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REGULATION OF ACTINOMYCIN SYNTHESIS IN *STREPTOMYCES ANTIBIOTICUS*[1]

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ABSTRACT.—Phenoxazinone synthase (PHS) is one enzyme that has been implicated in the biosynthesis of actinomycin in *Streptomyces antibioticus*. The gene for the 88,000 M_r subunit of PHS has been cloned and has been used to study the regulation of the enzyme in *S. antibioticus*. The expression of the enzyme in growing cultures is regulated at the transcriptional and post-transcriptional levels, and glucose repression of PHS synthesis also involves control at the level of mRNA synthesis. Interestingly, the transformation of *S. antibioticus* with a multicopy plasmid containing the cloned PHS gene leads to the premature cessation of mycelial growth and actinomycin production. Two other fragments of the *S. antibioticus* genome have been cloned and these fragments may be involved in the regulation of antibiotic synthesis in the donor organism and in other streptomycetes. These fragments appear to function by activating a normally silent PHS gene in the cloning host employed in these experiments, *Streptomyces lividans*. The implications of these results are discussed.

The actinomycins (see Figure 1 for the structure of actinomycin D) are a family of chromopeptide antibiotics that are elaborated by several members of the genus *Streptomyces* (1). Because of their toxicity, these antibiotics have been employed to only a limited extent in the clinical setting (2,3), but their activity as potent inhibitors of DNA-dependent RNA synthesis has made them valuable as biochemical tools (4,5). With regard to the biosynthesis of actinomycin, a putative biosynthetic pathway has been proposed (6), and at least two enzymes involved in the production of the antibiotic have been identified. One of these enzymes catalyzes the activation of 4-methyl-3-hydroxyanthranilic acid, the precursor of the chromophore of the antibiotic (7). The second enzyme, phenoxazinone synthase (PHS), participates in reactions of the type shown in Figure 2 (8). In the biosynthesis of actinomycin, PHS is thought to catalyze the oxidative condensation of two molecules of 4-methyl-3-hydroxyanthraniloyl pentapeptide to

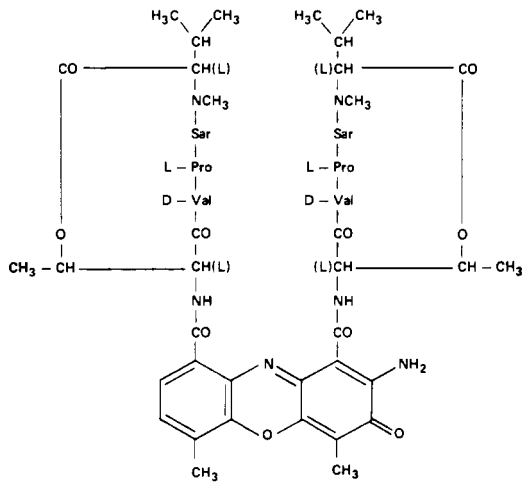

FIGURE 1. The structure of actinomycin D

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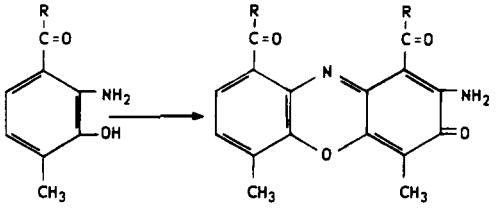

FIGURE 2. The reaction catalyzed by phenoxazinone synthase

produce actinomycin or the penultimate precursor in the pathway, actinomycinic acid (6).

Phenoxazinone synthase was first identified in *Streptomyces antibioticus* extracts by Katz and Weissbach (9). It was subsequently shown that PHS is not produced constituitively in *S. antibioticus*, but that its synthesis is initiated at a particular time in cultures grown on antibiotic production medium with galactose as a carbon source, and that its specific activity increases until some time after actinomycin synthesis has begun (10,11). Gallo and Katz (12) have also shown that PHS synthesis and overall actinomycin production are subject to catabolite repression. When *S. antibioticus* is grown on glucose rather than galactose as carbon source, the appearance of PHS and of actinomycin are delayed, presumably until all the glucose in the growth medium is utilized. PHS recently has been purified to homogeneity in the author's laboratory (13). It was observed that the enzyme exists in two native forms in *S. antibioticus* cells, a large form with a molecular weight of 528,000 and a small form with an M_r of 176,000. Both forms are composed of a single subunit with an M_r of 88,000. More recently, gene cloning experiments have led to the identification of the structural gene for the PHS subunit and of sequences that may be involved in the regulation of actinomycin biosynthesis. These studies are summarized below.

CLONING OF THE PHS GENE AND OF PUTATIVE REGULATORY SEQUENCES.—The cloning strategy employed in these experiments involved the use of the plasmid vector, pIJ702, developed at the John Innes Institute by Katz *et al.* (14). *S. antibioticus* DNA was digested with the restriction enzyme *Sph*I, and the digests were ligated into the corresponding site in the mel (tyrosinase) gene of pIJ702. After transformation of *Streptomyces lividans* TK 24, melanin-negative, thiostrepton-resistant colonies were selected. In screening these colonies for the PHS structural gene, the assumption was made that the gene, if present on a recombinant plasmid, would be expressed in *S. lividans*. This did not seem to be an unreasonable assumption since the mel gene of pIJ702 was itself derived from the *S. antibioticus* genome and is efficiently expressed in *S. lividans* transformed with that plasmid (14). By using a sib selection procedure and the in vitro assay for PHS, it was possible to isolate five transformant colonies that produced significant levels of active enzyme (15). On the basis of the sizes and orientations of the DNA inserts present in the plasmids obtained from those transformants, it was determined that the five could have represented only three transformation events, since two sets of pairs contained identical inserts. The three classes of PHS clones contained inserts of approximately 1.8, 2.4, and 4.3 kb, and the corresponding plasmids will generally be referred to below as PHS 1.8 (or pIJ2500), PHS 2.4 (pIJ2501), and PHS 4.3 (pIJ2502). Restriction maps of the cloned fragments are shown in Figure 3.

IDENTIFICATION OF THE PHS STRUCTURAL GENE AND REGULATION OF ITS EXPRESSION.—*The 2.5 kb insert in PHS 2.4 is the PHS structural gene.*—Evidence supporting this conclusion is derived from a number of experiments. First, high levels of PHS

activity are observed when the 2.4 kb insert is present in either orientation in *S. lividans* transformants. Second, those transformants produce a protein that has immunological and electrophoretic properties indistinguishable from authentic PHS. Third, PHS 2.4 functions quite efficiently as a template in a streptomycete- coupled transciption-translation system (16). Examination of the products of coupled synthesis confirmed that the 2.4 kb insert codes for a protein with the immunological and electrophoretic properties of the PHS subunit (15).

The direction of transciption of the PHS gene.—The direction of transcription of the PHS gene has been determined by RNA dot blotting, using total RNA isolated from *S. antibioticus* mycelium and end-labeled restriction fragments derived from the cloned PHS gene. The gene is transcribed from right to left, given the orientation shown in Figure 3 (17). In more recent experiments, fragments with promoter activity have been isolated from the right end of the PHS gene (author's unpublished results), suggesting that in addition to the PHS coding sequence, the 2.4 kb insert contains at least part of the genomic region involved in the initiation of transcription of the PHS gene.

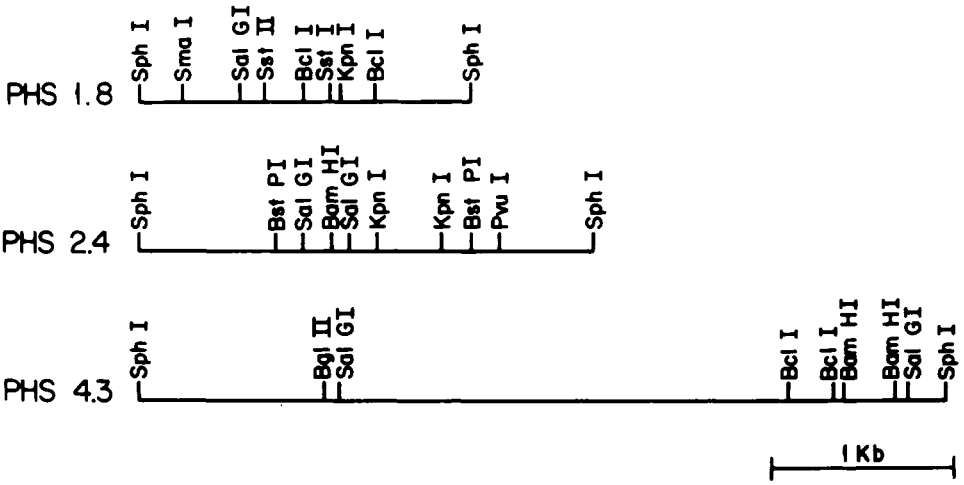

FIGURE 3. Partial restriction maps of the cloned 1.8, 2.4, and 4.3 kb DNA fragments from the *Streptomyces antibioticus* genome. The corresponding recombinant plasmids are properly designated as pIJ2500 (PHS 1.8), pIJ2501 (PHS 2.4), and pIJ2502 (PHS 4.3).

PHS expression is controlled at the transcriptional and post-transcriptional levels in S. antibioticus.—The concentration of PHS mRNA in *S. antibioticus* cultures was measured by RNA dot blotting using total RNA isolated from cultures of varying ages and the cloned PHS gene as a probe. It was observed that the increase in PHS specific activity occurring during growth of the organism on actinomycin production medium is due, at least in part, to an increase in the synthesis of PHS mRNA. Thus, at early times post-inoculation, PHS specific activity and the concentration of PHS mRNA are relatively low. At later times, the increase in PHS specific activity is reflective of a corresponding increase in the concentration of PHS message in the total RNA population. Similarly, glucose inhibition of PHS synthesis is also mediated at the transcriptional level. PHS specific activities and mRNA concentrations are lower in cultures grown on glucose than in cultures of the same age grown on galactose as the carbon source (17).

Evidence for post-transcriptional regulation of PHS expression derives from studies in which the degradation of that enzyme and of total mycelial protein were compared. These experiments revealed that PHS is relatively stable to degradation under conditions where most mycelial proteins are broken down. Thus, in vivo, the specific activity

of the enzyme (activity/total protein) in older cultures increases because of a decrease in the ratio of total protein:DNA with no change (or perhaps an increase) in the ratio of PHS:DNA (17).

S. lividans *transformed with PHS 2.4 produces both the large and small forms of the enzyme.*—These results (15) suggest that both forms of the enzyme are composed of the subunit encoded by the cloned 2.4 kb insert.

Overproduction of PHS in S. antibioticus *does not lead to overproduction of actinomycin.*—In these experiments, *S. antibioticus* was transformed with PHS 2.4. Specific activity levels equivalent to those observed in *S. lividans* transformants were obtained as a result (about 1600 units/mg protein), but there was no accompanying increase in the level of actinomycin production (Figure 4). Indeed, in *S. antibioticus* transformants containing PHS 2.4, mycelial growth and actinomycin synthesis ceased prematurely as compared with the untransformed organism. Since the same observation was made with transformants containing pIJ702 (or PHS 1.8 or 4.3, Figure 4), there is a strong possibility that these phenomena are a result of the high copy number of the plasmids that were used in the cloning experiments. This possibility is currently under investigation.

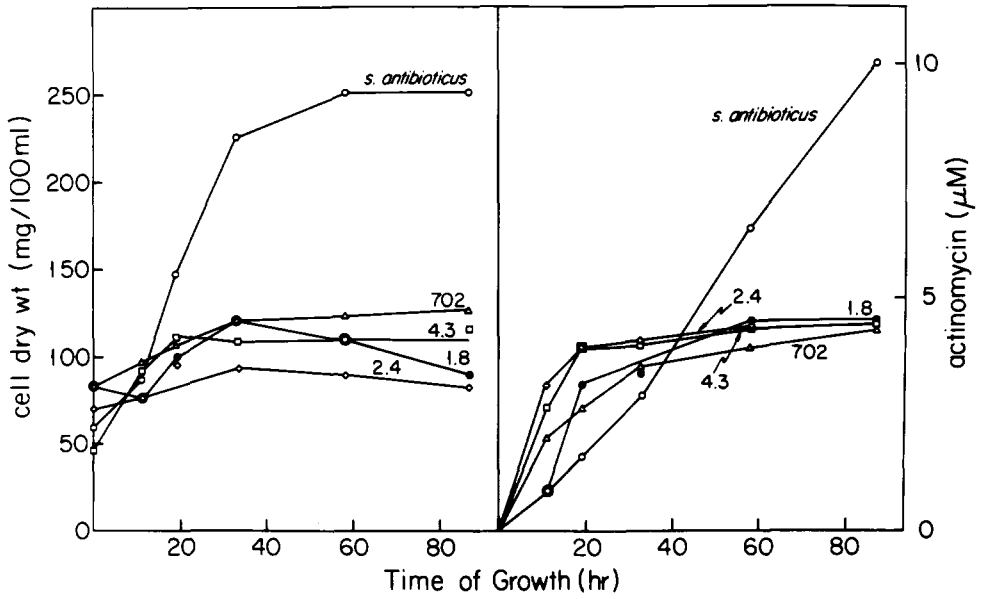

FIGURE 4. Effects of transformation of *Streptomyces antibioticus* with PHS plasmids on growth and actinomycin production. In the experiments depicted, *S. antibioticus* protoplasts were transformed with PHS 1.8, 2.4, and 4.3, and as a control with pIJ702. Cell dry weight and actinomycin production were measured as described previously (21). It is noteworthy that the effects of transformation with pIJ702 are identical to those observed after transformation with the PHS plasmids.

The size of the PHS mRNA.—Northern blotting experiments revealed that the putative PHS mRNA is much larger than is required to encode a protein of 88,000 M_r (17). Given the recent observations of polycistronic transciption of genes involved in the biosynthesis of other antibiotics (18,19), it seems possible that the PHS gene is also transcribed as a part of a polycistronic message and that the transcript contains sequences corresponding to other genes required for the production of actinomycin.

The successful cloning of the PHS gene demonstrates the melding of biochemical and recombinant DNA techniques in the examination of an antibiotic biosynthetic pathway. A major advantage of the cloning approach described above is that it requires

no genetic information about the biosynthetic pathway of interest. Blocked mutants are not required nor is there any need for knowledge of the genomic organization of the pathway genes. As long as a suitable assay for a protein of interest can be devised, this assay can, in principle at least, be used to identify the gene for that protein in a transformant pool. Successful use of this approach does, of course, require that the cloned gene be expressed in the cloning host. This approach is currently being utilized in the author's laboratory to identify other genes involved in the biosynthesis of actinomycin.

ACTIVATION OF A "SILENT" PHS GENE IN *S. ANTIBIOTICUS*.—As was indicated above, two recombinant plasmids were isolated, in addition to PHS 2.4, which were capable of transforming *S. lividans* with resultant production of an enzyme which functions in the PHS assay. It was further observed that *S. lividans* transformed with PHS 1.8 and 4.3 produce a protein quite similar in its biochemical properties to *S. antibioticus* PHS. Although both these plasmids function as templates in the coupled transcription-translation system, neither codes for proteins related to PHS (20). A possible explanation for these observations was suggested by the results of experiments in which extracts of ^{35}S-methionine labeled, untransformed *S. lividans* were treated with antibody to PHS. It was demonstrated that the untransformed cloning host synthesizes, albeit at very low levels, a protein with the properties of PHS. Southern blotting experiments further demonstrated the presence in the *S. lividans* genome of sequences that hybridize to the cloned *S. antibioticus* PHS gene (20).

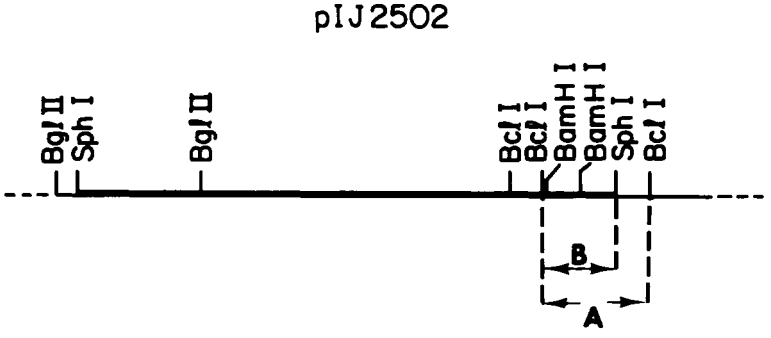

FIGURE 5. Partial restriction map of PHS 4.3 showing the fragment of this plasmid which was subcloned into the *Bgl*II site of pIJ702. Segment A, approximately 850, base pairs (bp) in size, includes a portion of the cloning vector and a 560 bp fragment of the 4.3 kb insert (segment B).

While the mechanism by which PHS 1.8 and 4.3 activate PHS expression in *S. lividans* has not been elucidated, two general classes of such mechanisms can be envisioned. Mechanisms of one class would require that the 1.8 and 4.3 kb inserts encode a protein or proteins and that the protein(s) interact with the *S. lividans* genetic machinery in such a way as to turn on the PHS gene. Alternatively, the 1.8 and 4.3 kb fragments might not perform a coding function, but might be equivalent to operator sequences, and bind a repressor protein that normally prevents the expression of the *S. lividans* PHS gene. Evidence bearing on these two general possibilities has been obtained in recent experiments. A fragment of PHS 4.3, approximately 850 base pairs in size and which includes a portion of the cloning vector pIJ702, has been subcloned in that same plasmid vector (Figure 5). In one orientation, this smaller fragment retains the ability of the 4.3 kb insert to activate PHS expression in *S. lividans*. Preliminary data indicate that the smaller fragment is not transcribed in *S. lividans* transformants. Further, preliminary nucleotide sequence results reveal the presence of numerous terminator codons, in all possible reading frames, dispersed throughout the cloned fragment. These data would seem to argue against the first of the two classes of mechanisms suggested above.

Two other interesting observations have been made relative to the activation of the *S. lividans* PHS gene. First, a number of other streptomycetes have been examined for the presence of sequences homologous to the PHS gene and the cloned 4.3 kb "activator" sequence. Several species (*Streptomyces chrysomallus, Streptomyces parvulus, Streptomyces erythreus*), in addition to *S. lividans*, contain homologs that hybridize quite efficiently to the *S. antibioticus* PHS gene (author's unpublished results). These species and several others (*Streptomyces albus, Streptomyces ambofaciens, Streptomyces glaucescens, Streptomyces fradiae, Streptomyces coelicolor*) all contain homologs of the *S. antibioticus* 4.3 kb "activator" fragment. It should be noted that *S. chrysomallus, S. parvulus,* and *S. fradiae* are known to produce actinomycins. Second, preliminary results indicate that the *S. lividans* genome contains not just one but two different homologs of the PHS gene.

The detection of a "silent" PHS gene in *S. lividans* has important implications for the interpretation of cloning experiments designed to identify antibiotic production genes, for the regulation and evolution of antibiotic biosynthetic pathways and for the possible production of new antibiotics by strains that normally produce others or none at all. The implications for cloning experiments are related to the approaches commonly used to identify cloned sequences as biosynthetic genes. It is possible that silent genes exist in some (perhaps all) streptomycetes for enzymes involved in the synthesis of antibiotics other than actinomycin. Thus, when cloned sequences lead to the appearance of a product possessing antibiotic activity, it must be established that this product is synthesized by enzymes encoded by genes that are part of the cloned fragment rather than as a result of the activation of a set of silent genes in the cloning host.

The presence of homologs of the PHS gene in other streptomycetes suggests the interesting possibility that silent pathways may be common in members of the genus. Perhaps such pathways represent evolutionary vestiges of experiments that were attempted and whose results were discarded, or perhaps the silent pathways can, in fact, still be expressed by these organisms under appropriate nutritional conditions. The observation that sequences homologous to the cloned 4.3 kb insert appear to be distributed widely among members of the genus is consistent with a role for this sequence as a general regulator of secondary metabolism. It seems possible that the 4.3 kb sequence functions to regulate antibiotic production in a fashion common to many or all the members of the genus, while other sequences may be required for more specific regulation of particular antibiotic production pathways. In any event, the existence of silent PHS genes would seem to augur well for the potential to produce novel antibiotics by genetic manipulation of organisms which do not normally make them. It will be of considerable interest to determine the extent to which the phenomena described here are repeated in other streptomycetes and to examine the mechanism of gene silencing in those organisms in which it is observed.

ACKNOWLEDGMENTS

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