Molecular cloning and in vitro expression of a silent phenoxazinone synthase gene from *Streptomyces lividans*

(Recombinant DNA; antibiotic; actinomycin biosynthesis; homology; hybridization; restriction mapping; in vitro expression)

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SUMMARY

Phenoxazinone synthase (PHS) catalyzes a step in actinomycin D biosynthesis in *Streptomyces antibioticus*. Two sequences from *Streptomyces lividans* that hybridize to the *phs* gene of *S. antibioticus* have been cloned in *Escherichia coli* K-12 using the plasmid pBR322. Although there was some similarity in the restriction maps of the two cloned fragments, neither insert appeared to be a direct subset of the other nor of the *S. antibioticus* *phs* gene. In vitro expression studies, in a streptomycete coupled transcription-translation system, showed that a 3.98-kb *SphI* fragment encoded a PHS-related protein. These observations provide additional support for the existence of silent genes for antibiotic production in streptomycetes.

INTRODUCTION

Phenoxazinone synthase (PHS) has been implicated in the enzymatic condensation of a variety of o-aminophenols to form phenoxazinones (Katz and Weissbach, 1962). In the actinomycin biosynthetic pathway in *S. antibioticus*, this enzyme is thought to be responsible for the synthesis of the chromophore of the antibiotic and for the conversion of 4-methyl-3-hydroxy-anthraniloyl pentapeptides to produce actinomycin or the penultimate precursor in the pathway, actinomycinic acid (Troost and Katz, 1979). The structural gene for this enzyme has been cloned from *S. antibioticus* (Jones and Hopwood, 1984a). At the same time, two other nt sequences, 1.8 kb and 4.3 kb in size, and which may be involved in regulating actinomycin biosynthesis in this organism, were also cloned. When the appropriate recombinant plasmids (pIJ2500 and pIJ2502, respectively) containing those inserts were used in transformation experiments, the two sequences were shown to have the interesting ability to induce PHS production in *S. lividans*, an organism which ordinarily does not...
produce either actinomycin or PHS (Jones and Hopwood, 1984b). Southern blotting studies, using the cloned PHS structural gene as probe, revealed that *S. lividans* does contain sequences which hybridize to that probe, thereby suggesting the existence of a cryptic or silent PHS gene in *S. lividans*.

To further characterize the activation phenomenon previously observed, we have cloned and partially characterized these sequences from *S. lividans*.

MATERIALS AND METHODS

(a) Organisms and growth conditions

The *S. lividans* 66 derivative, TK24 (Hopwood et al., 1983), was generally grown in tryptone soya broth (TSB) for isolation of chromosomal DNA, *E. coli* strains HB101 (Maniatis et al., 1982) and CSR603 (Sancar and Rupert, 1978) were routinely grown at 37°C in LB medium (Luria et al., 1960), and were used as recipient hosts in transformations. After transformation with pBR322 (Bolivar et al., 1977) and its derivatives, growth medium contained Cb (100 µg/ml) or Tc (10 µg/ml) as required.

The recombinant plasmids, pIJ2501 and pIJ2505, consist of the *S. antibioticus* structural gene for PHS cloned in the single SphI sites of the streptomyces vector, pIJ702, or of pBR322, respectively (Jones and Hopwood, 1984a; Katz et al., 1983).

(b) Isolation of total and plasmid DNAs

Total DNA was isolated from *S. lividans* strain TK24 essentially as described by Chater et al. (1982). Plasmid DNAs were obtained from *E. coli* according to the procedure of Birnboim and Doly (1979), usually following amplification with Cm (170 µg/ml). For some experiments, plasmids were purified by CsCl centrifugation (Maniatis et al., 1982) prior to use.

(c) Restriction digestion and agarose-gel electrophoresis

Restriction digestions were performed in appropriate buffers essentially as recommended by the suppliers of restriction enzymes. Procedures for agarose gel electrophoresis were essentially as described previously (Jones and Hopwood, 1984a).

(d) Construction and identification of recombinant plasmids

The shotgun approach utilized in this study has been described elsewhere (Jones and Hopwood, 1984a). Chimeric plasmids were constructed from the ligation of SphI-digested *S. lividans* chromosomal DNA into the corresponding site of pBR322, and were introduced into *E. coli* strain HB101 or CSR603 by transformation. Colonies appearing on LB plates supplemented with 100 µg Cb/ml (LB-Cb plates) after 16–24 h incubation at 37°C were screened for the presence of recombinant plasmids containing sequences similar to the *S. antibioticus* phs gene.

The CbR transformants were replicated on Millipore filters placed on LB-Cb plates. After 3–4 h incubation at 37°C, filters were transferred, colony-side up, to LB-Cb plates that had been supplemented with 170 µg/ml Cm, and incubation was continued for additional 12–16 h. The filters were then prepared for colony hybridization according to the procedure described by Grunstein and Hogness (1975), using 32P-labeled pIJ2501 as probe. Hybridization was performed in 3 x SSC at 68°C, and filters were washed twice with 2 x SSC/0.1% SDS and twice with 1 x SSC/0.1% SDS.

(e) Expression of the cloned *Streptomyces lividans* gene

The synthesis of proteins encoded by the cloned fragments was examined by both the maxicell procedure originally described by Sancar et al. (1979) and modified by Calhoun and Gray (1981) following transformation of appropriate plasmids into CSR603, and an in vitro coupled transcription-translation system (Jones and Hopwood, 1984a; Thompson et al., 1984).

(f) Miscellaneous methods

Nick-translation of probes with [α-32P]dCTP (Rigby et al., 1977) and Southern (1975) hybridization were carried out according to the procedures previously described (Jones and Hopwood, 1984a).
Restriction mapping was performed by standard procedures (Lawn et al., 1978; Fitch et al., 1983). DNA fragments were recovered from agarose gels according to the procedure described by Benson (1984).

(g) Enzymes and reagents

Restriction endonucleases and other enzymes were obtained from Bethesda Research Laboratories, Bethesda, MD, or from Boehringer-Mannheim Biochemicals, Indianapolis, IN. Radionuclides and other radioisotopes were supplied by DuPont New England Nuclear Products, Boston, MA. Antibiotics were purchased from Sigma Chemical Company, St. Louis, MO. All chemicals were of reagent grade or the highest purity commercially available.

RESULTS

(a) Identification of positive clones

SphI fragments of *S. lividans* strain TK24 DNA were introduced into the corresponding site of pBR322. Following ligation, transformation of *E. coli* strain HB101 and selection on LB Cb plates, CbR colonies were screened by colony hybridization for the presence of sequences similar to the *phs* gene of *S. antibioticus*, using plasmid pIJ2501 probe and hybridization conditions that should permit detection of only those clones that shared at least 75% homology with the probe. From a total of about 20000 primary transformants, two colonies were identified which hybridized to this probe, suggesting the presence in those clones of plasmids harboring

![Fig. 1. Recombinant plasmids pJSM208 and pJSM210 and construction of subclones. Circular restriction maps have been abbreviated to reveal only strategic restriction sites. Heavy lines represent DNA regions derived from the *S. lividans* genomic DNA, and borne on the cloning vector pBR322 (thin lines).](image-url)
inserts similar to the phs gene. These clones were designated PHS208 and PHS210 and the recombinant plasmids from them, pJSM208 and pJSM210 (Fig. 1). pJSM208 contained three chromosomal SphI fragments (3.98, 3.14 and 1.20 kb), suggesting an insert size of 8.32 kb, while the insert in pJSM210 was 2.61 kb (containing SphI fragments of 2.21 and 0.40 kb). To verify that these inserts originated from S. lividans, plasmids pJSM208 and pJSM210 were radiolabeled and used to probe filter-bound SphI restriction fragments of TK24 DNA. Each cloned insert hybridized to fragments of the appropriate size in both the SphI digest of S. lividans chromosomal DNA and the respective recombinant plasmid. In addition, partial SphI digests of TK24 DNA did not contain any fragments that hybridized to both plasmids in Southern blots (not shown), indicating that the cloned inserts in pJSM208 and pJSM210 were not immediately contiguous to one another in the S. lividans genome.

Fig. 2 shows detailed (but incomplete) restriction maps of the cloned inserts. A partial map of the PHS structural gene from S. antibioticus is included for comparison. It is evident that neither cloned sequence is a direct subset of the other or of the S. antibioticus phs gene, and they appear to be non-overlapping.

(b) Further characterization of pJSM208 and pJSM210

To identify which fragments of the chromosomal inserts of pJSM208 and pJSM210 hybridize specifically to PHS2.4 (and are therefore phs-related), 32P-labeled plasmid pIJ2501 (containing phs from S. antibioticus) was hybridized to filter-bound fragments from an SphI digest of the two clones. One filter was prepared from a gel of total SphI fragments of the two clones, whereas the target DNAs on the other filter were the individual fragments isolated from the gel and purified. This latter approach was deemed necessary to eliminate contributions from the cloning vector to the hybridization profiles, and to reduce potential interactions between the cloned fragments during hybridization. In both experiments (Fig. 3), the probe hybridized exclusively to the 3.98-kb fragment from pJSM208, and to the 2.21-kb fragment of pJSM210. The hybridization signal detected from the 3.98-kb fragment was weaker than that from the 2.21-kb fragment, perhaps reflecting a greater degree of similarity between the 2.21-kb fragment and the S. antibioticus phs gene than is the case for the 3.98-kb fragment.

(c) Expression of the cloned inserts

The ability of the plasmids containing the cloned S. lividans nt sequences to serve as templates for DNA-dependent protein synthesis was examined both in vitro and in vivo. In a typical in vitro analysis, the levels of [35S]methionine incorporation in the presence of S. lividans recombinant plasmids were at least eight times that of the control without added DNA, and two to four times the stimulatory effect of plasmid pIJ2501 (see legend to Fig. 4). However, SDS-PAGE analysis of aliquots of the reaction mixtures followed by fluorography (Fig. 4) showed no apparent difference between products synthesized under the direction of plasmid pJSM208 (lane 5) and those encoded by pBR322 (lane 9). A 24-kDa polypeptide was uniquely present among the polypeptides derived from pJSM210 (see arrow in lane 7). None of the products from either of the
Fig. 3. Southern blot showing hybridization of the *S. antibioticus* *phs* gene to specific *SphI* fragments of the cloned *S. lividans* sequences. Approx. 500 ng of each DNA sample was digested to completion with *SphI* and fractionated by electrophoresis at 75 V through a 0.7% agarose gel for 4 h. Following transfer and prehybridization, filters were probed with pIJ2501 (the *S. antibioticus* *phs* gene borne on the streptomycete vector pIJ702). Wash conditions were adjusted to the 75% stringency level. DNA sources for the respective autoradiograms were: panel A, *SphI* digests of pJSM208 (lane 1), pJSM210 (lane 2), and pIJ2505, the pBR322 derivative of pIJ2501 (lane 3); panel B contained the purified *SphI* fragments of the *S. lividans* inserts from plasmids pJSM208 and pJSM210, in the order 3.98 kb (lane 1), 3.14 kb (lane 2), 1.20 kb (lane 3), 2.21 kb (lane 4), and 0.4 kb (lane 5). For the maps of the pJSM plasmids see Fig. 1. The positions of the *HindIII*-generated λ fragments (in kb) are shown on the left margins.

Fig. 4. SDS–PAGE of 35S-labeled polypeptides encoded by the *S. lividans* recombinant plasmids in the coupled transcription-translation system. Assay conditions were as described in Jones and Hopwood (1984a). Aliquots containing approx. 100 000 cpm of the cell-free reaction mixtures or the immunoprecipitates from the reaction of such aliquots with antiserum to the PHS subunit were fractionated in a 10% separating gel. Electrophoresis was at 40 V for 16 h. Templates used in each reaction mixture were as follows: lanes: 1, pIJ2501 (9560); 3, pIJ2505 (27039); 5, pJSM208 (24735); 7, pJSM210 (48 804); 9, pBR322 (40 664). Numbers in parentheses represent acid-insoluble cpm/μl of reaction mixtures, and have been corrected by subtraction of a zero time control. The immunoprecipitates of each reaction mixture are shown in the even-numbered lanes in the corresponding order. The approx. 24-kDa polypeptide apparently encoded by the *S. lividans* insert in pJSM210 is identified by the arrow. Numbers on the left margin represent the sizes (kDa) of the markers, phosphorlase B, bovine serum albumin, ovalbumin, and carbonic anhydrase (top to bottom). PHS, the 88-kDa PHS subunit.
S. lividans recombinant plasmids reacted with antibody to the S. antibioticus PHS protein. The relatively high levels of incorporation observed in the coupled system with plasmids containing inserts from S. lividans, therefore, derived primarily from transcription and translation of genes borne by the vector, pBR322.

It was noted earlier that the cloned SpI fragments in either plasmid pJSM208 or pJSM210 are not contiguous in the S. lividans genome. Therefore, the possibility that the presence of the other cloned fragment(s) in the same plasmid might influence the expression of fragments bearing PHS-related sequences deserved some consideration. The S. lividans inserts in pJSM208 and pJSM210 were subcloned in pBR322 (Fig. 1), and tested for their ability to function in the coupled transcription-translation assay. Results from this analysis are presented in Fig. 5. Of particular interest in this figure is the protein profile for the products synthesized under the direction of plasmid pJSM221 (lane 1 of Fig. 5A). Proteins of 92 and 88 kDa (and some smaller polypeptides) are present among the products of the coupled synthesis with pJSM221 as template. Both the 92-kDa and 88-kDa proteins apparently encoded by the chromosomal insert in pJSM221 are immunoprecipitable with antibody to S. antibioticus PHS (lane 6 of Fig. 5B). Note also that the ability of the 3.98-kb insert to function as a template appears to be dependent on its orientation (compare lanes 1 and 2 of Fig. 5A). No insert-derived products were detected from the use of any of the other subclones.

The subunit size of S. antibioticus PHS is 88 kDa (Jones and Hopwood, 1984a). Thus, the 88-kDa protein observed with pJSM221 as a template is likely to represent the S. lividans PHS subunit, while the 92-kDa polypeptide could result from readthrough synthesis initiated from a promoter.

Fig. 5. SDS–PAGE of products of the coupled transcription-translation assay using plasmid templates derived from subclones of plasmids pJSM208 and pJSM210. Between 20000 and 100000 acid-insoluble cpm were applied per lane of a 0.1% SDS–10% polyacrylamide gel. Fractionation was at 120 V for approx. 4 h. The lines on the left margin (panel A) and right margin (panel B) mark the positions of the non-radioactive molecular standards in the order 92.5, 69, 46, and 30 kDa. Arrows and arrowheads indicate the positions of the 92-kDa and 88-kDa products of the cloned S. lividans gene(s), respectively. The following templates were used. (Panel A) lanes: 1, pJSM221; 2, pJSM220; 3, pJSM222; 4, pJSM223; 5, pJSM224; 6, pJSM225. (Panel B) Autoradiogram of total cell-free products (odd-numbered lanes), and immunoprecipitates from the reaction of such aliquots with antiserum to the PHS subunit (even-numbered lanes), derived from the following templates: pIJ2501 (lanes 1 and 2), pIJ2505 (lanes 3 and 4), and pJSM221 (lanes 5 and 6). The 88-kDa band in lanes 1–4 is the S. antibioticus PHS subunit.
upstream from the physiological tsp. These data, therefore, argue convincingly for the identity of the 3.98-kb insert in pJSM221 as an S. lividans homologue of the S. antibioticus structural gene for PHS.

We were unable to detect in the maxicell system any radiolabeled protein from the cloned Streptomyces sequences (including PHS2.4). This finding was not totally surprising since it has been shown that certain streptomycete promoters failed to function in E. coli (Horinouchi et al., 1980; Bibb and Cohen, 1982; Bibb et al., 1985). The in vivo expression of the S. lividans 3.98-kb fragment is being examined in appropriate Streptomyces hosts.

**DISCUSSION**

In this report, evidence has been presented confirming the presence of a normally silent gene for PHS in S. lividans. The 88-kDa product of in vitro transcription and translation of this gene is similar in size and immunological reactivity to the S. antibioticus PHS subunit.

As shown in Fig. 5A, the use of pJSM221 as a template in the coupled transcription-translation system also led to the synthesis of a 92-kDa protein precipitable with antibody to native PHS. While the precise origin of this protein has not yet been determined, one likely possibility is that its message is derived by transcription initiating outside the cloned 3.98-kb SphI fragment of pJSM221, perhaps from the tet promoter. The 92-kDa protein might then be a fusion of the N terminus of the tet gene product with the phs gene product. Preliminary observations indicate that deletion of the region of pBR322 which bears the tet promoter abolishes the synthesis of the 92-kDa protein in the coupled system (to be published). The lower Mr products in lane 6 of Fig. 5B may represent polypeptides resulting from non-physiological initiation or termination of transcription or translation, or even proteolytic degradation of the primary translation product in this cell-free system. Similar observations have been made previously (Jones and Hopwood, 1984a).

The cloned insert in pJSM224, bearing the 2.21-kb fragment with homology to the S. antibioticus phs gene (Fig. 1), did not appear to encode any protein products detectable in the fluorogram of Fig. 5A. There are at least three possible explanations for this result. First, since the parent plasmid, pJSM210, was responsible for the synthesis of a protein approx. 24 kDa in size in the in vitro assay (Fig. 6, lane 5), it is possible that the expression of the 2.21-kb fragment might be influenced by the 0.4-kb fragment in pJSM210. Second, the expression of this fragment might be dependent on the orientation of insertion. We have not yet succeeded in cloning the 2.21-kb fragment in the orientation opposite to that found in pJSM224. Third, since the 88-kDa PHS subunit requires a DNA fragment of approx. 2.4 kb to encode it, the 2.21-kb insert in pJSM224 possibly lacks some sequences required for in-frame translation of the resulting transcript. The organization of this and the other cloned fragments in plasmids pJSM208 and pJSM210 (Fig. 1) relative to the 3.98-kb fragment and how they all may relate to the expression of the structural gene is being investigated.

The existence of a PHS gene in S. lividans further establishes that portions of the Streptomyces chromosome can harbor silent genes. We refer to these genes as silent for PHS activity rather than cryptic because we do not know whether they have specific functions in their silent state, presumably in the biosynthesis of antibiotics. It is clear, however, that the PHS activity associated with the S. antibioticus homologue is not normally expressed in S. lividans. The existence of genes for biosynthetic enzymes in certain strains of Streptomyces which do not ordinarily produce the products in question is not unique to S. lividans. Other instances have been reported (Martin et al., 1984; Malpartida et al., 1987). These observations of sequence similarities among different species of Streptomyces may imply conservation of certain aspects of antibiotic production and/or its control in this group of organisms. This sequence conservation might most easily be explained by natural selection especially if these silent genes actually serve other functions related to antibiotic biosynthesis.

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