Activation of phenoxazinone synthase expression in *Streptomyces lividans*: characterization of the activator fragment from *Streptomyces antibioticus*

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We have isolated an active 719 bp fragment from the 4.3 kb region of the genome of *Streptomyces antibioticus* that activates a silent phenoxazinone synthase (PHS) gene in *Streptomyces lividans*. Sequencing of the 719 bp fragment revealed several potential open reading frames (ORFs); however, the distribution of G + C in these putative ORFs was uncharacteristic of streptomycete genes. No RNA products transcribed from the active sequence were detected by dot-blot hybridization and no proteins corresponding in size to the predicted products from the ORFs were observed when appropriate plasmids were used as templates in a streptomycete coupled transcription-translation system. Fragments of 249 and 243 bp, respectively, were obtained from the 719 bp fragment from *S. antibioticus* and from the *S. lividans* genome by PCR cloning. Both fragments activated *phs* in *S. lividans* when cloned on a high copy number plasmid.

Keywords: silent genes, *Streptomyces*, antibiotic, actinomycin, phenoxazinone synthase

INTRODUCTION

Actinomycins are chromopeptide antibiotics produced by several different actinomycetes (Waksman, 1968). The enzymes involved in activation of the precursor of the actinomycin chromophore and in polymerization of the amino acids of the pentapeptide chains have been isolated and characterized (Keller *et al.*, 1984; Keller, 1987; Keller & Schlumbohm, 1992; Stindl & Keller, 1993). In the pathway for actinomycin biosynthesis in *Streptomyces antibioticus*, phenoxazinone synthase (PHS) may catalyse the penultimate step, namely the oxidative condensation of two molecules of 4-methyl 3-hydroxyanthraniloyl pentapeptide lactone to generate actinomycinic acid (Katz & Weissbach, 1962). The structural gene for PHS (*phsA*) has been cloned in *S. lividans*, an organism that does not normally produce either PHS or actinomycin D (Jones & Hopwood, 1984a). Another cloned sequence, 4.3 kb in size and also derived from *S. antibioticus*, was shown to activate an otherwise silent *phs* gene in *S. lividans* (Jones &

Hopwood, 1984b). The silent gene has been cloned in *Escherichia coli* (Madu & Jones, 1989).

Cryptic genes have been defined as phenotypically silent DNA sequences that are not normally expressed during the life cycle of an organism but which can be activated as a result of rare genetic events such as point mutations, transposition of a mobile genetic element or recombination (Hall *et al.*, 1983). These cryptic genes are retained through cycles of decryptification and cryptification in response to environmental changes (Hall *et al.*, 1983). Silent genes, on the other hand, have been defined as unexpressed genes originating from duplications that do not confer a phenotype, and which are therefore not subject to selection pressure (Beacham, 1987). The *phs* gene from *S. lividans* will be referred to as silent since no PHS activity has been detected except upon the introduction of the *S. antibioticus* 4.3 kb sequence.

Several mechanisms by which the 4.3 kb sequence could activate the *phs* gene of *S. lividans* can be envisaged. The sequence could code for a protein or RNA activator that acts at the transcriptional or translational level, or even at the post-translational level (for example by modifying an inactive PHS precursor). Alternatively, the 4.3 kb sequence could act as an operator capable of titrating a repressor in the mycelium, resulting in the derepression of *phs*.

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Abbreviation: PHS, phenoxazinone synthase.

The GenBank accession number for the sequence (719 bp insert from pJSM550) reported in this paper is L14560.

To characterize further the 4.3 kb sequence and distinguish between these possibilities, we used a subcloning analysis followed by DNA sequencing, dot-blot assays of total RNA and the use of an *in vitro* coupled transcription-translation system. Our results indicate that no protein or RNA gene product is needed to activate the silent gene in *S. lividans*. Furthermore, the activating sequence has been localized to a 249 bp fragment with a region of strong dyad symmetry; disruption of this region destroys the activation phenotype.

METHODS

Organisms, growth conditions and plasmids. The *S. lividans* 66 derivative TK24 (Hopwood *et al.*, 1983) was cultured and used to prepare protoplasts and transformed as described previously (Hopwood *et al.*, 1985; Jones & Hopwood, 1984a, b). The PHS assay was done with mycelium grown on galactose-glutamic acid (GGA) agar (Gallo & Katz, 1972). Replicate PHS assays varied by no more than 4%.

Recombinant plasmids used or developed in the present study are listed in Table 1. *E. coli* strains XL1-Blue-2 (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F'*proAB lacI*^qΔM15 Tn10 (*tetR*)]; Bullock *et al.*, 1987) and M147 (*dam dcm gal ara lac thr leu thi tonA tsx rpsL supE*), obtained from Brian Spratt, University of Sussex, UK, were cultured in L broth and L agar.

DNA isolation and manipulation. Plasmid and chromosomal DNAs were prepared and manipulated as described in earlier publications (Hopwood *et al.*, 1985; Jones & Hopwood, 1984a, b).

PCR cloning. Primers for the polymerase chain reaction were obtained from the DNA Synthesis Core at the Biomedical Research Core Facilities of the University of Michigan. The sequences of primers pB1 and pB2 were 5'GAGGCATGCTTCGTGACGATCATGAA3' and 5'GAGTGATCATGAACCGCTTGCTGAT3', respectively. The underlined bases represent an *SphI* site in the case of pB1 and a *BclI* site in the case of pB2. PCR was done as described in Gould *et al.* (1989) using 1 µg of each of the primers, and 200 ng in the case of plasmid DNA or 1 µg of genomic template DNA. pJSM550 (Table 1) was used as the template for the synthesis of the *S. antibioticus* sequence spanning the two *BamHI* sites of the 719 bp *BclI*-*SphI* fragment of pIJ2502 (Fig. 1b, c). The same primers were used in a PCR reaction with *S. lividans* genomic DNA as the template. The PCR reaction products were digested in turn with *SphI* and *BclI*, then cloned into the modified KS+ and SK+ Bluescript vectors that harbour both sites and propagated in *E. coli* M147, a *dam* strain unable to methylate *BclI* sites. (The *BclI* and *SphI* sites in the vectors were obtained by inserting *BclI* and *SphI* linkers in the *SmaI* and *EcoRV* sites of the polylinkers, respectively.) Once the desired clone was identified, that plasmid was retransformed into XL1-Blue-2 for DNA sequencing. The PCR products were released from the M147 derived recombinant plasmids and cloned between the *BglII* and *SphI* sites of pIJ702 in *S. lividans*.

DNA sequencing. DNA was sequenced by the chain termination method of Sanger *et al.* (1977) using a Sequenase kit as described by the supplier (United States Biochemical), combined in some cases with Taq polymerase (Khambaty & Ely, 1990).

RNA isolation and dot-blot analysis. RNA was isolated from 48 h liquid cultures grown either in yeast extract/malt extract (YEME) medium supplemented with sucrose or in GGA medium as described in Hopwood *et al.* (1985) except that the

high salt precipitation step was omitted. Thus the RNA preparations would be expected to contain both high and low molecular mass RNAs. Increasing amounts of RNA were then spotted on a Nytran membrane using a filtration manifold (Bio-

Table 1. Plasmids used or referred to in the present study

Plasmid	Description
pBluescript KS+, SK+	Phagemid cloning vector (Stratagene). The vector was modified by inserting linkers containing sites for <i>BclI</i> or <i>SphI</i>
pIJ2501	2.4 kb <i>phs</i> structural gene (<i>phsA</i>) from <i>S. antibioticus</i> cloned into the <i>SphI</i> site of pIJ702 (Jones & Hopwood, 1984a)
pIJ2502	4.3 kb activating sequence cloned into the <i>SphI</i> site of pIJ702 (Jones & Hopwood, 1984b)
pIJ2505	<i>phsA</i> cloned into the <i>SphI</i> site of pBR322 (Jones & Hopwood, 1984a)
pIJ2506	4.3 kb activating sequence in pIJ702 in the opposite orientation to pIJ2502
pJSM500	Approximately 1.6 kb <i>BglII</i> fragment from pIJ2502 cloned into the <i>BglII</i> site of pIJ702 (present study)
pJSM510	Approximately 2.4 kb <i>BglII</i> - <i>BamHI</i> fragment from pIJ2502 cloned into the <i>BglII</i> site of pIJ702 (present study)
pJSM520	Approximately 1 kb <i>BclI</i> fragment derived from pIJ2502 cloned into the <i>BglII</i> site of pIJ702 (present study)
pJSM521	300 bp <i>SstI</i> - <i>BamHI</i> fragment derived from pJSM550 cloned into <i>SstI</i> - and <i>BglII</i> -digested pIJ702 (present study)
pJSM522	180 bp <i>BamHI</i> fragment derived from pJSM550 cloned into the <i>BglII</i> site of pIJ702 (present study)
pJSM523	298 bp <i>BamHI</i> - <i>SphI</i> fragment from pJSM550 cloned into <i>BglII</i> - and <i>SphI</i> -digested pIJ702 (present study)
pJSM524	249 bp <i>S. antibioticus</i> PCR product cloned between the <i>BglII</i> and <i>SphI</i> sites of pIJ702 (present study)
pJSM525	243 bp <i>S. lividans</i> PCR product cloned between the <i>BglII</i> and <i>SphI</i> sites of pIJ702 (present study)
pJSM550	719 bp <i>BclI</i> - <i>SphI</i> fragment from pIJ2502 cloned into <i>BamHI</i> - and <i>SphI</i> -digested, modified KS+ Bluescript vector (present study)
pJSM551	719 bp <i>BclI</i> - <i>SphI</i> fragment from pIJ2502 cloned into <i>BamHI</i> - and <i>SphI</i> -digested, modified SK+ Bluescript vector (present study)
pJSM620	Approximately 1 kb <i>BclI</i> fragment derived from pIJ2502 cloned into the <i>BamHI</i> site of the low copy number vector pIJ922 (present study)

Fig. 1. (a) Subcloning strategy for the 4.3 kb sequence from the recombinant plasmid pIJ2502. Plasmids pJSM500, pJSM510 and pJSM520 were constructed by inserting a 1.6 kb *Bgl*II fragment, a 2.4 kb *Bgl*II–*Bam*HI fragment and an approximately 985 bp *Bcl*I fragment, respectively, from pIJ2502 into the *Bgl*II site of the multicopy streptomycete vector pIJ702. Individual clones containing each of the recombinant plasmids were assayed for PHS activity. Only pJSM520 was found to activate the silent *phs* gene from *S. lividans*. (b) Further subcloning of the pJSM520 insert in pIJ702 to yield plasmids pJSM521, pJSM522 and pJSM523 resulted in loss of PHS activity. (c) Cloning of the region overlapping the two *Bam*HI sites of pIJ2502 using PCR. When reintroduced in *S. lividans* the PCR product was shown to activate *phs*. B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; N, *Nco*I; S, *Sph*I. The bars at the extreme right of the figure indicate the size scale for the cloned fragments represented in (a), (b) and (c).

Rad). Filters were prehybridized overnight and hybridized for 40 h at 68 °C with ³²P-labelled probes as described previously (Jones & Hopwood, 1984a, b).

In vitro coupled transcription-translation. Reaction mixtures for coupled transcription-translation typically contained 2.25 µg of template DNA, the other components indicated by Thompson *et al.* (1984) and [³⁵S]methionine. Samples were analysed by denaturing polyacrylamide gel electrophoresis and fluorography exactly as described by Jones & Hopwood (1984a).

Miscellaneous methods. PHS was assayed as described previously (Choy & Jones, 1981; Jones & Hopwood, 1984a). Protein was determined as described by Bradford (1976). The copy number of pJSM620 was estimated by fractionation of total DNA from *S. lividans* transformants on CsCl (Hopwood *et al.*, 1985), using a value of 10000 kb as the size of the *S. lividans* genome (Goodfellow & Cross, 1983).

RESULTS

Identification of a region of the 4.3 kb insert retaining the ability to activate PHS expression

The sequence responsible for activating PHS expression in *S. lividans* was localized to a specific region of the 4.3 kb fragment cloned in pIJ2502 from *S. antibioticus*. Advantage was taken of several restriction sites in pIJ2502 that furnished fragments that could be cloned in the *Bgl*II site of pIJ702. These experiments (Fig. 1 and Table 2)

identified a 985 bp *Bcl*I fragment that retained the ability to activate *phs* (the insert of pJSM520, Fig. 1a). The fragment, which contained 266 bp from pIJ702 and 719 bp from the 4.3 kb insert of pIJ2502 (Jones &

Table 2. PHS activity of subclones of pIJ2502

PHS activity was measured as described in Methods. Specific activities (averages of three replicates) were calculated as in Choy & Jones (1981).

Plasmid	Enzyme specific activity [nmol min ⁻¹ (mg protein) ⁻¹]
pIJ702	ND
pIJ2501	52.44
pIJ2502	2.33
pJSM520	1.19
pIJ922	ND
pJSM620	1.89
pJSM524	0.71
pJSM525	0.42

ND, No PHS activity detected.

<i>Bcl</i> I	10	20	Orf5 →	40	50
AGATC	AGT	TTT	CGC	GCC	GGG
60	70	80	90	100	
GCG	CCG	GAAG	GG	ACG	ATT
110	120	130	140	150	
CCG	CGG	AG	GAC	CA	GT
160	170	180	190	200	
CAC	GAT	CGG	GCA	CCG	CGG
210	220	Orf1 →	240	250	
TTCT	CTCT	CGAT	ATG	CGG	CA
	<i>Sph</i> I				
	260	Orf2 →	280	290	300
	CAC	CCC	CGC	TC	CGC
	310	320	330	340	350
	GGC	CTC	TGC	GCT	ATC
	360	370	380	390	400
	CGG	GCT	TGG	ACC	GGC
	410	420	430	440	450
	GCG	GGC	CGC	TT	GAC
	460	470	480	490	500
	CCG	GGC	ACC	CGC	ACT
	510	520	530	540	550
	ATC	GTC	AC	CG	CG
	560	<i>Bam</i> HI	570	580	590
	AC	CGG	ATC	CGC	CGG
	610	620	630	640	650
	CCT	CA	CGC	GGC	TT
	660	670	680	690	700
	CCT	CA	CGC	GGC	TT
	710	720	730	740	750
	GAG	GAC	CGC	AGC	TC
	760	770	780	790	800
	GG	AAG	AGG	GAC	ATC
	810	820	830	840	850
	CCG	GCT	GGG	GC	TAT
	860	870	880	890	900
	ACC	GCC	CAT	CC	CG
	910	920	930	940	950
	GCG	GTG	TGC	TC	CG
	960	970	980	990	
	CC	TGG	GCG	GAT	

Fig. 2. Sequence of the 985 bp *Bcl*I activating fragment. The sequence of the 266 bp *Bcl*I-*Sph*I fragment derived from pIJ702 is taken from Bernan *et al.* (1985). The rest of the sequence (719 bp) was determined in the present study. The sequence is numbered starting at the recognition site for the restriction endonuclease *Bcl*I in pJSM520. Relevant restriction sites are indicated on the figure. The ATGs corresponding to putative ORFs 1-4 are shown and the terminator codons for each putative ORF are overlined. The 249 bp sequence obtained by PCR cloning (Fig. 4) is shown in **bold** and the sequences of the primers used for the PCR are *italicized*.

Hopwood, 1984b), was cloned into the *Bgl*II site of pIJ702 in the same orientation as in pIJ2502 (Fig. 1a). When this fragment was further subcloned to yield recombinant plasmids pJSM521, pJSM522 and pJSM523 (Fig. 1b), the activation phenotype was lost.

Effect of copy number on the activation of PHS

To study the dosage effect on the activation of the *S. lividans phs* gene, the 985 bp *Bcl*I fragment cloned from pIJ2502 in pJSM520 was subcloned into the low copy number plasmid pIJ922 to give pJSM620. pIJ922 is present at three to five copies per genome (Lydiat *et al.*, 1985). *S. lividans* strains harbouring the vectors pIJ702 or pIJ922 were used as negative controls. Transformants carrying the recombinant plasmid pIJ2501 (the pIJ702 derivative bearing the *phs* structural gene from *S. antibioticus*; Jones & Hopwood, 1984a) were used as a positive

control. Extracts of mycelium transformed with either the high or low copy number derivatives gave similar levels of PHS activity (Table 2). Moreover, the observed values were comparable to the PHS specific activity in pIJ2502 transformants. We estimate the copy number of pJSM620 to be five to eight copies per chromosome.

Sequence analysis

Both strands of the 985 bp *Bcl*I fragment described above were sequenced on the average three to four times. The sequence of the vector-derived portion was as reported by Bernan *et al.* (1985). The sequence of the entire fragment is shown in Fig. 2. Similarly to other streptomycete sequences, the 719 bp *Bcl*I-*Sph*I fragment from *S. antibioticus* was found to be 74% G + C. Four potential open reading frames (ORFs) of at least 26 codons were identified. The positions of the initiation and termination codons of the four ORFs (Fig. 2) are: ORF1 (ATG/position 229, TAG/position 594); ORF2 (ATG/position 269, TGA/position 550); ORF3 (ATG/position 935, TGA/position 545); ORF4 (ATG/position 548, TGA/position 240). A fifth ORF (ORF5) in frame with ORF1 but starting further upstream than ORF1 and with GTG as the start codon was also detected.

The putative ORFs would encode proteins with molecular masses between 10 and 21 kDa. ORF1 and ORF5 would require sequence information from the cloning vector (nt 1-266 of the *Bcl*I-*Sph*I fragment, Fig. 2) for expression. The sequence of ORF2 is completely contained in pJSM523 (Table 1); that plasmid, however, does not activate PHS expression in *S. lividans*. ORF1, ORF3, ORF4 and ORF5 were disrupted by further subcloning the *Bcl*I sequence into pJSM521, pJSM522 and pJSM523 (Fig. 1b). As shown in Table 3, the G + C distribution in ORFs 1-5 is unlike that normally observed in streptomycete genes (Bibb *et al.*, 1984). Finally, no similarity of the predicted products of ORFs 1-5 to any protein in GenBank was discerned.

S. lividans transformants do not contain messenger or structural RNAs related to the 719 bp fragment

Total RNA was isolated from *S. lividans* mycelium transformed with pJSM520 or pIJ2501 (see Table 1). Cultures were grown for 48 h in YEME medium or, in some cases, in actinomycin production (GGA) medium. RNA from the pJSM520 derivative was probed with both pIJ702 and pJSM550; RNA from the pIJ2501 derivative was probed with pIJ2505. pIJ2505 consisted of the active *Bcl*I-*Sph*I fragment in pBluescript KS+ (Table 1). pIJ702 and pIJ2505 were used as positive controls; the former should detect the mRNA for the thiostrepton resistance gene (Thompson *et al.*, 1982) as well as other messenger RNAs encoded by the plasmid (Kendall & Cohen, 1988), and the latter should detect *phs* mRNA. Increasing amounts, up to 50 µg of RNA from mycelium cultured on YEME or GGA, were spotted on a Nytran membrane. The appropriate DNA positive control was included in each case. As shown in Fig. 3(b), no signal was observed

Fig. 3. Dot-blotting of total RNA from *S. lividans* transformed with pJSM520. Cells were cultured for 48 h in YEME or in GGA. Total RNA was prepared as described in Methods. Increasing amounts of RNA (5, 10, 20, 40 and 50 µg) were analysed (lanes 1 through 5). RNA was probed with pIJ702 (a), pJSM550 (b) and pIJ2505 (c). Appropriate DNA positive controls were spotted on each filter (not shown). Further exposure (up to a week) did not modify the pattern observed above.

with RNA probed with pJSM550. Under the same conditions positive signals were obtained with the DNA control and with RNA from transformed *S. lividans* probed with pIJ702 (Fig. 3a) or pIJ2505 (Fig. 3c). The filters shown in Fig. 3 were overexposed to facilitate identification of any hybrids that might have been formed with pJSM550 as the probe (Fig. 3b). This probably accounts for the apparent failure of the signal strength to vary linearly with RNA concentration in Fig. 3(a, c). Exposure of the filters for even longer periods did not modify the results shown in Fig. 3.

To compare results from this experiment to a negative control, pBluescript KS+ was used to probe the same amounts of RNA from *S. lividans* transformed with pJSM520 (data not shown). The appropriate DNA

Table 3. G + C distribution in putative ORF codons

The table summarizes the occurrence of G + C in the indicated codon positions for each of the four ORFs referred to in the text and for 27 *Streptomyces* genes (Seno & Baltz, 1989).

	Mol % G + C at given codon position:		
	1	2	3
ORF1	76.2	71.9	68.5
ORF2	68.9	71.1	81.1
ORF3	85.7	57.1	69.1
ORF4	73.5	85.3	70.6
<i>Streptomyces</i>	69.7	49.9	90.6

SA	GCATGCTTCG	TGACGATCAT	GAACACGGAC	-GGATCCGCT
SL				
SA	GCATGCTTCG	TGACGATCAT	GAA--C-GAC	GGGATCCGCT
SL				
SA	GGACCCACCC	GGACGAGAAG	GAGATAGGCC	GGCCCTTCGG
SL				
SA	GGACCCACCC	GGACGAGAAG	GAG-TAGGCC	GGCCCTT-CG
SL				
SA	CGGCAACACC	GCGCCGCGCT	GCGGGGCCGC	ACCTTCACCG
SL				
SA	CGGCAACACC	GCGGGCGGCT	GCGGGGCCGC	ACCTTCACCG
SL				
SA	AGACCTACAC	CGGCACCTC	GGCGCCTCGG	TCCGCGCGGT
SL				
SA	AGACCTACAC	CGGCACCTC	GGCGCCTCGG	TCCACGCGGT
SL				
SA	CACCCCGATC	GAGGAGGGCG	ACGGACGGAT	CATCGGCCTG
SL				
SA	CACCCCGATC	GAGGAGGGCG	ACGGACGGAT	CATCGGCCTG
SL				
SA	GTCAGCGCCG	GGATCCGGGT	CGAGGAGATC	AGCAAGCGGG
SL				
SA	GTCAGCGCCA	GGATCCGGGT	CGAGGAGATC	AGCAAGCGGG
SL				
SA	TTCATGATCA			
SL				
SA	TTCATGATCA			
SL				

Fig. 4. Sequence comparison of the *S. antibioticus* and *S. lividans* PCR products. SA, *S. antibioticus*; SL, *S. lividans*. Excluding the sequence of the two primers (included in this figure), the two sequences are 95% identical.

positive controls were also spotted on the filters. The two filters, probed with pBluescript KS+ and with pJSM550, showed similar results after exposure for 3 d (data not shown). Exposure of the filters for 7 d still did not reveal RNAs that hybridized specifically to the pJSM550 insert. The results of these experiments have other implications. Since total RNA was probed with the insert of interest, RNA products such as rRNA and tRNA should have been detected. They were not. Similar results were obtained in control experiments like those just described but using the insert of pIJ2502 as the probe (data not shown). Moreover, no similarity to any known RNA was revealed when the 719 bp sequence was used to search GenBank.

Finally, in other experiments (not shown), using the streptomycete coupled transcription-translation system,

the insert of pJSM520 did not encode polypeptides of sizes that corresponded to those of the putative ORFs described in the preceding section. Therefore, neither RNA nor protein products of the *Bcl*I–*Sph*I fragment appear to be involved in the activation of *phs* in *S. lividans*.

It should be noted here that Southern Cross hybridization experiments (Potter & Dressler, 1986) ruled out the possibility of rearrangement of the activating fragment or the region of the *S. lividans* genome corresponding to the activating fragment (Madu & Jones, 1989) in transformants containing pIJ2502 (unpublished results).

Only a 249 bp fragment derived from the *Bcl*I sequence is necessary for the activation phenotype

The results presented above suggested that the activating sequence overlapped one or both *Bam*HI sites of pIJ2502 (Fig. 1a). Since no convenient restriction sites for subcloning were available outside the two *Bam*HI sites, two primers were synthesized, each 26 bases long and containing 17 bases complementary to the sequence of interest (Fig. 2). The remaining bases were an *Sph*I site plus three randomly chosen bases for pB1, and a *Bcl*I site plus three other random bases for pB2 (see Methods). The two primers were used along with the DNA template pJSM550 in a PCR reaction. The amplified region spanning the two *Bam*HI sites was in turn cloned into pIJ702 to yield pJSM524. The relationship between the PCR fragment and the various subclones of pIJ2502 is shown in Fig. 1(c). Clones containing the PCR fragment were assayed for PHS activity. Results from this experiment indicated that the working hypothesis was correct and that this 249 bp fragment was able to activate *phs* expression in *S. lividans* (Table 2).

The sequence of the PCR product was determined for four independent clones and compared with the sequence of the 719 bp activator fragment. The cloned PCR fragment overlaps the rightmost (as depicted in Fig. 1) *Bam*HI site of pJSM524. If the region around that site is required for the function of the activating fragment, the loss of the activation phenotype (Fig. 1b) upon cloning of the *Bam*HI fragment into the *Bgl*II site of pIJ702 would be explained. No sequence similar to the 249 bp fragment was detected in GenBank.

PCR cloning of a fragment from *S. lividans* with homology to the *S. antibioticus* activator fragment

The two primers utilized in the previous experiment were used in a PCR reaction with *S. lividans* genomic DNA as the template. Annealing was done at 55 °C and 65 °C; the second reaction gave a product with a mobility similar to the *S. antibioticus* product on a 1.6 % agarose gel. The PCR product was in turn digested with *Sph*I and *Bcl*I and cloned in *E. coli* M147 in the modified KS+ and SK+ Bluescript vectors. The sequences of five different clones were determined and compared to the *S. antibioticus* sequence (Fig. 4). The two sequences were 95 % identical and contained a region around one of the *Bam*HI sites that exhibited dyad symmetry. This sequence, which starts at

S. antibioticus

CGGATCATCGGCCTGG TCAGCGCCGGGATCCG
 GCCTAGTAGCCGGACC AGTCGCGGCCCTAGGC

S. lividans

CGGATCATCGGCCTGG TCAGCGCCAGGATCCG
 GCCTAGTAGCCGGACC AGTCGCGGTCCTAGGC

Fig. 5. Region of dyad symmetry (arrows) overlapping one of the two *Bam*HI sites internal to the *S. antibioticus* and *S. lividans* PCR products. The two sequences are identical in both organisms except for a single bp change (**bold**). Two bases from one of the inverted repeats (*italics*) were lost when the *Bam*HI fragment of pIJ2502 was cloned to generate pJSM522. Complementary bases are underlined. pIJ522 (Fig. 1) does not activate *phs*.

nucleotide 713 and ends at nucleotide 744 (Fig. 2), might generate a stable hairpin structure that could function as a protein recognition sequence. One of the differences between the *S. lividans* and *S. antibioticus* sequences occurs in the region of dyad symmetry; this renders the *S. lividans* structure potentially less stable because of one fewer GC bond in the stem region (Fig. 5).

The 243 bp PCR product from *S. lividans* was cloned between the *Bgl*II and *Sph*I sites of pIJ702 giving pJSM525. The recombinant plasmid was used to transform *S. lividans* protoplasts. The PHS specific activity in these transformants was lower than that observed in transformants containing the *S. antibioticus* PCR product and was also lower than the activities observed in transformants containing pIJ2502, pJSM520 or pJSM620. However, PHS activities were easily measured in transformants containing the PCR products and replicate assay results varied by no more than 4 %. The reasons for the decreased levels of PHS in transformants containing the PCR products is not known at present.

DISCUSSION

Interest in the activation of the silent *phs* gene from *S. lividans* emerges from several interesting observations. First, PHS activity was detected in a number of streptomycetes, producers as well as non-producers of actinomycin D, upon transformation with the recombinant plasmid pIJ2502 (Jones & Hopwood, 1984b). Second, it was shown by both cloning (Madu & Jones, 1989) and Southern hybridizations that sequences similar to the PHS structural gene and the 4.3 kb activator fragment exist in the genomes of these organisms. These findings raised the exciting possibility that silent genes or even pathways for antibiotic production are present in members of this group. Streptomycetes possess a large genome, of the order of 8000–10000 kb (Goodfellow & Cross, 1983; Kieser *et al.*, 1992; Leblond *et al.*, 1993). Perhaps a significant fraction of these genomes harbour silent

biosynthetic and resistance genes for antibiotics that are not normally produced. If so, the reason for and mechanism of their silencing is as yet unexplained. The identification of silent genes in *S. lividans* may lead to answers to the questions posed by these observations.

The data presented above suggest that the silent *pbs* gene in *S. lividans* is not activated directly through a protein or RNA product of the activator fragment. Even though the *BclI* sequence that activates *pbs* includes four potential ORFs, FRAME analysis (Bibb *et al.*, 1984) showed that none had the properties expected of a streptomycete gene. Moreover, no protein products of the precise size predicted by the ORF analysis were found with an *in vitro* coupled transcription-translation system, and no sequence-specific messenger RNA was detected in a dot-blot assay of total RNA, even though *pbs* expression in *S. lividans* was activated by this sequence. The data presented are more consistent with a model in which the cloned 249 bp sequence activates *pbs* in *S. lividans* by interacting with a negatively acting DNA-binding protein; it would be similar to an operator-repressor system. Introduction of the cloned sequence would titrate the DNA-binding protein and activate *pbs* expression in *S. lividans*. If this model is correct, the DNA-binding protein must be present in only a few copies per chromosome since cloning the activator fragment on a low copy number plasmid vector leads to *pbs* activation in *S. lividans* (Table 1). Gel retardation experiments are in progress to examine this hypothesis.

When the two *BamHI* sites present in the pIJ2502 insert were used for subcloning, no PHS activity was detected in *S. lividans* transformants. This suggests that sequences around one or both sites are necessary for activation (Fig. 1). We speculate that the region of dyad symmetry observed near one of the two sites (Fig. 5) is involved in binding the putative repressor. Interestingly, this 32 bp long region is identical in *S. antibioticus* and *S. lividans* except for a single base pair. We have preliminary data suggesting the presence of a protein in *S. lividans* which binds to the 243 and 249 bp activator fragments (unpublished results). However, confirmation of the importance of this region in protein binding must await the results of further subcloning, purification of the putative protein, DNase footprinting experiments and site-directed mutagenesis.

While the mechanism of activation of the silent *pbs* gene in *S. lividans* appears thus far to be novel in the genus *Streptomyces*, similar systems are present in enteric bacteria. Thus, the *cel* operon of *E. coli* K12, which is involved in the utilization of β -glucoside sugars, consists of five genes, *celABCDF*, and is cryptic because it is in a constant state of repression (Parker & Hall, 1990a, b). The product of *celD* is a negative regulatory protein that prevents expression of the wild-type *cel* operon. Activation of this operon results from point mutations in *celD*, or from insertions in an upstream regulatory sequence, presumably the sequence normally recognized by the repressor product of *celD*. The putative presence of such a system in *S. lividans* may indicate that the phenomenon of cryptic

and silent genes is as widespread among streptomycetes as in other micro-organisms.

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