Isolation and characterization of a strong promoter element from the *Streptomyces ghanaensis* phage I19 using the gentamicin resistance gene (*aacC1*) of Tn1696 as reporter

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A promoter-probe shuttle plasmid (pGL7011) containing the promoterless aminoglycoside-O-acetyltransferase I gene (*aacC1*) of Tn1696 was used to isolate DNA fragments from *Streptomyces ghanaensis* phage I19 that possessed promoter activity in *Streptomyces lividans* TK23. Analysis of gentamicin (Gm) resistance levels in *Escherichia coli* and in *S. lividans* TK23, and of *aacC1* mRNA levels in *S. lividans*, identified a fragment (F14) that exhibited a high level of promoter activity in both species. Subsequent analysis revealed that the promoter activity of SF14 (a subcloned fragment of F14) was about twice that of *ermEp, one of the strongest characterized actinomycete promoters. SF14 contained two tandemly arranged promoters, 14-lp and p14-llp, with overlapping and adjacent –10 and –35 regions, respectively. Both promoters appear to be recognized with different efficiencies by the major RNA polymerase holoenzyme ($E\sigma^{hrdB}$) of *Streptomyces coelicolor* A3(2).**

Keywords: *Streptomyces ghanaensis* phage I19, promoter, mRNA quantification, S1 nuclease mapping, *in vitro* transcription

INTRODUCTION

Streptomyces are Gram-positive mycelial soil bacteria that undergo a complex process of morphological development (Chater, 1984). They also produce a large number of secondary metabolites, many of which have important applications in medicine and agriculture. Although the mechanisms that control the switch to secondary metabolism and the onset of differentiation

are not well understood (Hopwood, 1988; Chater, 1989), transcriptional regulation is likely to play a predominant role. Analysis of *Streptomyces* promoters has revealed a high degree of sequence heterogeneity (Strohl, 1992; Bourn & Babb, 1995), much of which may be attributable to the occurrence of multiple σ factors (Westpheling *et al.*, 1985; Chater *et al.*, 1989; Buttner *et al.*, 1990; Tanaka *et al.*, 1991; Lonetto *et al.*, 1994; Pótucková *et al.*, 1995). Interestingly, *Streptomyces coelicolor* A3(2), the most genetically characterized streptomycete, possesses a family of four very similar σ factors (Tanaka *et al.*, 1991) that includes the major and essential σ factor, HrdB (Buttner *et al.*, 1990; Brown *et al.*, 1992). HrdB is likely to recognize promoters with the consensus sequence TTGACN-17 nt -TAGAPuT (Strohl, 1992; Bourn & Babb, 1995). Comparison of the promoter sequences of 139 different *Streptomyces* genes revealed only 29 that appeared to have –10 and –35 regions similar to those recognized by σ^{70} of *Escherichia coli* (Strohl, 1992) and that are presumably recognized by σ^{hrdB} in *Streptomyces*. Much remains to be learnt about *Streptomyces* promoters, and the sequences that are recognized by the different holoenzymes, and the

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Abbreviations: AAC(3)-I, aminoglycoside-O-acetyltransferase I; Gm, gentamicin; Km, kanamycin.

The EMBL/GenBank accession number for the F14 sequence reported in this paper is A24557.

Table 1. Plasmids used in this study

Plasmid	Relevant characteristics	Reference/source
pUC19	<i>bla lacZ'</i> <i>mcs</i>	Yanisch-Perron <i>et al.</i> (1985)
pUC8 <i>neo</i>	<i>bla lacZ'</i> <i>neo</i>	W. Klipp, Bielefeld, Germany
pUC8 <i>aacC1</i>	<i>bla lacZ'</i> <i>aacC1</i>	W. Klipp, Bielefeld, Germany
pSVB10	<i>bla lacZ'</i> <i>mcs</i> (sequencing vector)	Arnold & Pühler (1988)
pSVB30	<i>bla lacZ'</i> <i>mcs</i> (sequencing vector)	Arnold & Pühler (1988)
pTP105	<i>bla</i> , mutant <i>ermE</i> promoter with higher levels of transcription (<i>ermEp</i> *)	Schmitt-John & Engels (1992)
pIJ486/487	<i>tsr</i> , <i>fd</i> terminator, <i>mcs</i> , promoterless <i>neo</i> gene (<i>neo</i> *)	Ward <i>et al.</i> (1986)
pGM8	pSG5 replicon, <i>tsr aacC1</i>	Muth <i>et al.</i> (1989)
pGM103	pSG5/pACYC184 replicon, <i>cat neo</i>	Muth <i>et al.</i> (1988)
pUC19 <i>aacC1</i> *	pUC19 containing <i>aacC1</i> *	This study
pGM103 <i>aacC1</i>	pGM103 containing <i>aacC1</i>	This study
pGM10	Based on pGM103, containing cartridge with <i>fd</i> terminator, <i>mcs aacC1</i> * (<i>Streptomyces</i> promoter-probe plasmid)	This study
pSVB30F7	pSVB30 containing I19 fragment 7 (F7)	This study
pSVB30F12	pSVB30 containing I19 fragment 12 (F12)	This study
pSVB30F14	pSVB30 containing I19 fragment 14 (F14)	This study
pSVB30F1	pSVB30 containing I19 fragment 1 (F1)	This study
pGM10F14	pGM10 containing F14	This study
pGM10E	pGM10 containing <i>ermEp</i> *	This study
pSVB10SF14	pSVB10 containing subfragment of F14 (SF14) and <i>neo</i> * of pGL87SF14	This study
pSVB10F1	pSVB10 containing F1 and <i>neo</i> * of pGL87F1	This study
pGL7011	Based on pGM103 with <i>fd</i> terminator, <i>mcs aacC1</i> * (promoter-probe shuttle plasmid)	This study
pGL7011F7	pGL7011 containing F7	This study
pGL7011F12	pGL7011 containing F12	This study
pGL7011F14	pGL7011 containing F14	This study
pGL7011F1	pGL7011 containing F1	This study
pGL86N	pIJ486 containing <i>neop</i>	This study
pGL87E	pIJ487 containing <i>ermEp</i> *	This study
pGL87G	pIJ487 containing <i>aacC1p</i>	This study
pGL87SF14	pIJ487 containing SF14	This study
pGL87F1	pIJ487 containing F1	This study

application of promoter-probe vectors clearly has a role to play. Various reporter genes are available for the isolation of *Streptomyces* promoters (e.g. Ward *et al.*, 1986; Ingram *et al.*, 1989; Clayton & Bibb, 1990; Bruton *et al.*, 1991; Sohaskey *et al.*, 1992; Paget *et al.*, 1994). Most of the promoter-probe vectors containing these genes are based on the multicopy plasmid pIJ101 (Kieser *et al.*, 1982) and only a few have been constructed as *Streptomyces*-*E. coli* shuttle plasmids (e.g. Nazarov *et al.*, 1990).

Here we describe the use of the promoterless *aacC1* gene (*aacC1**) of Tn1696, a transposon first identified within the *Pseudomonas aeruginosa* plasmid R1033 (Rubens *et al.*, 1979), to isolate a particularly strong promoter from the *Streptomyces ghanaensis* phage I19. The *aacC1* gene confers resistance to gentamicin (Gm) in both *E. coli* and *Streptomyces* species (Rubin, 1987; Muth *et al.*, 1989). Detailed transcriptional analysis revealed an unusual structural arrangement with two tandemly arrayed and overlapping promoters that may be of value in the construction of improved expression vectors.

METHODS

Bacterial strains and plasmids, and conditions for culture, transformation and DNA isolation. The following strains were used: *E. coli* MM294 [*F*⁻ *endA1 hsdR*-17 (*r*_K⁻, *m*_K⁺)] (Meselson & Yuan, 1968); *E. coli* JM83 [*supE*-44 *thi*-1 *ara* Δ(*lac*-*pro*) *thi*-1 *strA* (φ80 *lacZ*ΔM15)] (Vieira & Messing, 1982); *S. lividans* TK23 (*spc*-1) (Hopwood *et al.*, 1983). Plasmids used in this study are listed in Table 1. Restriction maps of pGL7011 and pGM10 are given in Fig. 1 and details for their construction are available upon request from G. Labes.

Streptomyces manipulations were as described by Hopwood *et al.* (1985). *E. coli* cultivation and transformation were as described by Sambrook *et al.* (1989); *E. coli* plasmid DNA was isolated as described by Arnold & Pühler (1988). Unless noted otherwise, plasmids were selected and maintained using antibiotics at the following final concentrations (μg ml⁻¹): (for *E. coli*) Gm, 10; kanamycin (Km), 50; tetracycline, 10; chloramphenicol, 50; (for *Streptomyces* spp.) Gm, 5; neomycin, 30; Km, 50; thiostrepton, 50.

Determination of MIC. For *E. coli*, MICs were determined using cultures grown in 10 ml Penassay liquid medium

(inoculated with 0.1 ml of a stationary phase culture) supplemented with different amounts of Gm. The MIC was defined as the concentration of antibiotic that reduced bacterial growth to half its maximal level, determined by OD measurement after 16 h of growth. For *S. lividans*, MICs for Gm were determined using solid medium supplemented with different amounts of the antibiotic. MICs for Km were determined using microtitre plates with each well containing 200 µl R3 medium (Shirahama *et al.*, 1981) supplemented with different amounts of the antibiotic. After spore inoculation, the plates were incubated at 28 °C for 3 d and the MIC defined as the concentration of antibiotic that reduced colony number by 50%.

Construction of the promoterless Gm resistance gene (*aacC1) of Tn1696.** A 3.15 kb *Bam*HI restriction fragment containing the sequenced Gm resistance gene (*aacC1*) of Tn1696 (Wohlleben *et al.*, 1989), which encodes aminoglycoside-O-acetyltransferase I [AAC(3)-I; gentamicin acetyltransferase I, EC 2.3.1.60], was digested with *Fok*I (a *Fok*I recognition site is located between the putative promoter region and the ribosome binding site of *aacC1* and another is located downstream of the *aacC1* coding region). After blunt-ending the *Fok*I segment with the Klenow fragment of DNA polymerase I and *Hind*III-digestion (a *Hind*III site lies about 550 bp downstream of the *aacC1* coding region within the *Fok*I fragment), the 1.13 kb fragment containing the promoterless gene (*aacC1**) was cloned in the opposite orientation to *lac*p in dephosphorylated, *Sma*I/*Hind*III-digested pUC19, resulting in pUC19*aacC1**. The elimination of the *aacC1* promoter (*aacC1*p) was verified by DNA sequencing.

and purity of RNA after DNaseI treatment (0.2 Units DNaseI μl^{-1} (RNase-free, Boehringer Mannheim); 30 min at 37 °C) were determined spectrophotometrically (Spectronic 88) as described by Maniatis *et al.* (1982).

PCR primers and conditions. The following PCR primers were used to generate the probes used in the S1 nuclease mapping experiments: *fd*-tail 5' CATGGATCCCTAACGTCTGGA-AAGACGACAAAAC 3', *neo* 5' AGAACCTGCGTGCAA-TCCATCTTG 3' and *rev* 5' GTCATAGCTGTTTCTG 3'. The nucleotide sequence at the 5' end of the *fd*-tail primer (in italics) does not correspond to sequences present in the subcloned fragment containing the *fd* terminator, permitting discrimination between transcriptional read-through past the *fd* terminator and probe reannealing.

All PCR reactions (final volume of 50 μl) were performed using a Perkin Elmer DNA Thermal Cycler 480. PCR standard buffer (Pharmacia) was supplemented with 5% (v/v) glycerol, 1.5 mM MgCl_2 and 200 nM of each primer. All reactions were subjected to 30 cycles with a final extension of 10 min at 72 °C. The following amounts of DNA and primer pairs were used: 50 ng pGL87SF14 with primers *fd*-tail/*neo*, 50 ng pGL87F1 with primers *fd*-tail/*neo*; 450 ng pSVB10SF14 with primers *rev*/*neo*; 450 ng pSVB10F1 with primers *rev*/*neo*. Cycle conditions: for pGL87SF14 and pGL87F1, 1 min at 94 °C for denaturation, annealing for 2 min at 67 °C and extension for 1 min at 72 °C; for pSVB10SF14 and pSVB10F1, 1 min at 94 °C for denaturation, annealing for 2 min at 47 °C and extension for 1 min at 72 °C.

The PCR products were purified using Nick-Sephadex-G50 columns (Pharmacia). To generate a uniquely 5'-end-labelled DNA fragment for high-resolution S1 nuclease mapping, about 25 pM labelled *neo* primer was used per PCR reaction.

DNA labelling and sequencing. For Southern blot analysis, digoxigenin-11-dUTP labelled DNA of pGL7011 derivatives was used. All steps were performed as recommended by the manufacturer (Boehringer Mannheim). For dot-blot analysis, 1 μg of the relevant DNA fragment (a *Bam*HI–*Hind*III fragment from pUC8*aacC1* for *aacC1* and a *Bgl*II–*Sal*I fragment from pUC8*neo* for *neo*), previously isolated from agarose gels using a GeneClean kit (Bio 101), was labelled with 7.4×10^5 Bq [α - ^{32}P]dATP (Amersham) using the random primer method (Feinberg & Vogelstein, 1983). To generate the probes for S1 nuclease mapping, 100 pM of the *neo* primer were 5'-end-labelled using 3.7×10^6 Bq [γ - ^{32}P]dATP (New England Nuclear) and T4 polynucleotide kinase (Gibco BRL). Unincorporated label was removed from each of the reactions using Nick-Sephadex-G50 columns (Pharmacia). The nucleotide sequences of the promoter fragments were determined using the dideoxy-chain termination method (Sanger *et al.*, 1977) and a T7 DNA Polymerase Sequencing Kit (Pharmacia) using restriction fragments cloned in pSVB30 and universal and reverse M13 primers (Amersham). For high-resolution S1 nuclease mapping, aliquots of the 5'-labelled PCR products were sequenced by the method of Maxam & Gilbert (1980) using the C, G + A and G reactions.

Quantification of transcripts. For mRNA quantification, all probes were adjusted to a final volume of 100 μl in 7.4% formaldehyde, 6 $\times$ SSC buffer (1 $\times$ SSC is 0.15 M sodium chloride, 0.015 M sodium citrate). RNA samples were denatured at 65 °C for 5 min prior to transfer to the nylon filter (Amersham, Hybond N).

To quantify the *aacC1* transcripts with respect to the level of *neo* mRNA, two identical hybridization filters were prepared. Each filter was loaded with 5–80 μg DNaseI-treated total RNA isolated from different plasmid-bearing *S. lividans* TK23 strains by vacuum transfer using a dot-blot chamber. One filter was probed with ^{32}P -labelled *neo* DNA, the other with ^{32}P -labelled *aacC1* DNA. Hybridization conditions were as described by Hopwood *et al.* (1985) in the presence of formamide. Signal intensities were determined by scanning the resulting X-ray film using the software programme Quantity One (Microsystems). After identifying RNA samples that gave comparable signal intensities for *neo* mRNA, *aacC1* transcript levels were determined from the corresponding dots of the second filter. Two independent hybridization experiments and computer calculations were performed for each promoter.

High-resolution S1 nuclease mapping. High-resolution S1 nuclease mapping (Sharp *et al.*, 1980) was performed as described by Baylis & Bibb (1988) with the following exceptions: 80 μg *S. lividans* RNA and 750 000 Cerenkov c.p.m. of the appropriate end-labelled PCR-generated probe were heated for 30 min at 70 °C, while 50 μg of *E. coli* RNA and 50 000 Cerenkov c.p.m. of labelled probe were incubated for 15 min at 65 °C in sodium-TCA buffer (Summerton *et al.*, 1983) prior to slowly cooling each to 45 °C overnight. For digestion of single-stranded nucleic acid, 90 Units (in the case of *S. lividans* RNA) or 120 Units (in the case of *E. coli* RNA) of S1 nuclease (Sigma) were added and the mixture incubated at 37 °C for 30 min. RNA-protected fragments were resolved on 6% polyacrylamide/7 M urea gels (Sanger & Coulson, 1978) together with Maxam–Gilbert sequencing ladders (Maxam & Gilbert, 1980) of the same end-labelled fragment (C, G + A and G reactions). Before assigning precise transcription start points, 1–1.5 nt were subtracted from the length of the protected fragments (Hentschel *et al.*, 1980).

In vitro run-off transcription assays. *In vitro* run-off transcription assays were performed on PCR-generated fragments 14 (433 bp) and 1 (668 bp), and on a 589 bp *Sma*I–*Ava*II restriction fragment containing the p4 promoter of *dagA* (Buttner *et al.*, 1987). Transcription initiation from *dagAp4* resulted in a run-off transcript of 285 nt. RNA polymerase fractions 3 and 4 kindly provided by Kelly Brown (University of Pittsburgh, PA), gave the highest level of p4 transcription. Fraction 4 was used for *in vitro* transcription of the *dagA* promoter fragment. Run-off transcription assays were performed as described by Brown *et al.* (1992) using 1 μg template DNA per reaction. All products were analysed on denaturing 6% (w/v) polyacrylamide gels using end-labelled *Hpa*II restriction fragments of pBR322 (Sutcliffe, 1979) as size markers, together with appropriate aliquots of RNA-protected DNA fragments generated during S1 nuclease mapping experiments.

RESULTS

Use of the promoterless Gm resistance gene (*aacC1**) of Tn1696 as a new reporter gene

Insertion of the *lacZ*, *tac* (Bagdasarian *et al.*, 1983) and native *aacC1* promoters in front of *aacC1**, which retains its natural ribosomal binding site (see Methods), revealed a good correlation between the level of Gm resistance and reported promoter strength in *E. coli* JM83 (data not shown). In contrast to *E. coli*, the level of Gm resistance conferred by the wild-type *aacC1* gene in *Streptomyces* is relatively low (e.g. pGM103*aacC1*,

Table 2. Gm and Km resistance levels conferred by different promoters in *S. lividans* TK23

Plasmid	Promoter	Gm resistance level ($\mu\text{g ml}^{-1}$)†
pGM10	–	< 2
pGM10F14	F14	20
pGM10E	<i>ermEp*</i>	20
pGL7011	–	< 2
pGM103 <i>aacC1</i>	<i>aacC1p</i>	3–5
pGM8	<i>aacC1p</i>	5–8
pGL7011F12	F12	5
pGL7011F7	F7	5–10
pGL7011F1	F1	10–15
pGL7011F14	F14	15–20
Km resistance level ($\mu\text{g ml}^{-1}$)‡		
pIJ486/487	–	< 5
pGL87F1	F1	85
pGL87G	<i>aacC1p</i>	180
pGL86N	<i>neop</i>	260
pGL87E	<i>ermEp*</i>	450
pGL87SF14	SF14	750

† The mean resistance levels of three independent MIC determinations using agar plates.

‡ The mean resistance levels of three independent MIC determinations performed as described in the legend to Fig. 3.

25–35 copies per chromosome, and pGM8, 50–60 copies per chromosome; Table 2). We speculated that the presence of two TTA codons – known to be rare in *Streptomyces* (Wright & Bibb, 1992) – located near the beginning of the *aacC1* coding region may render *aacC1** a particularly useful reporter gene for the isolation of strong *Streptomyces* promoters. Two promoter-probe vectors (pGL7011 and pGM10; Fig. 1) were constructed using the *Streptomyces*–*E. coli* shuttle vector pGM103, which is based on the pSG5 replicon (Muth *et al.*, 1988). Each plasmid carries the neomycin resistance gene (*neo*) of Tn5 (Beck *et al.*, 1982) as a selectable marker, the promoterless *aacC1* gene located downstream of the major transcriptional terminator of phage *fd* (Gentz *et al.*, 1981) and a multiple cloning site (*mcs*). In *S. lividans* TK23, the shuttle plasmid pGL7011, which was used for most of the work reported here, had a copy number of 25–35 ($\pm 5\%$) per chromosome, while pGM10 had a copy number of approximately 50–60 ($\pm 5\%$), in good agreement with earlier data for pSG5-derived plasmids (Labes *et al.*, 1990).

Isolation of *Streptomyces* phage promoters using pGL7011 and characterization of promoter strength in *S. lividans* TK23

Streptomyces ghanaensis phage I19, isolated previously in our department, was chosen as a target for promoter isolation. Restriction analysis using a series of endo-

Fig. 2. Restriction map of *S. ghanaensis* I19 DNA. An additional *BstEII* restriction site creating a *BstEII* fragment of about 0.1 kb could not be mapped. The double-stranded DNA was not cut by *Bam*HI, *Bgl*II, *Bcl*I and *Sau*3A. The shaded areas at the end of the phage genome represent terminally duplicated DNA sequences as determined by hybridization studies. The location of identified promoter regions (boxes I and II) is indicated.

nucleases revealed that the phage genome was a linear DNA molecule of 14.2 (± 0.5) kb (Fig. 2). I19 DNA was partially digested with *Taq*I and fragments inserted in the *Cla*I site of pGL7011. Fifteen plasmids were isolated that conferred Gm resistance in *S. lividans* TK23. Hybridization experiments revealed that the promoter-bearing fragments originated from two different regions (I, II) of the linear phage genome (Fig. 2). Subsequent DNA hybridization studies and sequence analyses revealed the insertion of four different I19 DNA fragments (Table 2) named F1 (423 bp), F7 (305 bp), F12 (196 bp) and F14 (358 bp), three of which also conferred Gm resistance in *E. coli* MM294 (in $\mu\text{g Gm ml}^{-1}$: F7, 140; F12, 430; F14, 375). There was no obvious relationship between the levels of Gm resistance conferred upon the two bacterial species by each of the DNA fragments.

While SDS-PAGE of total protein isolated from *S. lividans* TK23 derivatives containing each of the recombinant plasmids failed to reveal the 19.45 kDa AAC(3)-I protein (Wohlleben *et al.*, 1989), a good correlation was observed between the resistance level conferred in *E. coli* and the amount of AAC(3)-I (data not shown).

Estimation of relative promoter strengths in *S. lividans* TK23 by mRNA quantification

The relative strengths of the different promoter fragments in *S. lividans* TK23 were determined by RNA dot-blot analysis using the level of mRNA transcripts derived from the *neo* gene of the vector as an internal standard. *neo* should be transcribed at a constant level from its own constitutive promoter and its presence on the vector served as a control for changes in plasmid copy number during growth. F14 appeared to contain the strongest promoter (defined to be 100%). Relative values ($\pm 5\%$) for the other promoter-active fragments were: F1, 57%; F7, 48%; F12, 29%. These estimates did not take into account any differences in the stability of the *aacC1* fusion transcripts.

Fig. 3. Comparison of the strength of different *Streptomyces* promoters measured by *neo* expression. The promoters *neop*, *aacC7p* and *ermEp**, as well as the 119 DNA fragments SF14 and F1 were cloned separately in pIJ486 or pIJ487 resulting in plasmids pGL86N, pGL87G, pGL87E, pGL87SF14 and pGL87F1, respectively. Spores (10^8) of each of the *S. lividans* TK23 derivatives were inoculated into each well. The medium was supplemented with different amounts of Km. The Km MIC value (see also Table 2) was determined as described in Methods.

Comparison of F1 and F14 promoter activities with that of previously characterized *Streptomyces* promoters using a different reporter system

Subcloning experiments revealed that the promoter activity of F14 was located on a 253 bp *Hind*III–*Taq*I fragment (SF14; see Fig. 4b). For comparison, F1, SF14, *aacC1p*, *neop* (promoter of the *neo* gene of Tn5; Beck *et al.*, 1982) and *ermEp** (mutant promoter of the erythromycin resistance gene; Bibb & Janssen, 1986; Schmitt-John & Engels, 1992; Bibb *et al.*, 1994) were cloned separately in front of the promoterless *neo* gene (*neo**) of pIJ487 in *S. lividans* TK23. Spores (10^8) of each of the *S. lividans* TK23 derivatives were used for inoculation. SF14 conferred almost twice the level of Km resistance attributable to *ermEp**, followed by *neop*, *aacC1p* and F1 (Fig. 3; Table 2).

Determination of transcription start points by high-resolution S1 nuclease mapping

High-resolution S1 nuclease mapping (Fig. 4a) revealed three putative transcription start sites in SF14 that were observed with RNA isolated from *S. lividans* TK23(pGL87SF14) and from *E. coli* MM294-(pSVB10SF14): one transcript of 247 nt was allocated to the putative promoter 14-Ip, the strongest signal (252–254 nt) to the potential promoter 14-IIp and the third transcript (301 nt) to a promoter whose

Fig. 4. (a) High-resolution S1 nuclease mapping of the transcriptional start sites present in 119 SF14. The first three lanes contain Maxam–Gilbert sequence ladders (C, G+A and G) derived from the same end-labelled fragment that was used in the S1 nuclease protection assays. Lanes 4 and 5 contain 10 and 20 μ l, respectively, of RNA-protected fragments isolated from *S. lividans* TK23(pGL87SF14). Lane 6 shows the protected fragments obtained using 10 μ g total RNA isolated from *E. coli* JM83(pSVB10SF14). The transcriptional start points of 14-Ip and 14-IIp are indicated by asterisks adjacent to the nucleotide sequence derived from the ladders. The protected fragment of 301 nt corresponds to a promoter whose putative –35 region partially overlaps the *Hind*III cloning site. (b) Nucleotide sequence of SF14. Transcriptional start points (+1) are marked with arrows, which also indicate the direction of transcription. Putative –10 and –35 regions are indicated in boxes. Short inverted repeats are indicated by converging arrows. (c) Nucleotide sequence around the transcriptional start point of F1. The transcriptional start point (+1) is marked with an arrow, which also indicates the direction of transcription. 1–3, short direct repeats.

Fig. 5. *In vitro* run-off transcription assays of SF14. *In vitro* run-off transcripts obtained with SF14 and six different protein fractions enriched with the major RNA polymerase holoenzyme. σ^{hrdB} occurred predominantly in fractions 3 and 4 (data not shown). The end-labelled PCR fragment (SF14*) is shown in lane 2, and the corresponding S1 nuclease mapping products are separated in lane S1. SM, end-labelled *Hpa*I-digested pBR322 size marker. The 258 nt transcript initiated at *dagAp4* (Buttner *et al.*, 1987) after incubation with protein fraction 3 is shown in the lane labelled *dagAp*.

expected -35 region would overlap the *Hind*III site of SF14, and which is therefore unlikely to represent an original phage promoter.

Interestingly, the putative -10 regions of 14-Ip and 14-IIp overlapped, while their putative -35 regions were adjacent (Fig. 4b). 14-IIp appeared to be stronger than 14-Ip in both species (Fig. 4a). Both 14-IIp and 14-Ip possess sequences in their putative -10 and -35 regions (Fig. 4b) that resemble the likely consensus sequences for vegetatively expressed streptomycete promoters (Strohl, 1992; Bourn & Babb, 1995).

S1 nuclease mapping experiments revealed a weak signal for F1 in RNA isolated from *S. lividans* TK23(pGL87F1) which was not observed in RNA isolated from *E. coli* MM294(pSVB10F1) (data not shown). The nucleotide sequence upstream of the transcription start point of F1 did not resemble typical *E. coli* σ^{70} -like *Streptomyces* promoters (Fig. 4c), nor any other defined promoter sequence.

In vitro run-off transcription experiments using σ^{hrdB} -associated RNA polymerase of *S. coelicolor* A3(2)

DNA sequence analysis suggested that 14-Ip and 14-IIp would be recognized by the major RNA polymerase holoenzyme of streptomycetes, a hypothesis supported by the fact that SF14 gave the same transcription start sites in *E. coli*.

Aliquots of six different protein fractions enriched for the major holoenzyme containing subunits α, β, β' and σ^{hrdB} (previously isolated from *S. coelicolor* A3(2); Brown *et al.*, 1992) were used in *in vitro* run-off transcription assays. σ^{hrdB} was present predominantly in protein fractions 3 and 4 (illustrated by *in vitro* transcription of *dagAp4*; K. Brown, personal communication). There was a good correlation between the amount of σ^{hrdB} and the strength of the signals corresponding to 14-IIp and 14-Ip (Fig. 5). As noted in the S1 nuclease protection assays, transcription initiation at 14-IIp appeared to be greater than that at 14-Ip.

Experiments performed with F1 revealed an extremely weak signal with all fractions; the size of the run-off transcript corresponded to the F1 transcription start site determined by S1 nuclease mapping (data not shown).

DISCUSSION

The promoter-probe plasmids described here are based on the *Streptomyces* broad-host-range replicon pSG5 (Muth *et al.*, 1988, 1989), which is compatible with the frequently used pIJ101-based vectors, enhancing their potential utility (Wohlleben *et al.*, 1993). *aacC1* is expressed from its own promoter in other bacterial species (Rubin, 1987) and in plants if transcription is initiated at a suitable promoter sequence (Carrer *et al.*, 1991). Consequently, the promoterless *aacC1* gene is likely to be a useful reporter gene in a variety of organisms. In *Streptomyces*, this gene may be particularly useful for the isolation of strong promoter elements.

Using the bifunctional pGL7011, carrying *aacC1**, we successfully isolated different *S. ghanaensis* phage promoters. During hybridization experiments we identified two promoter regions on the phage genome; F12 was located in the middle part of the linear I19 DNA (promoter region I, Fig. 2), whereas F1, F7 and F14 were found in region II which partially overlaps the terminally duplicated sequences of the phage genome (Fig. 2).

The isolated I19 promoters were characterized in *S. lividans* and *E. coli*. In *S. lividans* TK23, we observed a good correlation between the level of Gm resistance and transcript abundance.

Compared to *E. coli*, the level of Gm resistance conferred on *S. lividans* by *aacC1* is relatively low. This may reflect poor translation of the *aacC1* mRNA. Inspection of the nucleotide sequence of *aacC1* (Wohlleben *et al.*, 1989) reveals that 41% of codons possess adenine or thymine in the third position, compared to a mean of only 9% for 64 analysed *Streptomyces* genes (Bibb *et al.*, 1984; Wright & Bibb, 1992). Furthermore, TTA codons are located at positions two and nine of the *aacC1* coding region, and there is evidence to suggest that the translation of these rare codons may be limiting in vegetatively growing cultures (Leskiw *et al.*, 1991a, b). In *S. coelicolor* A3(2) and in *S. griseus*, the efficient translation of UUA codons requires the leucyl-tRNA encoded by *bldA* (Piret & Chater, 1985; McCue *et al.*,

1992). However, the requirement for *bldA* can be bypassed by over-expression of at least some TTA-containing genes and/or by growth on a rich medium, presumably by a low level of mistranslation of UUA codons (Leskiw *et al.*, 1991b; Passantino *et al.*, 1991). Early expression of Gm resistance, a prerequisite when selecting for growth on the antibiotic, may therefore require high levels of *aacC1* transcription for mistranslation to provide a sufficient level of acetyltransferase. The use of *aacC1* as a reporter gene would consequently favour the isolation of strong promoters.

Although the promoter element on F14 conferred a significantly higher level of Km resistance than *ermEp** on *S. lividans* TK23 when cloned in pIJ487, the Gm resistance levels mediated by each of these two promoters when located in front of *aacC1** in pGM10 were the same (about 20 µg Gm ml⁻¹; Table 2). This might reflect the limited availability of the *bldA* tRNA, or limitations imposed by the frequency of mistranslation – perhaps the non-cognate tRNA(s) responsible for incorrect reading of UUA codons are also present in limiting amounts.

To date there have been few studies dealing with the quantification of plasmid-borne transcripts in streptomycetes (e.g. Jones *et al.*, 1992). In our studies, possible fluctuations resulting from changes in plasmid copy number were accommodated by using transcripts derived from the plasmid-borne *neo* gene as an internal control. We found a good correlation between the amount of the *aacC1* transcript and the level of Gm resistance. The level of Km resistance conferred by F1 was six to seven times lower than that derived from SF14, and a similar difference was observed in transcript levels by S1 nuclease mapping. Interestingly, the promoter element present in SF14 appears to be about two times stronger than *ermEp**, one of the strongest characterized *Streptomyces* promoters (Ward *et al.*, 1986; Schmitt-John & Engels, 1992). Consequently SF14, which is known to be highly active not only in *S. lividans* but also in other *Streptomyces* species (data not shown), may prove useful in the construction of high-level expression vectors for this group of bacteria.

Only 29 of 139 promoters isolated from different *Streptomyces* species possessed *E. coli* σ^{70} -like promoter regions (Strohl, 1992). The remainder revealed considerable sequence heterogeneity, attributable at least in part to a high degree of σ factor heterogeneity (summarized by Bourn & Babb, 1995). Since F1 was not active in *E. coli* and did not possess a σ^{70} -like promoter region upstream of the determined mRNA start point (Fig. 4c), it is difficult to conclude anything about the nature of this promoter, and the RNA polymerase holoenzyme that recognizes it. Several short direct repeats were noted in F1 (Fig. 4c) and palindromic sequences were found in SF14 (Fig. 4b), all of which may play a role in transcriptional regulation. Although tandemly arranged promoters are not uncommon in streptomycetes (examples cited in Strohl, 1992; Bourn & Babb, 1995), the arrangement shown by 14-Ip and p14-IIp appears unique and may explain the high

transcriptional activity of SF14. Each promoter possesses features of σ^{70} -like *Streptomyces* promoters (Bourn & Babb, 1995) with 14-IIp showing most similarity.

In conclusion, using *aacC1** we isolated a promoter-containing fragment from the *S. ghanaensis* phage I19 that is efficiently transcribed in both *E. coli* and in *S. lividans*. We believe that this fragment will prove useful for the high-level expression of genes in streptomycetes.

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