JOURNAL OF BACTERIOLOGY, Sept. 1985, p. 1215–1221
0021-9193/85/091215-07$02.00/0

Vol. 163, No. 3

# Regulation of Phenoxazinone Synthase Expression in *Streptomyces antibioticus*

GEORGE H. JONES

*Department of Cellular and Molecular Biology, Division of Biological Sciences, The University of Michigan, Ann Arbor, Michigan 48109-1048*

Received 15 April 1985/Accepted 24 June 1985

**The cloned gene for the subunit of phenoxazinone synthase (PHS), an enzyme implicated in the biosynthesis of actinomycin in *Streptomyces antibioticus*, was used as a probe to study the regulation of the enzyme. The direction of transcription of the PHS gene was determined with end-labeled restriction fragments derived from the gene. Low-resolution S1 mapping revealed that transcription was initiated at a position which may lie within the *SphI* restriction site, which represents the limit of the cloned sequence. Northern blotting allowed the identification of the putative PHS message. This RNA appeared to be significantly larger than the size required to encode the PHS subunit. RNA dot blotting showed that the increase in PHS specific activity observed in cultures grown on antibiotic production medium, with galactose as a carbon source, was due in part to an increased production of PHS mRNA. PHS was also more stable than most cellular proteins and appeared to be protected against degradation under conditions in which most other proteins are broken down. This protective effect also contributed to the increase in PHS specific activity observed in *S. antibioticus* cultures grown on production medium. The repression of PHS synthesis by glucose was also reflective of a transcriptional control mechanism. At early time points postinoculation, PHS mRNA levels were lower in cultures grown on glucose as a carbon source than in cultures of the same age grown on galactose. mRNA levels presumably begin to increase only after all the glucose in the medium is utilized. The ability of 5-fluorouracil to stimulate PHS production in young cultures was also due to the synthesis of new mRNA for the enzyme.**

Phenoxazinone synthase (PHS) is one enzyme thought to be involved in the biosynthesis of actinomycin in *Streptomyces antibioticus*. In the biosynthetic pathway, the enzyme catalyzes the oxidative condensation of 2 mol of 4-methyl-3-hydroxyanthraniloyl pentapeptide (Fig. 1) to produce actinomycin or the penultimate intermediate in the pathway, actinomycinic acid (22). PHS was first identified in *S. antibioticus* extracts by Katz and Weissbach (14). It was subsequently shown that PHS is not produced constitutively in *S. antibioticus* but that its synthesis is initiated at a particular time in cultures grown on antibiotic production medium and that its specific activity increases until sometime after actinomycin synthesis has begun (15, 19). Gallo and Katz (6) also showed that PHS synthesis and overall actinomycin production are subject to catabolite repression. When *S. antibioticus* cultures are grown on glucose as a carbon source, rather than on galactose (which is normally the carbon source used in production medium), the appearance of PHS and the production of actinomycin are delayed, presumably until all the glucose in the medium is utilized.

PHS has been purified to apparent homogeneity by Choy and Jones (3) and shown to exist in two native forms in *S. antibioticus* cells. The large form of the enzyme has an $M_r$ of about 530,000, whereas the small form has an $M_r$ of about 175,000 (3; unpublished results). Both forms of the enzyme are composed of a single subunit which has a molecular weight of about 88,000 (M. E. Johnson and G. H. Jones, manuscript in preparation). The appearance of the two forms of PHS is also regulated in *S. antibioticus*. Young, non-actinomycin-producing cultures predominantly contain the small enzyme form, whereas older, actinomycin-producing cultures contain more of the large than of the small enzyme form (3).

The structural gene for the 88,000-$M_r$ subunit of PHS has recently been cloned in *Streptomyces lividans* (10). The gene is contained on a 2.45-kilobase (kb) genomic fragment and is expressed in *S. lividans* when cloned in the plasmid vector pIJ702 (13). The availability of the cloned gene has made it possible to examine the regulation of PHS expression in the donor organism, *S. antibioticus*. The direction of transcription of the gene was determined, low-resolution S1 nuclease mapping was used to examine the transcription start site for the PHS gene, and the size of the PHS message was examined by Northern blotting. In addition, the cloned structural gene was used to examine the mechanism by which the synthesis of PHS is controlled during growth of *S. antibioticus* on actinomycin production medium and in medium containing glucose as the carbon source. The stability of PHS in growing *S. antibioticus* mycelium was also examined. The results of these studies are presented below.

## MATERIALS AND METHODS

**DNA manipulations.** Procedures for cloning of the PHS structural gene, restriction digestion, agarose gel electrophoresis, Southern blotting, nick translation, and hybridization were as described earlier (10) or as described below. Plasmids used in the experiments described were pIJ2501 (PHS 2.4, a derivative of pIJ702 [13] containing the cloned PHS structural gene), pIJ2503 (the pIJ702 derivative with the PHS gene in the orientation opposite to that of pIJ2501), and pIJ2505 (a derivative in which the 2.45-kb PHS gene is cloned into the *SphI* site of pBR322).

**Isolation of RNA.** *S. antibioticus* cultures were grown in glutamic acid-salts medium with either 1% glucose or 1% galactose as a carbon source, as described previously (6). Inocula were prepared with cells grown on NZ-amine medium (6). Portions were removed from growing cultures at 6, 12, 18, 24, and 48 h after inoculation. Cells obtained from the NZ-amine culture served as the source of zero-time RNA samples. In some experiments, RNA was isolated from

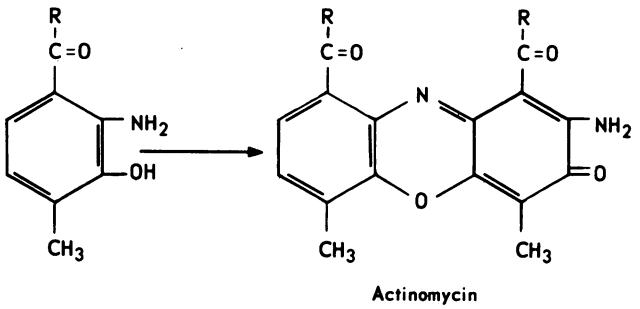


FIG. 1. Reaction catalyzed by PHS.

cultures grown in the presence of 1% galactose and 20 μg of 5-fluorouracil (FU) per ml or from *S. lividans* transformed with pIJ2501 or pIJ2503.

RNA was isolated from mycelia by a procedure to be described in detail elsewhere (C. P. Smith and K. F. Chater, manuscript in preparation). Briefly, the procedure involved softening of the washed mycelium by a short lysozyme digestion, extraction of the mycelial suspensions with Kirby mix (16), and repeated phenol extraction of the aqueous extracts. RNA was then precipitated with isopropanol, redissolved, and subjected to two rounds of precipitation in 3 M sodium acetate (pH 6). The final RNA pellet was dissolved in 50 mM Tris-hydrochloride–5mM $MgCl_2$ (pH 7.8) and incubated for 20 min at room temperature with RNase-free DNase (Bethesda Research Laboratories) at 0.5 μg/ml. After two phenol and two $CHCl_3$ extractions, the RNA was precipitated with ethanol and stored at −20°C. RNA concentrations were calculated from $A_{260}$ measurements, with 1 $A_{260}$ unit considered the equivalent of 50 μg of RNA.

**5′-End labeling of restriction fragments.** Fifteen micrograms of pIJ2505 was digested with *Bam*HI, and the resulting digest was fractionated by agarose gel electrophoresis. The bands containing the large and small *Bam* fragments from the PHS structural gene (see Fig. 2) were eluted from the gel (20), extracted with phenol and $CHCl_3$ as described above, and precipitated with ethanol. After being redissolved, the fragments were treated with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals), extracted again with phenol and $CHCl_3$, and precipitated with isopropanol. The phosphatase-treated fragments were then labeled at their 5′ ends with [$^{32}$P]ATP (3,200 Ci/mmol; New England Nuclear Corp.) and polynucleotide kinase (Bethesda Research Laboratories). After 30 min of incubation at 37°C (18), the reaction was terminated by phenol extraction and unincorporated label was removed from the fragments by passing them through small columns of Biogel P-100. Labeled fragments were then digested with *Sph*I to release the 1.41- and 1.04-kb arms of the PHS gene.

**Dot blotting and Northern analysis.** Portions of ethanol-precipitated total RNA samples (usually corresponding to 30 μg) were collected by centrifugation and dried in vacuo for 5 min. Samples were then dissolved in 2 μl (for dot blotting) or 10 μl (for Northern blots) of glyoxal-formamide-phosphate (21) and heated for 10 min at 55°C. For dot blots, samples were spotted onto nitrocellulose filters which had been soaked in 20× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate). Filters were baked for 2 h at 80°C in vacuo.

RNA samples for Northern blotting were fractionated on 1% agarose gels in 10 mM sodium phosphate buffer (pH 7.0). RNA was transferred to nitrocellulose as described previously (10), and the filters were baked as described above. Titration curves, which identified the range over which the strength of the hybridization signal was proportional to the amount of RNA spotted on filters, were prepared.

**Low-resolution S1 nuclease mapping.** Conditions for S1 nuclease mapping were essentially as described by Favaloro et al. (5). Usually, 30 μg of RNA was allowed to hybridize at 70°C with about 50 ng of the 3.01-kb *Sal*GI fragment obtained by digestion of pIJ2501 (see Fig. 2). Hybrids were treated with S1 nuclease (Sigma Chemical Co.), precipitated with isopropanol, and fractionated on alkaline agarose gels. In some experiments, RNA was omitted from reaction mixtures so that reannealing artifacts could be identified. DNAs were transferred to nitrocellulose filters as described previously (10).

All hybridizations to filter-bound nucleic acids were performed in 3× SSC for 36 h at 70°C. Dot blots and Northern blots were washed twice at 70°C in 2× SSC–0.1% sodium dodecyl sulfate before autoradiography, whereas DNA blots from S1 nuclease mapping were washed twice with 2× SSC–0.1% sodium dodecyl sulfate and twice with 0.2× SSC–0.1% sodium dodecyl sulfate. Autoradiography was performed with Kodak XAR-5 X-ray film. In some experiments autoradiograms were scanned with a Joyce-Loebl densitometer.

**Studies on the stability of PHS.** A 50-ml *S. antibioticus* culture growing on galactose was labeled with 1 mCi of [$^3$H]leucine (56.5 Ci/mmol; New England Nuclear Corp.) between 0 and 12 h postinoculation. A 30,000-fold excess of unlabeled leucine was then added, a 10-ml aliquot was removed from the flask, and the mycelium was collected by brief centrifugation. Additional 10-ml aliquots were removed at 18, 24, and 48 h postinoculation. Mycelium was stored at −70°C until use. The thawed mycelium was washed in 10 ml of 50 mM Tris-hydrochloride–1 M KCl (pH 7.6), followed by 10 ml of 0.9% NaCl. Mycelium was disrupted in phosphate-buffered saline by a single passage in a French press at 12,000 lb/in$^2$. Cell debris was removed by centrifugation, and the soluble extracts were concentrated by overnight dialysis against ammonium sulfate without stirring (9). Ammonium sulfate precipitates were collected by centrifugation and dissolved in phosphate-buffered saline. Samples of these solutions were subjected to trichloroacetic acid precipitation and scintillation counting, and other samples were treated with antibody to PHS (3). Antibody precipitates were collected, washed, and dissolved in NCS solubilizer for liquid scintillation counting. Titration experiments were performed with each protein sample to ensure maximum precipitation of radiolabeled PHS. DNA and protein concentrations were also determined on samples of the crude extracts (before ammonium sulfate precipitation). Results of the determination of total radioactivity are expressed as counts per minute per milligram of protein; results of the antibody analysis are

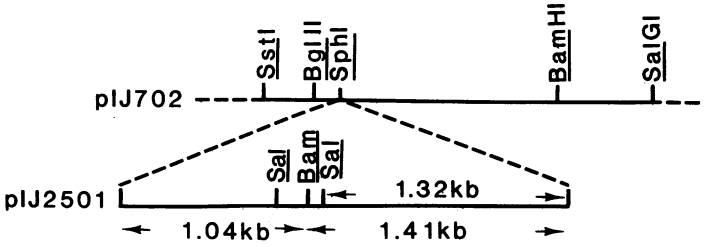


FIG. 2. Partial restriction maps of pIJ702 (after Katz et al. [13]) and pIJ2501. Only a portion of the pIJ702 vector is shown.

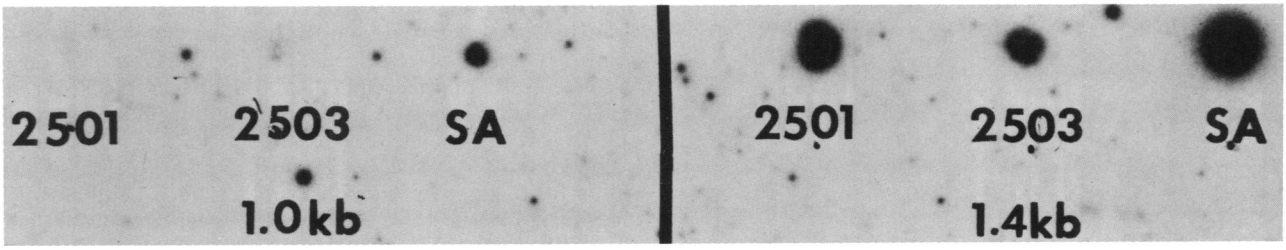


FIG. 3. Determination of the direction of transcription of the PHS gene. 5'-End-labeled restriction fragments were prepared from *Bam*HI-digested pIJ2505 as described in the text. In the experiments shown in the right panel, the end-labeled, 1.41-kb *Bam-Sph*I fragment was hybridized to total RNA from *S. lividans* transformed with pIJ2501 (2501) or pIJ2503 (2503) or to RNA from *S. antibioticus* (SA). In the left panel, the hybridization probe was the end-labeled, 1.04-kb *Bam-Sph*I fragment from the PHS gene.

expressed as percentage of total counts per minute which were specifically immunoprecipitable.

Protein was determined with the BioRad protein assay reagent; PHS was assayed as described previously (3); and DNA was determined as described by Ceriotti (1).

## RESULTS

**Direction of transcription of the PHS gene.** Partial restriction maps of pIJ702 and pIJ2501 (PHS 2.4, the plasmid containing the PHS structural gene [10]) are shown in Fig. 2. To determine the direction of transcription of the PHS gene, I prepared 5'-end-labeled restriction fragments, using a *Bam*HI site which is internal to the PHS gene, as suggested by calculations based on the coding capacity of the cloned sequence. The PHS subunit has a molecular weight of 88,000. It can be estimated that 2.38 kb of DNA are required to code for a protein of that size. Thus, the cloned 2.45-kb fragment is just large enough to code for the PHS subunit. Since the *Bam*HI site is some distance away from the ends of the cloned fragment and this fragment apparently codes for the full-length PHS subunit (10), it seems almost certain that the restriction site in question is internal to the PHS gene. Further evidence in support of this contention is provided by coupled transcription-translation experiments with *Bam*HI-digested pIJ2501. Although the intact plasmid serves as a very efficient template for synthesis of the PHS subunit in a streptomycete coupled system (10), the *Bam*HI-restricted plasmid does not direct the synthesis of the protein, even though the synthesis of other polypeptides (including the putative rRNA methylase, which specifies thiostrepton resistance) can be detected with that template (data not shown). Thus, *Bam*HI digestion abolishes the ability of the cloned 2.45-kb insert to direct PHS synthesis in vitro.

For these experiments, a pBR322 derivative containing the PHS structural gene (pIJ2505 [10]) was employed. The large and small (1.41- and 1.04-kb) *Bam* fragments were prepared from pIJ2505 and end labeled as described above. The labeled probes were then hybridized by dot blotting to RNA isolated from 24-h *S. antibioticus* cultures. The DNA strand which is the template for transcription will hybridize to the *S. antibioticus* RNA. Since the 5'-end-labeled fragments were generated from a restriction site which is internal to the PHS gene (Fig. 2), the direction of transcription must be 5' → 3', starting at the end of the gene which is opposite to the 5'-labeled end of the hybridizing restriction fragment. The 5'-end-labeled, 1.41-kb *Bam* fragment hybridized to the *S. antibioticus* RNA, whereas the 1.04-kb fragment did not (Fig. 3). Thus, the direction of transcription of the PHS gene is from right to left, given the orientation of the gene shown in Fig. 2. Figure 3 also shows the direction of transcription of the gene in *S. lividans* transformed with pIJ2501 (RNA dot labeled 2501 in Fig. 3) and in *S. lividans* transformed with pIJ2503 (the pIJ702 derivative containing the PHS gene in the orientation opposite to that shown in Fig. 2). In each case, transcription was initiated at the same end of the PHS gene.

**Low-resolution S1 nuclease mapping and Northern blotting of PHS mRNA.** Once the direction of transcription of the PHS gene had been established, it was of interest to determine whether the initiation site for transcription was contained within the cloned 2.45-kb PHS structural gene. The small size of the cloned fragment relative to the amount of DNA required to code for a protein with an $M_r$ of 88,000 (about 2.38 kb) made it likely that the transcription start site for the gene would be located near or outside the rightmost *Sph*I site bounding the PHS gene (Fig. 2). Low-resolution S1 nuclease mapping was performed to confirm this hypothesis. Total RNA was isolated from 12-h *S. antibioticus* cultures and hybridized to a 3.01-kb restriction fragment produced by *Sal*GI digestion of pIJ2501 (the fragment extends from the rightmost *Sal*GI site in the cloned PHS gene to the rightmost *Sal*GI site shown in Fig. 2 for pIJ702). Conditions for hybridization and S1 nuclease digestion were essentially as described by Favaloro et al. (5). S1 nuclease-resistant hybrids were fractionated on an alkaline agarose gel, transferred to nitrocellulose, and hybridized to nick-translated pIJ2505 (see above). Typical results are shown in Fig. 4. Lane 1 of Fig. 4 shows the products of a reaction mixture from which RNA was omitted; thus, the observed bands are presumably reannealing artifacts. Lane 2 shows the products obtained from a reaction containing *S. antibioticus* RNA, and it is apparent that a hybrid band is present in lane 2 (see star) which is absent from the control products shown in lane 1. The hybrid band has an estimated size of 1.27 kb. The distance from the rightmost *Sal* site in the PHS gene to the *Sph*I site, which bounds the gene, is 1.32 kb (Fig. 2). Thus, the transcription start site might lie within the cloned PHS gene, but if this is so, it must be fairly close to the rightward *Sph*I site shown in Fig. 2.

The size of the PHS mRNA was examined by Northern blotting. Total RNA from 12-h galactose- and glucose-grown *S. antibioticus* cultures was denatured (21) and fractionated by agarose gel electrophoresis. After transfer to nitrocellulose, the RNA blots were hybridized to nick-translated pIJ2505 (Fig. 5). The labeled probe hybridized to a single band on the RNA blots. That this band is the PHS mRNA is suggested by the observation that this band was present in lower concentrations in glucose-grown cultures than in galactose-grown cultures (see below). This band was also eliminated by prior treatment of the RNA sample with heated RNase (data not shown). Of greatest interest with regard to the data in Fig. 5 is the apparent size of the putative

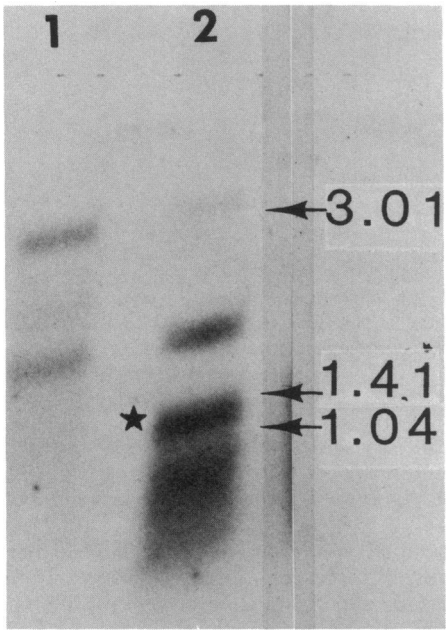


FIG. 4. Low-resolution S1 nuclease mapping of the PHS gene. Hybridization and digestion conditions were as described by Favaloro et al. (5) and in the text. Lane 1, S1 nuclease-resistant hybrids formed in the absence of added RNA; lane 2, hybrids formed in the presence of the 3.01-kb *Sal* fragment derived from pIJ2501. Standards electrophoresed in parallel were: 3.01, the 3.01-kb *Sal* fragment; 1.41 and 1.04, the *Bam-Sph* fragments from the PHS gene produced by double digestion of pIJ2501.

PHS message. It can be seen that the hybridizing band migrated well behind the 16S and 23S RNA markers in these experiments. This result suggests the interesting possibility that the PHS mRNA is polycistronic, although explanations related to mRNA structure cannot be eliminated at this time.

**Regulation of synthesis of PHS mRNA.** RNA dot blotting experiments were performed to examine possible transcriptional regulation of PHS synthesis in *S. antibioticus*. In these experiments, cultures were grown in actinomycin production medium containing galactose as a carbon source, and mycelium was harvested at 6, 12, 18, 24, and 48 h postinoculation. RNA was isolated from the mycelium and from mycelium grown on NZ-amine medium, in which actinomycin synthesis does not take place. RNA was denatured and spotted on nitrocellulose, and blots were hybridized to nick-translated pIJ2505 (Fig. 6). The zero time (NZ-amine RNA) points shown in Fig. 6 demonstrate the efficacy of DNase treatment in eliminating contaminating DNA from the RNA preparations. In the row containing RNA from galactose-grown cultures, an RNA preparation which had not been treated with DNase was spotted at the zero-time position, whereas in the row containing RNA from glucose-grown cultures, the same RNA preparation was treated with DNase, as described above, before being spotted at the zero-time position. It can be seen that no hybridization to the labeled probe occurred after treatment of RNA from NZ-amine grown cultures with DNase. This result was expected, since NZ-amine cultures contain barely detectable levels of PHS (see Fig. 7).

The levels of hybrid formation observed in Fig. 6 were quantitated by densitometry (Fig. 7B). The change in PHS specific activity over the course of the experiment is depicted in Fig. 7. It can be seen that, between 0 and 12 h postinoculation, there was an increase in the amount of PHS mRNA present in the total RNA population from galactose-grown *S. antibioticus* cells, and the increase in mRNA concentration corresponded to an increase in PHS specific activity (Fig. 7A). Between 12 and 24 h, the concentration of PHS mRNA declined, even though PHS specific activity continued to increase during this time. A possible explanation for this apparent inconsistency is provided below. The level of PHS mRNA stabilized between 24 and 48 h at a concentration which was about 30% of the maximum observed at 12 h in galactose-grown cultures (Fig. 7B).

The data in Fig. 7 also provide an explanation for the ability of glucose to repress PHS production. It can be seen that PHS specific activity was lower in glucose-grown cultures than in galactose-grown cultures at all time points (Fig. 7A). This lowered specific activity reflects lower concentrations of PHS mRNA in the glucose-grown cultures between 0 and 18 h postinoculation. The concentration of PHS mRNA began to increase in the glucose-grown cultures after about 18 h of growth, a time when the glucose in the medium had presumably been utilized. It is significant that the general shape of the RNA concentration curve between 0 and 18 h for the galactose-grown culture was similar to that for the glucose-grown culture between 18 and 48 h. This observation suggests that growth on glucose simply retards the synthesis of PHS mRNA.

The data in Fig. 6 and 7 also provide an explanation for an observation made by Jones and Weissbach some years ago (12). In those experiments, it was observed that growth of *S. antibioticus* in the presence of FU led to the premature synthesis of PHS in the mycelium. Figures 6 and 7 demonstrate that this premature synthesis is the result of the synthesis of new PHS mRNA. In Fig. 7A, it can be seen that

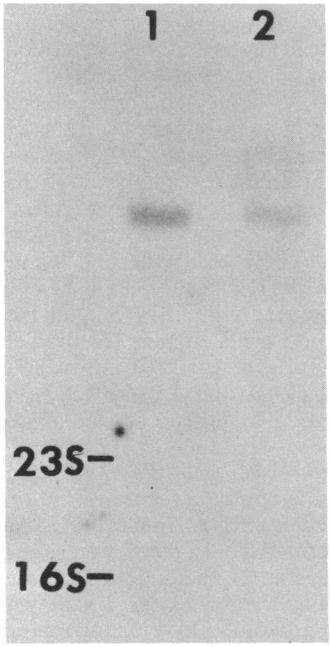


FIG. 5. Northern blotting of PHS mRNA. RNA samples (30 μg) were denatured and electrophoresed as described in the text. Lane 1, Hybridization of the labeled probe (pIJ2505) to RNA from 12-h galactose-grown *S. antibioticus* cells; lane 2, hybridization of the probe to RNA from 12-h glucose-grown cultures. The figure also shows the migration positions of the 16S and 23S rRNA species.

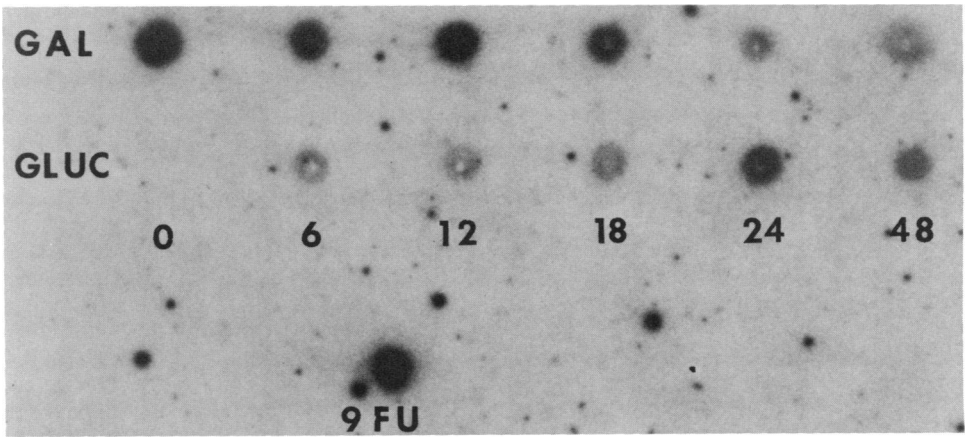


FIG. 6. Dot blotting of RNA from *S. antibioticus* cultures of various ages. RNA was isolated from growing *S. antibioticus* cultures as described in the text, and 30 μg of each RNA sample was spotted onto nitrocellulose. Blots were reacted with nick-translated pIJ2505 and subsequently exposed to X-ray film. GAL, RNA from galactose-grown cultures; GLUC, RNA from glucose-grown cultures. The numbers under each dot represent the age of the culture (in hours) at the time of harvest for RNA isolation. The strong signal from the zero-time GAL dot is explained in the text. The dot labeled 9FU represents RNA isolated from a culture which had been grown for 9 h in the presence of 20 μg of FU per ml.

the specific activity of PHS in a 9-h *S. antibioticus* culture grown in the presence of 20 μg of FU was comparable to the specific activity normally observed in an 18-h culture. Figure 7B shows that this increase reflects a corresponding increase in the concentration of PHS mRNA. In the experiment shown, the PHS specific activity in the FU-grown culture was about twice that in the control culture of the same age, whereas the concentration of PHS mRNA in the FU-grown culture was about 1.6 times that estimated for a 9-h culture grown in the absence of FU (Fig. 7).

**Stability of PHS in *S. antibioticus* cells.** It was somewhat surprising to discover (Fig. 7) that PHS specific activity increased in *S. antibioticus* cultures at a time when the concentration of PHS mRNA in those cultures was actually decreasing. Although this observation may reflect the operation of a translational control mechanism in the organism, there is an alternative explanation which was suggested by previous observations in this experimental system. Earlier work from my laboratory has demonstrated that there is a drastic decline in the rates of RNA and protein synthesis before the onset of actinomycin production in *S. antibioticus* (8). Further, experiments in which RNA polymerase was purified from young (non-actinomycin-producing) and old (actinomycin-producing) *S. antibioticus* cultures suggest

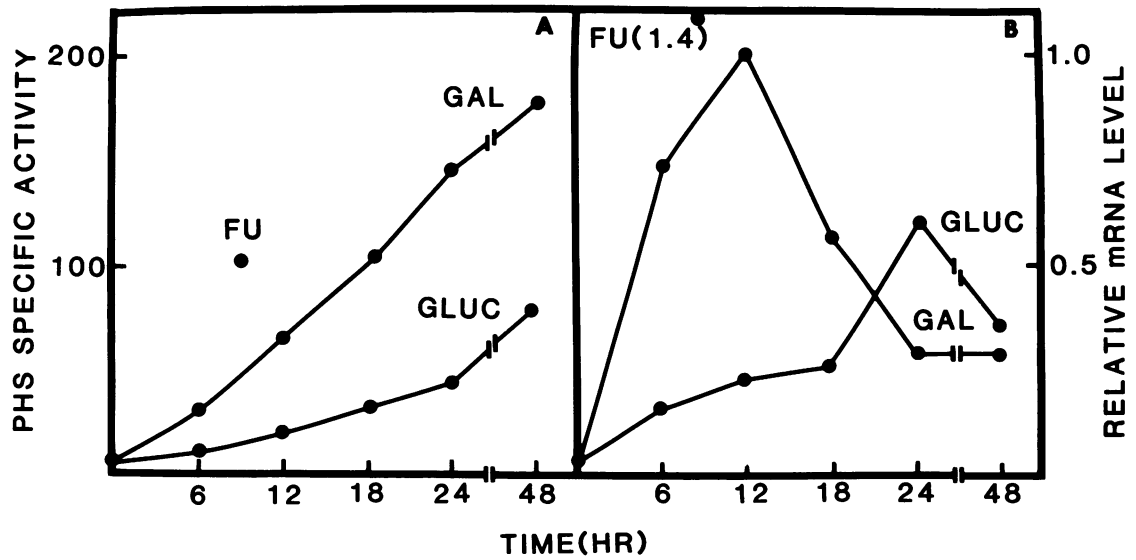


FIG. 7. (A) Change of PHS specific activity with time of growth of *S. antibioticus* in the presence of galactose (GAL) or glucose (GLUC). The enzyme assay was performed as described previously, and PHS specific activity is expressed as defined by Choy and Jones (3). The point labeled FU represents the PHS specific activity in the culture grown in the presence of FU (see legend to Fig. 6). (B) Densitometry of the dot blot shown in Fig. 6. Densitometric scanning was performed with a Joyce-Loebl densitometer. To generate the curves shown in panel B, the mRNA concentration in 12-h galactose-grown cultures was arbitrarily set at 1.0 and all other values were normalized to that concentration. The point at the very top of panel B represents the mRNA concentration in the FU-grown culture, which in this experiment had a relative value of 1.4. (Note that the FU point is plotted below the corresponding position on the vertical axis to fit it onto the graph.)

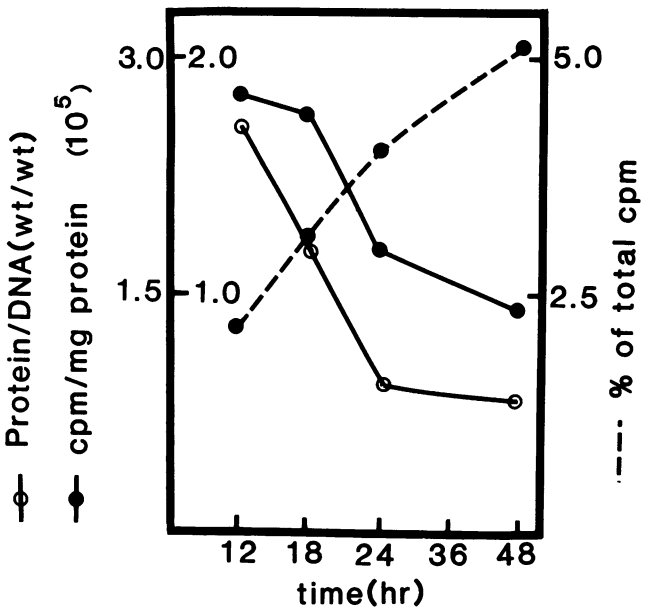


FIG. 8. Time course of changes in total protein and PHS labeling in *S. antibioticus* cultures. The culture was grown and labeled with [$^3$H]leucine as described in the text. Symbols: O——O, change in the ratio of protein to DNA (wt/wt) over the course of the experiment; ●——●, change in total radioactivity (counts per minute per milligram of protein); ●-----●, change in percentage of total protein at each sampling time which is PHS.

that there is a decrease in the ratio of mycelial protein to total mycelial mass as the cultures age (9). Studies from this laboratory have also shown that the native forms of PHS are extremely resistant to degradation by some proteases (M. E. Johnson, Ph.D. thesis, University of Michigan, Ann Arbor, 1982). Thus, the data suggest that mycelial protein is degraded during growth, but because of the decreased levels of RNA and protein synthesis, much of that protein is not resynthesized. The data in Fig. 7, then, could be explained if PHS were more stable than total cell protein and not degraded at the same rate as most cellular proteins.

To examine this possibility, I labeled an *S. antibioticus* culture with [$^3$H]leucine between 0 and 12 h postinoculation. A 30,000-fold excess of unlabeled leucine was then added, and growth was allowed to continue. Samples of the culture were removed at various times thereafter, the mycelium was disrupted, and the soluble extracts were analyzed by antibody precipitation for total radioactivity and radioactivity in PHS (Fig. 8). The results indicate that there indeed was a decrease in the ratio of protein to DNA in aging *S. antibioticus* cultures (Fig. 8, open circles). This result is consistent with the earlier observation suggesting a decreased protein content in the mycelium from actinomycin-producing cultures (9). The solid curve in Fig. 8 shows that the amount of radioactivity in total mycelial protein decreased over the course of the experiment, exactly as expected if the radioactive protein synthesized in the initial 12-h labeling period were degraded (the unlabeled leucine prevents the reincorporation of the released amino acid into protein). In contrast, the dashed curve in Fig. 8 shows that the percentage of the total radioactivity which was PHS actually increased over the course of the experiments. Thus, while total radioactive protein was being broken down, PHS appeared to be protected against degradation so that it increased in relative amount in the mycelium, even under conditions in which its synthesis may have been slowed.

## DISCUSSION

The results described in this report provide some interesting insights into the regulation of PHS production in *S. antibioticus*. Hybridization of end-labeled probes to *S. antibioticus* RNA showed that the PHS gene was transcribed from right to left (Fig. 2) and that, not unexpectedly, the direction of transcription in plasmid constructs containing the PHS gene was the same as that determined for the gene in *S. antibioticus*. S1 nuclease mapping showed that the transcription start site, if it lies within the cloned PHS gene, must be very close to the rightmost *Sph*I site, which bounds the cloned fragment (Fig. 2). Precise identification of the initiation and termination sites for transcription of the PHS gene must await the cloning of fragments which overlap the 5′ and 3′ ends of the gene; such experiments are in progress. There is an additional piece of evidence, however, which suggests that transcription may be initiated within the cloned 2.45-kb sequence. It has been shown that PHS activity is expressed at a high level, not only in *S. lividans* transformed with pIJ2501, but also in transformants containing pIJ2503, the plasmid bearing the cloned PHS gene in the orientation opposite to that of pIJ2501 (10). This result suggests the interesting possibility that the 2.45-kb sequence contains the PHS promoter. It can be estimated that about 2.38 kb of DNA is required to code for a protein with an $M_r$ of 88,000. The cloned fragment thus possesses some 70 to 80 bases more than are required to satisfy the coding function. These sequences might indeed house the signals required for the initiation of transcription.

It was surprising to observe that the size of the putative PHS mRNA apparently exceeds that required to code for the PHS subunit. This observation might simply reflect the fact that the RNA samples were not fractionated on denaturing gels or the possibility that the conditions used for denaturation of the RNA before electrophoresis were not sufficient to completely disrupt its secondary structure. The high guanine-plus-cytosine content of streptomycete DNA (7) makes this a possibility that cannot be ruled out at this time. If this is the correct explanation, the mobility of the RNA bands shown in Fig. 5 cannot reflect the true size of the mRNA. An alternative (and more interesting) explanation for the data in Fig. 5 would involve the possibility that the PHS message is polycistronic and bears the cistrons for other enzymes which are involved in the actinomycin biosynthetic pathway. Malpartida and Hopwood (17) have recently cloned the entire biosynthetic pathway leading to the biosynthesis of the antibiotic actinorhodin. Although the transcriptional analysis of the cloned sequences has not been published, it seems possible that such an analysis may also shed light on the question of the possible encoding of antibiotic biosynthetic genes with polycistronic mRNAs.

The data in Fig. 6, 7, and 8 demonstrate that PHS expression in *S. antibioticus* is controlled at both the transcriptional and posttranscriptional levels. The early appearance of the enzyme is the result of the synthesis of new PHS mRNA, whereas at later times it appears that the continued increase in PHS specific activity reflects, at least partially, the greater stability of PHS as compared with total cell protein. Thus, while the ratio of mycelial protein to total mycelial mass (or to total DNA; Fig. 8) is presumably decreasing in older cultures, the percentage of the total protein which is PHS (and thus, PHS specific activity) is increasing. If there is continued synthesis of PHS during this

time—and the data in Fig. 7B demonstrate the persistence of PHS mRNA in older cultures—this would also contribute to the increase in PHS specific activity depicted in Fig. 7A. It will be interesting to determine whether other enzymes in the actinomycin biosynthetic pathway are regulated in this fashion.

Figure 7 also provides an explanation for the ability of glucose to repress PHS synthesis in *S. antibioticus*. As in other catabolite-repressible operons (4), growth on glucose represses the synthesis of PHS mRNA. Unlike systems such as the *lac* operon of *Escherichia coli*, however, cAMP does not appear to mediate repression of actinomycin synthesis in *S. antibioticus*. Gallo and Katz (6) have shown that exogenous cAMP has no effect on the ability of *S. antibioticus* cultures to make PHS or actinomycin, and Chatterjee and Vining (2) have demonstrated that intracellular cAMP levels do not change appreciably in glucose-grown cultures after the glucose is exhausted and actinomycin synthesis begins. Thus, the intracellular mediator of the glucose effect in *S. antibioticus* remains to be identified.

The interesting effect of FU in stimulating premature synthesis of PHS in *S. antibioticus* cultures also reflects an action at the level of transcription. PHS specific activity is higher in 9-h FU-grown cultures than in control cultures of the same age, and the FU-grown cultures also contain higher concentrations of PHS mRNA (Fig. 7). In a previous report, it was suggested that growth on FU caused the inactivation of a repressor for the PHS gene (12). The availability of cloned probes for that gene should now make it possible to elucidate in molecular terms the details of the PHS regulatory system. Indeed, additional DNA sequences which may be involved in the regulation of PHS expression (and of actinomycin synthesis overall) have been cloned from the *S. antibioticus* genome (11). One way in which these sequences might function is as the equivalent of operators, regulating the PHS operon and perhaps others which might be involved in the actinomycin biosynthetic pathway.

## ACKNOWLEDGMENT

This work was supported by a grant from the Horace Rackham School of Graduate Studies, The University of Michigan.

## LITERATURE CITED

1. **Ceriotti, B.** 1952. A microchemical determination of desoxyribonucleic acid. J. Biol. Chem. **198**:297–303.
2. **Chatterjee, S., and C. Vining.** 1982. Intracellular adenosine triphosphate and cyclic adenosine 3′,5′-monophosphate concentrations during derepression of actinomycin biosynthesis. Can. J. Microbiol. **28**:1396–1399.
3. **Choy, H. A., and G. H. Jones.** 1981. Phenoxazinone synthase from *Streptomyces antibioticus*: purification of the large and small enzyme forms. Arch. Biochem. Biophys. **211**:55–65.
4. **de Crombrugghe, B., S. Busby, and H. Buc.** 1984. Cyclic AMP receptor protein: role in transcription activation. Science **224**:831–838.
5. **Favaloro, J., R. Treisman, and R. Kamen.** 1980. Transcription maps of polyoma virus-specific RNA: analysis by two-dimensional nuclease S1 gel mapping. Methods Enzymol. **65**:718–749.
6. **Gallo, M., and E. Katz.** 1972. Regulation of secondary metabolite biosynthesis: catabolite repression of phenoxazinone synthase and actinomycin formation by glucose. J. Bacteriol. **109**:659–667.
7. **Gladek, A., and J. Zakrzewska.** 1984. Genome size of *Streptomyces*. FEMS Microbiol. Lett. **24**:73–76.
8. **Jones, G. H.** 1976. RNA synthesis in *Streptomyces antibioticus*: in vitro effects of actinomycin and transcriptional inhibitors from 48-h cells. Biochemistry **15**:3331–3341.
9. **Jones, G. H.** 1979. Purification of RNA polymerase from actinomycin producing and nonproducing cells of *Streptomyces antibioticus*. Arch. Biochem. Biophys. **198**:195–204.
10. **Jones, G. H., and D. A. Hopwood.** 1984. Molecular cloning and expression of the phenoxazinone synthase gene from *Streptomyces antibioticus*. J. Biol. Chem. **259**:14151–14157.
11. **Jones, G. H., and D. A. Hopwood.** 1984. Activation of phenoxazinone synthase expression in *Streptomyces lividans* by cloned DNA sequences from *Streptomyces antibioticus*. J. Biol. Chem. **259**:14158–14164.
12. **Jones, G. H., and H. Weissbach.** 1970. RNA metabolism in *Streptomyces antibioticus*: effects of 5-fluorouracil on the appearance of phenoxazinone synthase. Arch. Biochem. Biophys. **137**:558–573.
13. **Katz, E., C. J. Thompson, and D. A. Hopwood.** 1983. Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. J. Gen. Microbiol. **129**:2703–2714.
14. **Katz, E., and H. Weissbach.** 1961. Studies on the biosynthesis of actinomycin: enzymatic synthesis of the phenoxazinone chromaphore. J. Biol. Chem. **236**:PC16–PC18.
15. **Katz, E., and H. Weissbach.** 1982. Biosynthesis of the actinomycin chromaphore; enzymatic conversion of 4-methyl-3-hydroxyanthranilic acid to actinocin. J. Biol. Chem. **237**: 882–886.
16. **Kirby, K. S., E. Fox-Carter, and M. Guest.** 1967. Isolation of deoxyribonucleic acid and ribosomal ribonucleic acid from bacteria. Biochem. J. **104**:258–262.
17. **Malpartida, F., and D. A. Hopwood.** 1984. Molecular cloning of the whole biosynthetic pathway of a *Streptomyces* antibiotic and its expression in a heterologous host. Nature (London) **309**:462–464.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Marshall, R., B. Redfield, E. Katz, and H. Weissbach.** 1968. Changes in phenoxazinone synthase activity during the growth cycle of *Streptomyces antibioticus*. Arch. Biochem. Biophys. **123**:317–323.
20. **Tautz, D., and M. Renz.** 1983. An optimized freeze-squeeze method for the recovery of DNA fragments from agarose gels. Anal. Biochem. **132**:14–19.
21. **Thomas, P.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. **77**:5201–5205.
22. **Troost, T., and E. Katz.** 1979. Phenoxazinone biosynthesis: accumulation of a precursor, 4-methyl-3-hydroxyanthranilic acid, by mutants of *Streptomyces parvulus*. J. Gen. Microbiol. **111**:121–132.