described by Hopwood *et al.* (1985c).

In vitro manipulation of DNA. Standard techniques were used throughout, as described by Maniatis *et al.* (1982) and Hopwood *et al.* (1985c). An oligonucleotide adapter sequence, converting restriction sites for *Bam*HI, *Mbo*II, *Bgl*II or *Bcl*I to sites for *Xho*I and *Bgl*II, has been previously described (Rodicio *et al.*, 1985).

Detection of β -galactosidase activity. For detection of β -galactosidase activity on plates we used several methods: (1) the development of blue coloration (the XG⁺ phenotype) in media containing X-gal (Bachem) (40 μ g ml⁻¹); (2) the rapid development of blue-green fluorescence (the MUG⁺ phenotype) after overlaying colonies or plaques with soft Y agar containing MUG (4-methylumbelliferyl β -D-galactoside; Sigma) (12.5 μ g ml⁻¹ final concentration from a stock solution at 50 mg ml⁻¹ in dimethyl sulphoxide), seen by irradiating open inverted plates at 310 nm on a transilluminator; (3) for *E. coli* colonies, the development of red colour on lactose McConkey agar. Quantitative assays of β -galactosidase activity in *Streptomyces* cell-free extracts and in 'soak-outs' from lysates in top layers used the cleavage of ONPG (*o*-nitrophenyl β -D-galactoside) (Miller, 1972): 1 ml mycelial extract was warmed to room temperature, 0.2 ml ONPG (4 mg ml⁻¹ in 0.1 M-phosphate buffer pH 7.0) was added, and the change in OD₄₂₀ against blanks of Z-buffer (Miller, 1972) was measured. In these conditions, 1 unit of β -galactosidase was defined as that which produced an increase in OD₄₂₀ of 0.001 per minute (light path length, 1 cm).

Preparation of mycelial extracts. The minimal (LMPC) and rich (YEME) media and the procedures for growth and lysis were essentially as described by Seno & Chater (1983), except that the buffer was Z-buffer (Miller, 1972). After sonication, extracts were clarified by low speed centrifugation (12000 g, 10 min, 4 °C). Extracts were kept at 4 °C until use (β -galactosidase activity in extracts was stable for at least 24 h at 4 °C or 20 °C). Protein content was assayed by the biuret method (Herbert *et al.*, 1971).

Preparation of 'soak-outs' from overlays containing plaques, for β -galactosidase assays. For the semi-quantitative estimation of β -galactosidase activity in lytically infected cells, 2.5 ml 100 mM-phosphate buffer pH 7.0 was added to 16–18-h-old phage overlay plates (5 cm diameter) containing 500–2000 plaques and allowed to soak for 2 h. Most of the fluid was then transferred to a test-tube using a Pasteur pipette. It was kept on ice for up to 2 h before use. To 1 ml samples, 0.2 ml ONPG (stock solution 4 mg ml⁻¹) was added (in some experiments, smaller volumes

were used). Spectrophotometric analysis (as above) was done against a blank of a phosphate buffer soak-out of a top layer not inoculated with phage. Consistent results were obtained when values were compared with an internal control, though there was day-to-day variation in the absolute levels of activity detected (perhaps due to varying host densities in the top layers). Activities decreased markedly if plates were confluent lysed or contained fewer than 500 plaques. Using these preparative conditions, β -galactosidase activity was stable for at least 2 h after soaking-out, and gave a linear response during the assay for at least 20 min.

RESULTS

*Endogenous β -galactosidase activity in *S. coelicolor* and *S. lividans**

S. coelicolor and its close relative *S. lividans* are the most widely used streptomycetes in genetic studies. *S. lividans* was shown by Eckhardt & Fare (1982) to produce intracellular and extracellular β -galactosidase activity. Like *S. lividans*, *S. coelicolor* (strains M130 and J1501) gave strong X-gal responses on glucose-supplemented MM after about 2 d incubation. The presence of the activity is not sufficient to allow significant growth of *S. coelicolor* on lactose (although *S. lividans* utilizes lactose well as sole carbon source). Eckhardt & Fare (1982) isolated a mutant (1326-9; our stock no. J1528) of *S. lividans* deficient in endogenous activity. This mutant was helpful in the work reported in this paper, and we have attempted to isolate similar mutants of *S. coelicolor*. Two mutants of *S. coelicolor*, J1510 and J1511, that did not hydrolyse X-gal on MM containing glucose were isolated from NTG-treated M130 and J1501 spore suspensions respectively. Surprisingly, neither mutant was deficient in ONPG-hydrolysing activity when cell-free extracts were assayed from cultures grown in liquid MM containing galactose or galactose plus lactose. On the other hand, when glucose was the sole carbon source the mutants had a 100-fold lower enzyme activity, whereas the wild-type level was approximately halved (Table 1). The nature of this interesting, apparently regulatory, abnormality has yet to be examined. β -Galactosidase activity was absent from cell-free extracts of all the *S. coelicolor* and *S. lividans* strains tested after growth in the complex YEME medium.

In the remainder of the work described in this paper, ONPG-based assays of *lacZ*-specified activity in cell-free extracts were done with cultures grown in YEME. The detection of β -galactosidase activity in plaques, using Difco nutrient agar medium containing glucose, was not usually affected by the *S. lividans* host activity, which developed later than the phage-specified activity (or not at all, when J1528 was used).

*Expression of *lacZ* in *Streptomyces* by using ϕ C31::lacZ derivatives*

The early steps of the construction of ϕ C31 derivatives carrying *lacZ* DNA (Fig. 1) took advantage of the ability of some ϕ C31::pBR322 derivatives to replicate both as plasmids in *E. coli* and as phages in *Streptomyces* (Suarez & Chater, 1980). In later steps, in addition to *in vitro* manipulation, recombinant phages generated *in vivo* were isolated from double lysogens (Chater *et al.*, 1982), and spontaneous deletion mutants were selected after exposure of phage suspensions to sodium pyrophosphate (Suarez & Chater, 1980; Chater *et al.*, 1981). In the course of these constructions, two small *E. coli* plasmids were made that could be of further use: pIJ2141 and pIJ2145 (Fig. 2), linearized at their *Bgl*III sites, provide 'portable cassettes' by

Table 1. *Specific activities of β -galactosidase in cell-free extracts of *S. coelicolor* cultures*

Results are given as units (mg protein)⁻¹, with the number of determinations given in parentheses. In similar experiments with YEME-grown *S. lividans*, values of 0.3 (strain 1326) and 0.5 (strain J1528) units (mg protein)⁻¹ were obtained.

<i>S. coelicolor</i> strain	Phenotype on MM + glucose + X-gal	YEME	LMPC		
			+ Glucose (0.6%)	+ Galactose (0.6%)	+ Galactose (0.6%) + lactose (0.6%)
J1501	Blue	0.5 (5)	18.4 (1)	36.4 (1)	45.7 (1)
J1510	White	0.5 (2)	0.6 (1)	64.3 (1)	62.1 (1)
J1511	White	0.7 (2)	0.5 (1)	57.1 (1)	56.7 (1)

Fig. 1. Construction of ϕ C31 *lacZ* fusion vectors. —, ϕ C31 DNA; ■, pBR322 DNA; ▨, *S. vinaceus* DNA; ▩, *S. azureus* DNA; ▤, *E. coli* DNA; □, fd terminator; Δ , site for transcriptional fusion; $\blacktriangle$, site for translational fusion; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; P, *Pst*I; SI, *Sst*I; X, *Xho*I. Restriction sites marked with daggers may be present in other constructs but are shown only where relevant to the legend.

1. The *Bam*HI *Pst*I pBR322 portion of KC117 DNA (ϕ C31 *c*⁺:pBR322 Δ W17; Chater *et al.*, 1981) was replaced by a *Bam*HI-*Pst*I fragment from pLG400 (Guarente *et al.*, 1980), which included a *lac'ZY'* sequence (suitable for the construction of a translational fusion of the *lacZ* gene) and pBR322 DNA corresponding to sequences between the *Pvu*II and *Pst*I restriction enzyme sites. KC650 thus retains the ability to function as a plasmid in *E. coli* (producing Ap^R transformants) as well as a phage in *Streptomyces* species.

2. KC651 was obtained from the transfection of protoplasts with a ligation mixture of three separate DNA fragments: the large fragment of *Pst*I-digested KC650 DNA, the *Pst*I-*Eco*RI *vph* fragment from pIJ36 (Thompson *et al.*, 1982a), and the right-hand end fragment of *Eco*RI-digested KC650 DNA (only the relevant *Eco*RI site is shown). Note that the *Bam*HI-*Bam*HI segment of KC651 and the *Bgl*II-*Bam*HI segment of KC656 were subject to frequent inversion (see Results).

3. The construction of KC656 (containing a transcriptional fusion) from KC651 was achieved in three steps via the *E. coli* plasmids pIJ2141 and pIJ2145 (see Fig. 2).

4. *In vivo* recombination between the prophages of a KC656/KC666 (Δ C10,::*tsr*, *att*⁺; King, 1983) double lysogen produced KC660, detected by its ability to produce clear XG⁺ plaques (see Fig. 3 for further explanation). The Δ C10 deletion spans 3 kb of the ϕ C31 genome in the region of the *c* gene, and produces a clear plaque phenotype.

5. KC659, a *c*⁺ derivative of KC660, was an *in vivo* recombinant (giving turbid XG⁺ plaques) released from a double lysogen containing KC400 (Harris *et al.*, 1983) and KC660.

Fig. 2. Construction of KC656. The 700 bp *HindIII* fragment of foreign DNA used in the first step originated from an *Agrobacterium tumefaciens* Ti plasmid (A.G. Hepburn, personal communication) and was used solely because it contained an internal *BglII* target site. The orientation of the synthetic adapter oligonucleotide in pIJ2145 was deduced from the presence of an *XhoI* target site in KC656 DNA. Symbols are defined in Fig 1, except for: , pACYC177; H, *HindIII*.

6. KC669 was produced by the introduction of a *BglII*-*XhoI* synthetic adapter oligonucleotide (of 9 bp flanked by 5' GATC single-stranded extensions; Fig. 2 and Rodicio *et al.*, 1985) into the *Bam*HI site of KC650 DNA. The orientation of the two restriction enzyme target sites was determined after the insertion of foreign DNA into the *BglII* site (data not shown).

7. The major transcription terminator of the phage fd was introduced as a 338 bp *Sau*3AI fragment (isolated from the plasmid pLBU-1; Gentz *et al.*, 1981) into the *BglII* site of KC669 DNA in the orientation such that the *BglII* site was retained between the terminator and the *lacZY'* sequence, producing KC670.

8. The left and right arms respectively of *BglII*-digested KC670 and KC659 DNA, combined *in vitro*, produced KC678 on transfection.

9. KC680, the *c⁺* derivative of KC678, was isolated from a KC400/KC678 double lysogen, identified as producing turbid XG⁺ plaques.

10. The *in vitro* combination of the large *SstI* fragment of KC659 DNA, the small (right-hand end) *PstI* fragment of the KC117 derivative KC515 (Rodicio *et al.*, 1985) and the *SstI*-*PstI* *lacZ* fragment from pMC1871 (Shapira *et al.*, 1983) produced KC681 on transfection.

11. KC684 was produced by the *in vitro* insertion, into the *Bam*HI site of the KC681 genome, of a *BclI* fragment of *S. azureus* DNA containing the *tsr* gene (Thompson *et al.*, 1982a).

12. KC685 was produced by the *in vitro* insertion, into the *Bam*HI site of the KC681 genome, of the *S. vinaceus vph* gene as a *Bam*HI fragment (Thompson *et al.*, 1982a).

which the selectable insertion (using the *vph* gene) of *lacZ* translational or transcriptional fusion probes, respectively, may be made into *Streptomyces* transcription units.

The earliest construction that could be expected to show β -galactosidase activity was KC656 (Figs 1 and 2). Its principal features are: (a) a ϕ C31 replicon, allowing plaque formation in a wide range of *Streptomyces* species; (b) the replication origin of pBR322, allowing it to be maintained in *E. coli* as a multicopy plasmid; (c) the *vph* gene of *S. vinaceus*, expressed both in *E. coli* and in *Streptomyces* by transcription from the adjacent *tet* promoter of pBR322 (M. R. Rodicio, personal communication); (d) a *lacZY* segment made up of the *trp-lac* region from pMC903 (transcribed from a second copy of the pBR322 *tet* promoter) but with its *lac* operon truncated in the *lacY* gene by replacement of the 3' end with that from pLG400; (e) unique sites for *Bgl*II and *Xho*I between the pBR322 *tet* promoter and the pMC903 *trp-lac* region. Interestingly, the presence in KC656 (and its progenitor KC651) of two copies of a 375 bp sequence containing the pBR322 *tet* promoter, in inverse orientation and separated by 7.5 kb, resulted in a population of phages of which about half carried each orientation of the intervening DNA.

With some – but not all – *Streptomyces* hosts tested, KC656 plaques gave an XG⁺ (and MUG⁺) reaction. The best results were with the XG⁻ *S. lividans* mutant J1528, in which the plaques became blue after about 16 h (i.e. a few hours after becoming visible), and the blue colour remained confined to plaques. A similar result was obtained with *S. albus* G J1074 (a restrictionless mutant of *S. albus* G; Chater & Wilde, 1980), but plaques were smaller and reduced in number, probably because of an interaction between the pBR322 *tet* promoter region and this host (Chater *et al.*, 1981). In *S. lividans* 1326, the host lawn itself developed an XG⁺ reaction after about 24 h, some 8 h after the development of blue plaques. With *S. parvulus* ATCC 12434 (strain 2283; Hopwood & Wright, 1973) a similar XG⁺ reaction of the host developed too early to allow the XG reaction of the plaques to be determined. Hosts which gave XG⁻ plaques were the XG⁻ strains *S. albus* P (CMI 52766) and *S. clavuligerus* ATCC 27064, and the XG⁺ derivative of *S. coelicolor* A3(2), J1501. In this last case it was shown that progeny phages subsequently gave XG⁺ plaques on *S. lividans* J1528.

Transformation of *E. coli* by KC656 DNA was more difficult than with most of the bifunctional phages described in this paper, for reasons that are not understood, but which may conceivably be connected with the presence of inverted repeats of the 375 bp *Eco*RI to *Bam*HI fragment of pBR322. Of 81 transformants obtained with 0.5 μ g KC656 DNA, only one was XG⁺. All of the XG⁻ colonies examined contained deleted plasmids lacking the *Bgl*II and *Xho*I sites. The single XG⁺ derivative contained a plasmid (pIJ556) with the predicted *Bgl*III, *Cla*I, *Eco*RI and *Eco*RV restriction patterns. *E. coli* was transformed uniformly to XG⁺ by pIJ556 at a frequency of about 100 colonies μ g⁻¹. Transformants were weakly Lac⁺ and red on lactose McConkey agar. pIJ556 transfected *S. lividans* to give XG⁺ plaques. The resultant phages had DNA indistinguishable from that of KC656.

ϕ C31::lacZ promoter-probe vector development

Although KC656 demonstrated that *lacZ* expression could occur in *Streptomyces*, it was not a convenient vector for making *lacZ* fusions. The route of construction of more useful ϕ C31::*lacZ* fusion vectors is given in Fig. 1. The vectors KC659 and KC680 (see Fig. 4 for a detailed restriction map of KC659) were designed and constructed so that the insertion of a vegetatively expressed promoter next to the *lacZ* gene could be detected rapidly in plaques by the use of X-gal or MUG. [For some purposes KC660 (Figs 1 and 3), the Δ C10 derivative of KC659, may be useful, since it will accommodate larger DNA inserts and also gives somewhat more β -galactosidase activity in plaques (Table 2).] The KC659 genome contains the amino-terminal end of the *lacZ* gene derived from pMC903 (Casadaban *et al.*, 1980), in which the *lacZ* promoter has been replaced by a segment of the *trp* operon which does not block transcription (Mitchell *et al.*, 1975). Plaques produced by KC659 in *S. lividans* J1528 top layers were XG⁺ (and MUG⁺; Fig. 5). KC680 was constructed in an attempt to eliminate this expression of *lacZ*. In KC680, the major transcriptional terminator of the coliphage fd was inserted as a 338 bp *Sau*3AI fragment (Beck *et al.*, 1978) into the *Bgl*II site of KC659 in the orientation which regenerated a *Bgl*II site

Fig. 3. Construction of KC660 by *in vivo* recombination in a double lysogen (see Fig. 1). *S. coelicolor* J1501 was simultaneously infected with KC656 and KC666, and Vio^R Thio^R lysogens selected. Phages released from these lysogens were plated on *S. lividans* 1326-9 using medium containing X-gal. KC660 was identified as a clear, XG⁺ plaque. KC666, containing the 3 kb Δ C10 deletion, was described by King (1983). Symbols are defined in Fig. 1, except for: ▼, most probable site involved in homologous recombination event.

Fig. 4. Restriction maps of ϕ C31::*lacZ* constructs. Only the right-hand end of each construct is shown. The left-most *Eco*RI site shown corresponds to *Eco*RI site 13 (co-ordinate 32.9 kb) on the ϕ C31 restriction map of Rodicio *et al.* (1985). Symbols are defined in Fig. 1, except for: C, *Cla*I; Hp, *Hpa*I; PvI, *Pvu*I; PvII, *Pvu*II; SII, *Sst*II; Sp, *Sph*I; V, *Eco*RV.

between the terminator and *lacZ* (Fig. 1, steps 7–9). The β -galactosidase activity in KC680 plaques was still clearly detectable (Fig. 5), although reduced when compared with KC659 (Table 2).

Both KC659 and KC680 are potentially suitable for the cloning, into their *Bgl*II or *Xho*I sites, of promoters strong enough to increase the background *lacZ* expression. Since they have a functional *c* gene and contain the ϕ C31 *att* site, stable lysogens can then be made in which the regulation of activity of the cloned promoter can be assessed at single copy number. Spores from lawns of *S. coelicolor* strains J1501, J1510 or J1511 infected with KC659 contained 99%, 97% and 29% lysogens, respectively. The J1501 and J1510 lysogens were 100% stable, while the Pgl⁺ lysogen tested was apparently less stable (23%) (but possibly the procedure used in this estimation leads to under-estimations, since it depends on detecting phage release from lysogens by replica plating). *S. lividans* was lysogenized by KC659 with more difficulty, fewer than 1% of the spores of an infected lawn being stable lysogens. Similar results were obtained previously with *S. lividans* and KC300 (ϕ C31 *c*⁺ Δ M Δ 23::pBR322 Δ W12::vph; Chater *et al.*, 1982), which contains no *lac* DNA but has the same DNA organization around the *attP* site as KC659.

Assays of β -galactosidase activity in KC659 and KC680 lysogens are given in Table 2. Marked activity was found only with *S. lividans* 1326(KC659). *S. coelicolor* J1501(KC659) gave very low expression, consistent with the XG⁻ phenotype of KC659 plaques on *S. coelicolor* derivatives. In contrast to the 40% reduction of β -galactosidase activity in plaques caused by

Table 2. *β -Galactosidase activities in lysogens and plaques associated with derivatives of KC659*

The β -galactosidase activities are given as units (mg protein)⁻¹ for cell-free extracts of lysogens (less the values obtained with the non-lysogen), and as units ml⁻¹ for soak-outs. The number of determinations is given in parentheses. Three sets of results (*a*, *b* and *c*) are given for the soak-outs to show the reproducibility within experiments compared with the overall two-to threefold variation in the absolute values obtained in different experiments. KC679 was identical to KC680 except for the *Bgl*III site, which was replaced by a *Bam*HI site.

		β -Galactosidase activity		Relative activity associated with lysogens or plaques (as a fraction of KC659 result)				
		No phage	KC659	KC680 (KC659::fd ter)	KC679	KC674 (KC659::aphP)	KC687 (KC659:: ϕ C31 DNA)	KC660 (KC659 Δ C10)
YEME-grown J1501								
lysogens								
		0.32 (9)	0.97 (7)	0.05 (2)	-	3.8 (3)	0.22 (3)	-
YEME-grown 1326								
lysogens		0.33 (6)	7.24 (6)	0.15 (2)	-	6.2 (2)	ND	-
Soak-outs from	(a)	-	18 (3)	-	0.33 (1)	2.3 (3)	3 (3)	2.2 (3)
J1528 overlays	(b)	-	20 (1)	0.6 (1)	0.75 (1)	2.4 (1)	3.4 (1)	1.6 (1)
	(c)	-	47 (1)	0.6 (1)	0.36 (1)	1.7 (2)	2.1 (2)	1.6 (1)
Means		-	28	0.6	0.5	2.1	2.8	1.8

Fig. 5. β -Galactosidase activity in plaques (on *S. lividans* 1326-9) of ϕ C31 derivatives containing *lacZ*. MUG-stained plates were photographed through a Wratten gelatin filter no. 45 (Kodak) using a Polaroid CU5 Land Camera, during irradiation at 310 nm (see Methods); the photograph is shown at $\times 1$ magnification. The sequences immediately upstream of the *trp-lacZ* region (itself common to all the phages except KC675) were: KC659 and KC681, pBR322 *tet* promoter; KC679, KC680, *tet* promoter followed by *fd* terminator; KC674, *tet* promoter followed by *aph* promoter; KC686, KC687, *tet* promoter followed by fragments of ϕ C31 DNA. KC675 (King, 1983) is a 'control' ϕ C31 derivative carrying no *lacZ* DNA.

introducing the *fd* terminator into KC659, there was an 85–95% reduction in the enzyme activity in KC680 lysogens.

A functional aph-lacZ transcriptional fusion

The aminoglycoside phosphotransferase gene (*aph*) from *S. fradiae* appears to be highly expressed in *S. lividans* (Thompson & Gray, 1983). A 527 bp *Sau*3AI fragment has been identified which includes the first 128 bp of the *aph* structural gene and the 399 bp immediately upstream to this, and which exhibits moderately strong promoter activity as determined both by RNA analysis and in plasmid promoter probe vectors (Bibb *et al.*, 1985; G. R. Janssen, M. J. Bibb and J. M. Ward, personal communication). This *Sau*3AI fragment was used to demonstrate the feasibility of fusing a *Streptomyces* promoter to *lacZ* in ϕ C31. The fragment was positioned directly upstream from and reading into *lacZ* in KC659 by subcloning the *Sau*3AI fragment into the *Bgl*II site of the vector (giving rise to KC674). The insert was detected by plaque hybridization and its orientation determined by the position of the *Bgl*II target site reconstituted at the 'downstream' end of the fragment (because the relevant *Sau*3AI site is preceded by an A residue).

Plaques produced by KC674 in *S. lividans* J1528 top layers gave stronger colour reactions with X-gal (and were MUG⁺⁺; Fig. 5) compared with those of KC659, and gave correspondingly higher activity in soak-outs (Table 2). *S. coelicolor* and *S. lividans* lysogens of KC674 appeared to be quite stable, and sonicated cell-free extracts also exhibited increased β -galactosidase activities when compared with KC659 lysogens, again most markedly in *S. lividans* (Table 2).

The use of KC659 to clone ϕ C31 DNA fragments with apparent promoter activity

In the hope of cloning strong ϕ C31 promoters, *Sau*3AI-cleaved ϕ C31 wild-type DNA was treated with calf intestine alkaline phosphatase (to avoid multiple insertion events) and inserted into *Bgl*II-cleaved KC659 DNA. Plaques obtained after transfection with the ligation mixture were transferred to master plates containing X-gal. A few plaques (6/116 and 2/180 in two experiments) were more intensely blue (XG⁺⁺) (they also gave a MUG⁺⁺ reaction – e.g. KC686 and KC687 in Fig. 5), and a few (8/116, 25/180) were white (XG⁻). Restriction analysis of isolated DNA showed that all but one of the XG⁺⁺ phages contained inserted DNA (0.2–1.2 kb)

in the *Bgl*II site (which was not usually regenerated at the *Sau*3A1-*Bgl*II junction). All of the eight XG⁻ phages examined contained deletions affecting *lacZ*. In a similar experiment using the translational fusion vector KC670 (Fig. 1), no XG⁺ plaques were obtained.

We anticipated that most ϕ C31 promoters should be subject to repressor control, and should, therefore, be expressed only during vegetative infections and not in lysogens. This assumption was examined with one of the XG⁺⁺ phages, KC687, which contained a 1.1 kb insert. A *S. coelicolor* J1501(KC687) lysogen had lower β -galactosidase activity than a J1501(KC659) lysogen. However, soak-outs from semi-confluently lysed J1528 overlays infected with KC687 had consistently higher activities than those from overlays infected with KC659 (Table 2). This indicated that *lacZ* was fused to a ϕ C31 promoter subject to repressor control. However, in addition to the obvious possibility that a promoter had been cloned, there was circumstantial evidence pointing to an alternative possibility; namely, that recombination took place during lytic infections, between the cloned fragment and its homologue in the phage genome, to place *lacZ* under the control of the promoter of a transcription unit of which the cloned fragment was a part. Complex patterns were obtained with restriction digests of virion DNA, indicating that DNA rearrangements occurred frequently during propagation of this phage and of several of the other XG⁺⁺ derivatives obtained in this promoter-probing experiment.

Vectors designed to place lacZ under the control of promoters in situ in the Streptomyces genome

Rodicio *et al.* (1985) used an *att*-deleted *c⁺* ϕ C31 derivative to place the *vph* (viomycin phosphotransferase) gene of the vector under the transcriptional control of a chromosomally located promoter for the glycerol operon of *S. coelicolor*. This was done by placing a correctly oriented fragment of the glycerol operon immediately upstream of the promoterless *vph* gene in the vector KC515, and then allowing this fragment to direct integration of the recombinant phage genome into the glycerol operon. As a consequence, growth in glycerol medium resulted in an increase in viomycin resistance. This principle can, potentially, be used to place other appropriate probe genes, such as *lacZ*, under the control of other chromosomal promoters. A suitable vector for this purpose would also have to contain a selectable resistance gene to allow detection of transductants. Such vectors (KC684 and KC685; Fig. 4) were obtained from KC656, as shown in Fig. 1, by steps which included: a reduction in genome size by removing redundant *lacY* and pBR322 sequences (achieved using *lacZ* sequences from pMC1871); removal of the inverted repeat of pBR322 DNA; and, in the case of KC684, the introduction of the thiostrepton-resistance gene (*tsr*) as an alternative selective marker to *vph*. Restriction maps of the right-hand ends of KC684 and KC685 are given in Fig. 4.

The vectors described above all contain a translationally functional *lacZ* structural gene. Equivalent vectors containing *lacZ* genes devoid of translation initiation sites, derived from pLC400 and pMC931, can also presumably be constructed for use in translational fusions.

Difficulty in cloning lacZ in Streptomyces plasmids

We attempted to introduce *lac* DNA, from pMC903 and its translational fusion equivalent pMC931 digested with *Bam*HI or *Bam*HI plus *Bgl*II, into *Bam*HI-cleaved pIJ41 (a *Streptomyces* plasmid with copy number 4-5; Thompson *et al.*, 1982a) and *Bgl*II-cleaved pIJ702 (a *Streptomyces* plasmid with copy number 30-300; Katz *et al.*, 1983). *S. lividans* Thio^R transformants were selected and plasmid DNA was isolated from colonies with the relevant insertional inactivation phenotype (loss of Neo^R for pIJ41 and loss of melanin production for pIJ702). Numerous inserts were detected by restriction analysis. All fragments of the donor DNA were well-represented except the one sought - i.e. that containing *lac* operon DNA. However, some plasmid preparations contained a mixture of deletion derivatives, as if deletions had been selected after transformation. Among these, some contained recognizable parts of the desired *lac* fragment.

We next ligated the *lac*'IZ-containing *Bam*HI fragment of ϕ C31 KC651 (Fig. 1) into the *Bam*HI site of pIJ41. This fragment lacked about 25% of *lacY* and all of *lacA*, and contained the *vph* gene. Vio^R derivatives of *S. lividans* were selected after transformation with the ligation

mixture. Once again, the plasmids with inserts contained deletions extending into the *lac* region for various distances, irrespective of the orientation of the inserted DNA.

Finally, *Bgl*II-cleaved pIJ2141 (Fig. 2) was ligated into the *Bam*HI site of pIJ41 and the ligation mixture was used to transform *E. coli*. Vio^R transformants containing the co-integrated plasmids with both possible configurations (pIJ2143 and pIJ2144) were obtained. These were expected to be able to replicate in *S. lividans* to give Vio^R Thio^R growth. However, only nine and 53 *S. lividans* Thio^R transformants were obtained with 1 µg of pIJ2143 and pIJ2144, respectively, and nine of these 62 were Vio^S. Restriction analysis of plasmid DNA from the transformants again revealed that there were always deletions in the *lac* region. The plasmid content of the transformants depended on whether selection of transformants was by early overlaying of regenerating protoplasts with thiostrepton or by replication to thiostrepton medium of spores from mature sporulating growth on regeneration plates: in the former case, multiple deleted species were present in each preparation, and in the latter case, each transformant yielded a homogeneous population of plasmid deletion derivatives. Presumably in the latter case a single, more 'fit' plasmid species had been able to out-replicate others during the lag before selection was applied.

We concluded at this point that if *lacZ* were to be maintained at all in these plasmids, it would need to be closely tailored. We have not attempted further to determine what tailoring might be suitable, nor whether other sites in the plasmid could be used with more success, nor whether other *Streptomyces* hosts might be more tolerant of *lac* DNA.

DISCUSSION

Our results suggest that ϕ C31::*lacZ* vectors may be useful in analysing *Streptomyces* gene expression in several respects. First, the use of X-gal (or MUG) to assess β -galactosidase activity in plaques of KC659 or, better, of KC680 (Figs 1, 4 and 5) allows the cloning of strong promoters. It may also be possible to use a reduction in X-gal staining to aid the rapid identification of down-promoter mutations after *in vitro* mutagenesis (the assay of β -galactosidase activity in soak-outs of semi-confluently lysed top layers will be a helpful adjunct to this). Second, assays of β -galactosidase activity in cell-free extracts of lysogens containing *lacZ* fused to the promoters of *Streptomyces* genes, either as a direct result of *in vitro* manipulations (i.e. cloning of promoters into KC659 or KC680; Figs. 1 and 4; Table 2) or through insert-directed recombination (using KC684 or KC685; Figs 1 and 4), should now be applicable to the study of the regulation of various kinds of genes, including those for antibiotic production and morphological differentiation. In these applications, ϕ C31::*lacZ* vectors would be expected to be complementary to the sophisticated high copy number promoter-probe plasmids which use the Tn5 *neo* gene (Ward *et al.*, 1986). The possibility of using KC670 to construct translational fusions that express β -galactosidase activity in *Streptomyces* remains to be established.

However, the general use of *lacZ* in *Streptomyces* is not without problems. The introduction of *lacZ*-containing DNA into oligo- and multi-copy plasmids in *S. lividans* was unsuccessful, for reasons that we do not understand, even though the sequences proved to be acceptable in ϕ C31. Similar observations have also been made by S. Grant and M. J. Bibb (personal communication). A second problem, previously described by Eckhardt & Fare (1982), was the presence of endogenous β -galactosidase-like activity in some species, notably in *S. coelicolor* and *S. lividans*. For some experimental purposes this difficulty can be circumvented either physiologically, by the use of growth conditions minimizing the endogenous activity, or genetically, by the use of mutants [e.g. *S. coelicolor* J1510 and J1511, in which the regulation of β -galactosidase activity is apparently altered, and *S. lividans* J1528 (Eckhardt & Fare, 1982) which lacks the activity]. A third difficulty is the relatively low level of *lacZ*-specified β -galactosidase activity detectable either in plaques or in lysogens, especially with *S. coelicolor* hosts. This does not seem to be due to instability of the enzyme in *Streptomyces* (activities were stable in cell-free extracts over several hours at 4 °C or room temperature). More probably the problem arises from inefficient translation of *lacZ* mRNA and/or mRNA instability. The low levels of β -

galactosidase may be responsible for the failure of colonies, even of lysogens containing *lacZ* fused to the moderately strong *aph* promoter, to stain with X-gal (results not shown; this effect may also be due to poor X-gal uptake by *S. lividans* and *S. coelicolor*). Whatever the reason, this difficulty currently prevents some important genetic applications of *lacZ*-fusions in *S. coelicolor*, though a recent report by Fayerman *et al.* (1985) suggests that other *Streptomyces* host-vector combinations will allow maintenance and expression of *lacZ* in plasmids.

Using the transcriptional fusion vector KC659, we attempted to clone ϕ C31 promoters. Increased β -galactosidase activity in some plaques but not (in the one case tested) in lysogens implied that a ϕ C31 repressor-controlled promoter had been fused to *lacZ*, though whether this was a direct result of the *in vitro* manipulation, or whether the fusion required *in vivo* recombination during lytic phage multiplication, was not resolved.

The insertion of the fd terminator (e.g. in KC680) was apparently effective in eliminating read-through transcription in lysogens. This result provides further confirmation of the conclusion of Jaurin & Cohen (1984) and Ward *et al.* (1986) that *E. coli* transcriptional termination signals function in *Streptomyces*. However, the terminator appeared to be less effective during lytic infections, a result which suggests the operation of an anti-termination factor in ϕ C31 lytic infections. If it is assumed that the operation of such factors requires that RNA polymerase should traverse specific signals (such as *nut* sites in coliphage λ ; Friedman & Gottesman, 1983), then the expression of *lacZ* in KC659 and KC680 plaques is probably largely from a ϕ C31 promoter reading rightwards from a position somewhere to the left of the pBR322-based cloning region of the vectors. This possibility deserves further investigation, since there appear to be no examples of anti-termination of transcription in Gram-positive organisms in the literature.

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