Actinomycin Production Persists in a Strain of *Streptomyces antibioticus* Lacking Phenoxazinone Synthase

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Truncated fragments of the phenoxazinone synthase gene, *phsA*, were prepared by the PCR. The resulting fragments were cloned into conjugative plasmid pKC1132 and transferred to *Streptomyces antibioticus* by conjugation from *Escherichia coli*. Two of the resulting constructs were integrated into the *S. antibioticus* chromosome by homologous recombination, and each of the resulting strains, designated 3720/pJSE173 and 3720/pJSE174, contained a disrupted *phsA* gene. Strain 3720/pJSE173 grew poorly, and Southern blotting suggested that genetic changes other than the disruption of the *phsA* gene might have occurred during the construction of that strain. Strain 3720/pJSE174 sporulated well and grew normally on the medium used to prepare inocula for antibiotic production. Strain 3720/pJSE174 also grew as well as the wild-type strain on antibiotic production medium containing either 1 or 5.7 mM phosphate. Strain 3720/pJSE174 was shown to be devoid of phenoxazinone synthase (PHS) activity, and PHS protein was undetectable in this strain by Western blotting. Despite the absence of detectable PHS activity, strain 3720/pJSE174 produced slightly more actinomycin than did the wild-type parent strain in medium containing 1 or 5.7 mM phosphate. The observation that strain 3720/pJSE174, lacking detectable PHS protein or enzyme activity, retained the ability to produce actinomycin supports the conclusion that PHS is not required for actinomycin biosynthesis in *S. antibioticus*.

The actinomycins are chromopeptide antibiotics produced by a number of *Streptomyces* strains and by some strains of *Micromonospora* (14, 17). In actinomycin D, the pentapeptide chains contain several methylated amino acids and one D-amino acid, but it was shown some years ago that the L-forms of the relevant amino acids serve as precursors for the synthesis of the forms found in the antibiotic (19). These pioneering studies by Katz and Weissbach also demonstrated that the chromophore of actinomycin D, a phenoxazinone ring, is derived from the catabolism of tryptophan (19). Thus, 3-hydroxykynurenine is converted to 3-hydroxyanthranilic acid, and the latter intermediate is methylated to form 4-methyl-3-hydroxyanthranilic acid, the precursor of the phenoxazinone ring (28).

The enzymes required for the methylation of 3-hydroxyanthranilic acid, for the activation of the chromophore precursor, and for the activation of the amino acids in the pentapeptide chains and their incorporation into those chains have been isolated and characterized (7, 22, 23). Schauwecker and co-workers have recently cloned the gene cluster for actinomycin production from *Streptomyces chrysomallus* (27). Thus, many of the biochemical and molecular genetic details regarding the biosynthesis of actinomycin have been elucidated. One unanswered question regards the synthesis of the actinomycin chromophore. Some years ago, Katz and Weissbach (18) identified an enzyme in *Streptomyces antibioticus* that catalyzes the oxidative condensation of 2-aminophenol derivatives to produce phenoxazinones (Fig. 1). This enzyme, phenoxazinone synthase (PHS), was subsequently purified to homogeneity (5), and its gene has been cloned and sequenced (11, 13). PHS production in *S. antibioticus* is subject to glucose repression, as is overall actinomycin production (9, 12), and this observation, along with the catalytic activity of PHS, suggested strongly that

PHS was the enzyme responsible for the production of the actinomycin chromophore. However, PHS has been isolated only from *S. antibioticus*. Repeated attempts to identify the enzyme in other actinomycin producers have been unsuccessful (16).

It was therefore important to determine definitively whether PHS is required for the production of actinomycin in the one actinomycin-producing organism in which it has been identified, *S. antibioticus*. To this end, the *phsA* gene, encoding the PHS subunit, has been disrupted, and the properties of the resulting disruptant strain have been analyzed. This strain lacks detectable PHS activity and PHS protein. Despite the absence of detectable PHS, this strain produces actinomycin as effectively as the wild type. The results lead to the conclusion that PHS is not required for actinomycin production in *S. antibioticus*.

MATERIALS AND METHODS

Growth of organisms. The strains and plasmids used or constructed in this study are described in Table 1. *S. antibioticus* IMRU 3720 was grown on NZ-amine and galactose-glutamic acid (GGA) media as described previously (5, 9). *Escherichia coli* strains XL-1 Blue (Stratagene, La Jolla, Calif.) and ET12567/pUB307 (8) were grown in L broth containing antibiotics as necessary. Conjugation mixtures were plated on SFM agar (8).

Construction of strains containing a disrupted *phsA* gene. Primers were prepared from the sequence of the *phsA* gene (11), and PCRs were performed to produce fragments truncated at their 5′ and 3′ ends as indicated in Fig. 2. The PCR primers were designed to contain *Eco*RI sites to facilitate cloning into *Eco*RI-digested pKC1132, a 3.5-kb conjugative plasmid bearing an apramycin resistance gene (2) (Table 1). The relevant ligation mixtures were used to transform *E. coli* XL-1 Blue. Transformants containing the desired recombinant plasmids were identified, and the plasmids were isolated and used to transform *E. coli* ET12567/pUB307 (8). ET12567/pUB307 is a conjugative strain of *E. coli* in which the transfer functions and a kanamycin resistance (Kan^r) gene are borne by plasmid pUB307 (8). Appropriate transformants were then used for conjugation with *S. antibioticus* spores as described previously (10, 15). Plates containing putative exconjugants were overlaid with apramycin. As pKC1132 cannot replicate in *Streptomyces*, apramycin-resistant exconjugants could be produced only by homologous recombination between the inserts of the plasmid derivatives used for conjugation and the *phsA* gene in the *S. antibioticus* chromosome. *S. antibioticus* IMRU 3720 produces the pigment melanin, and this property was used to identify putative exconjugants on SFM agar. Melanin-producing colonies appeared approximately 7 days following plating. These colonies were streaked

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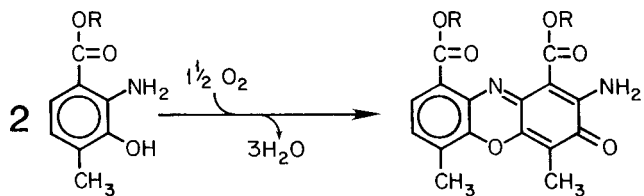
FIG. 1. The reaction catalyzed by phenoxazinone synthase. The reaction involves the oxidative condensation of 2-aminophenols and their derivatives, the reactants shown in the figure, to produce the phenoxazinone ring.

on GGA agar, and the resulting cultures were allowed to sporulate. Spores were then isolated from the plates and streaked on GGA agar containing apramycin (50 μg/ml). These plates were incubated until spores were formed, and the spores were isolated and used for the studies described herein. Exconjugants were obtained from only two of the conjugations. The strain containing the integrated plasmid bearing the 1,688-bp fragment of *phsA* was designated 3720/pJSE173, and the strain containing the integrated 1,325-bp fragment was designated 3720/pJSE174.

Preparation of mycelial extracts and assays for PHS. *S. antibioticus* mycelium was harvested from GGA cultures 12, 24, 48, 72, and 96 h postinoculation. Mycelium from 10-ml portions of relevant cultures was disrupted by sonication in 1 ml of a buffer composed of 50 mM Tris-HCl (pH 7.5), 5% glycerol, and Complete protease inhibitor cocktail at the concentration specified by the supplier (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Mycelial debris was removed from sonic extracts by centrifugation for 5 min at $12,000 \times g$ and 4°C. The PHS assay was performed as described previously (5), except that the total reaction volume was 1.0 ml, the final concentration of sodium acetate used was 50 mM, and the substrate was 2-aminophenol. An extinction coefficient of 23,200 was used to calculate concentrations of 2-aminophenoxazinone (1). The protein concentration was determined with dye-binding assay reagent (Bio-Rad, Hercules, Calif.) and bovine serum albumin as the standard. We followed the protocol specified by Bio-Rad.

Miscellaneous procedures. Southern blotting was performed as described previously (13) with the cloned *phsA* gene as a probe. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed essentially as described by Laemmli (24). PAGE samples were boiled for 15 min in sample buffer prior to electrophoresis. Following electrophoresis, proteins were electroblotted to Hybond P membranes (Amersham Pharmacia Biotech, Piscataway, N.J.). Western blotting was performed with a Bio-Rad Opti-4CN kit and a 1:1,000 dilution of anti-PHS antibody. Actinomycin concentrations were estimated by extracting culture media with ethyl acetate. The absorbance of the extracts was then determined at 452 nm, and actinomycin concentrations were

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Properties	Reference or source
Strains		
S. antibioticus IMRU 3720	Actinomycin-producing wild-type strain	9
E. coli XL-1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* (F′ *proAB lacI*q *Z*Δ*M15* Tn*10*) (Tet[r])	Stratagene
E. coli ET12567/ pUB307	*dam-13*::Tn*9 dcm-6 hsdM*	8
Plasmids		
pUB307	Conjugative plasmid bearing a Kan[r] gene	8
pKC1132	Conjugative plasmid bearing Apr[r] *rep*[pUC]	2
pJSE173	pKC1132 bearing 1,688-bp fragment of *phsA*	This study
pJSE174	pKC1132 bearing 1,325-bp fragment of *phsA*	This study
pJSE175	pKC1132 bearing 1,032-bp fragment of *phsA*	This study
pJSE176	pKC1132 bearing 735-bp fragment of *phsA*	This study

calculated with an extinction coefficient of 24,800 (18). To label actinomycin, 5-ml portions of 72-h cultures were incubated for 60 min with 5 μCi of [*methyl-*^{14}C]methionine. At the end of the incubation, the reaction mixtures were extracted with ethyl acetate, the extracts were evaporated to dryness, and the resulting residues were dissolved in methanol. Radioactive products were separated by thin-layer chromatography in ethyl acetate-methanol-water (100:5:5 [vol/vol/vol]). Plates were subjected to autoradiography.

RESULTS

Construction and characteristics of strains 3720/pJSE173 and 3720/pJSE174. Although gene disruption by gene replace-
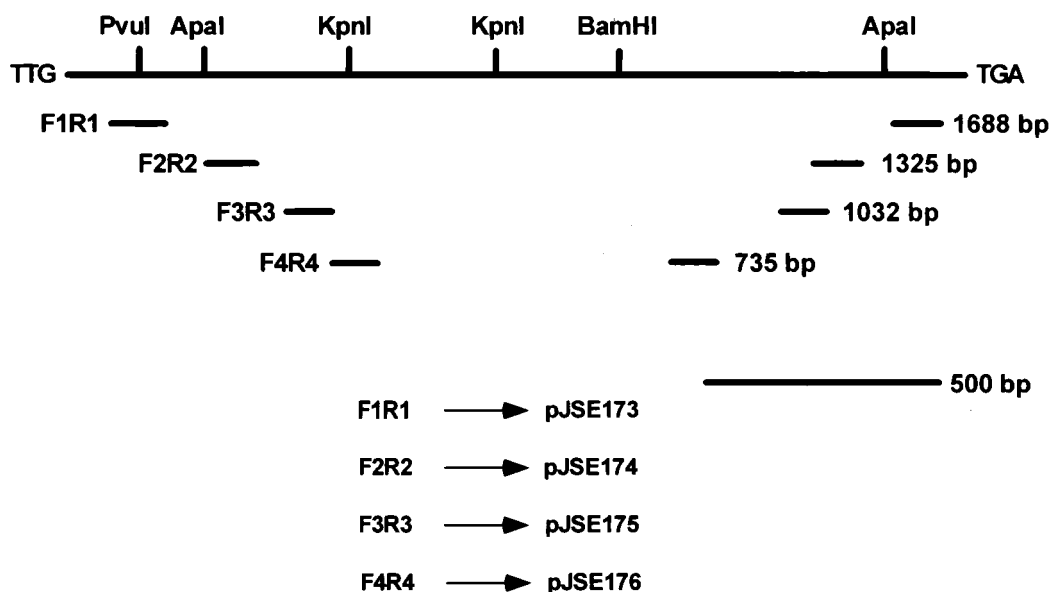

FIG. 2. Partial restriction map of the *phsA* gene and positions of the primers used to generate PCR fragments for mutational cloning. The sizes of the PCR primers are not shown to scale. The numbers to the right of the primers, at the 3′ end of the gene, represent the sizes of the fragments generated by each primer pair. pJSE173 and other plasmid designations represent the plasmids obtained by cloning the PCR fragments into pKC1132.
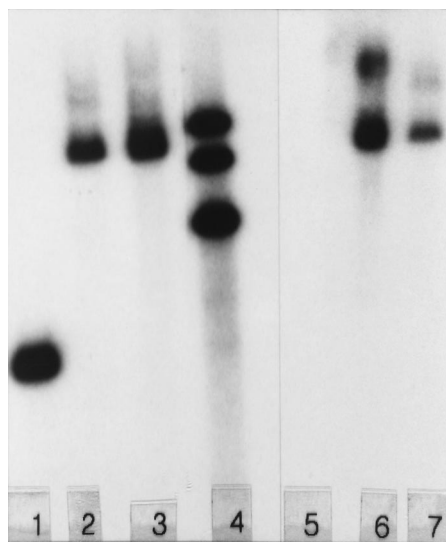
FIG. 3. Southern blot of digests of chromosomal DNAs from *S. antibioticus* strains IMRU 3720, 3720/pJSE173, and 3720/pJSE174. Approximately 2 μg of DNA was digested with *Sph*I. The filter containing the digests depicted in lanes 1 to 3 was probed with the radioactively labeled *phsA* gene, while the digests in lanes 5 to 7 were probed with pKC1132. Lanes 1 and 5, IMRU 3720 DNA; lanes 2 and 6, DNA from 3720/pJSE173; lanes 3 and 7, DNA from 3720/pJSE174. Lane 4 contains a set of markers (9.8, 6.3, and 4.4 kb).

ment was used effectively to inactivate the *relA* gene in *S. antibioticus* (10), it was not possible in initial experiments to disrupt the *phsA* gene by this technique. Thus, attempts were made to disrupt the *phsA* gene by using the technique of

mutational cloning (4). As indicated in Materials and Methods, four PCR products, each representing forms of *phsA* truncated at the 5′ and 3′ ends (Fig. 2), were cloned into conjugative plasmid pKC1132 (2). *E. coli* strains containing the four plasmids were conjugated with *S. antibioticus*, and two of the plasmids, pJSE173 and pJSE174, were successfully transferred via a single crossover to the *S. antibioticus* chromosome by homologous recombination with the resident *phsA* gene. No exconjugants were obtained when the other two plasmids were used, presumably because the cloned fragments were too small to support recombination in *S. antibioticus*. The presence of the relevant plasmids in the chromosomes of the disruptant strains was verified by Southern blotting (Fig. 3). In the experiments depicted, chromosomal DNAs were digested with *Sph*I. As pKC1132 contains no *Sph*I site, digestion with this enzyme should produce only one band containing the wild-type or disrupted *phsA* gene. Lanes 2 and 3 of Fig. 3 show that the 2.3-kb *phsA* band (lane 1) is replaced by a band of the expected size (ca. 7 kb) in strains 3720/pJSE173 and 3720/pJSE174, respectively. Lanes 6 and 7 of Fig. 3 show that this band hybridizes to cloning vector pKC1132, while chromosomal DNA from the wild-type *S. antibioticus* strain (lane 5) does not. Thus, the *phsA* gene was disrupted in strains 3720/pJSE173 and 3720/pJSE174.

As shown in Fig. 3, lane 6, pKC1132 hybridized strongly to a second band in the digest of DNA from strain 3720/pJSE173. This observation, coupled with the fact that the strain grew very poorly on actinomycin production medium (GGA) (data not shown), suggested that changes in addition to the disruption of the *phsA* gene occurred during the construction of this strain. Moreover, restoring an intact copy of the *phsA* gene to this strain using either high- or low-copy-number plasmids (21, 25) had no effect on growth or on the ability of the strain to
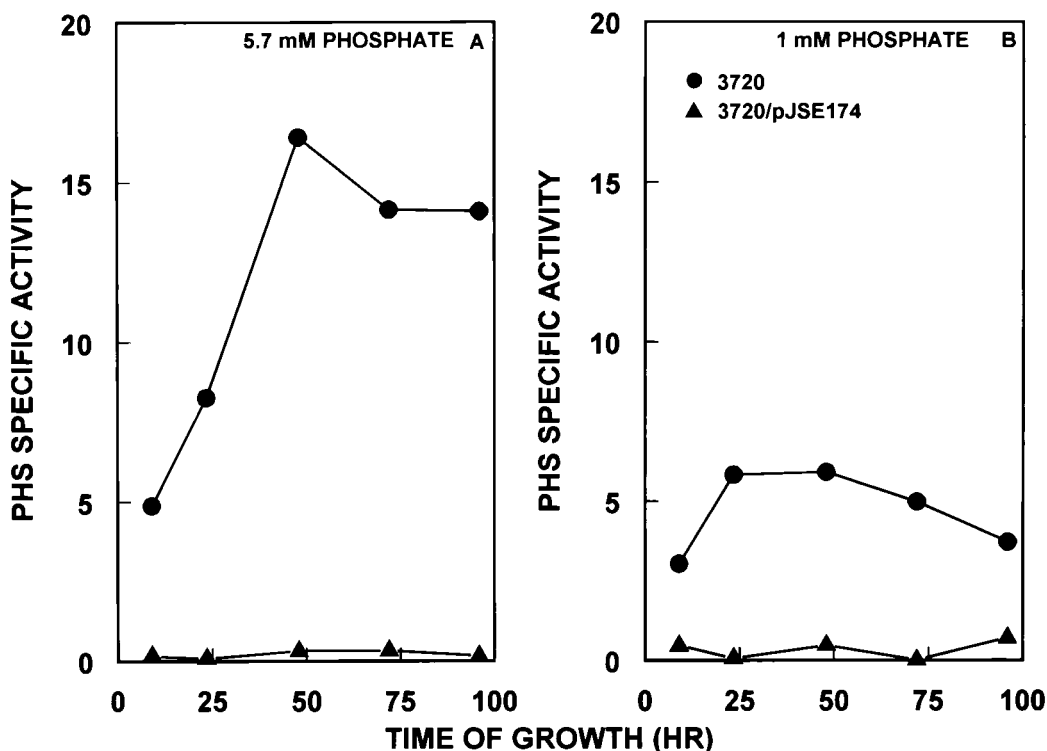

FIG. 4. PHS activity in *S. antibioticus* strains IMRU 3720 and 3720/pJSE174. PHS assays were performed as described in Materials and Methods. Results are expressed as nanomoles of 2-aminophenoxazinone formed per minute per milligram of protein under the assay conditions described in the text.
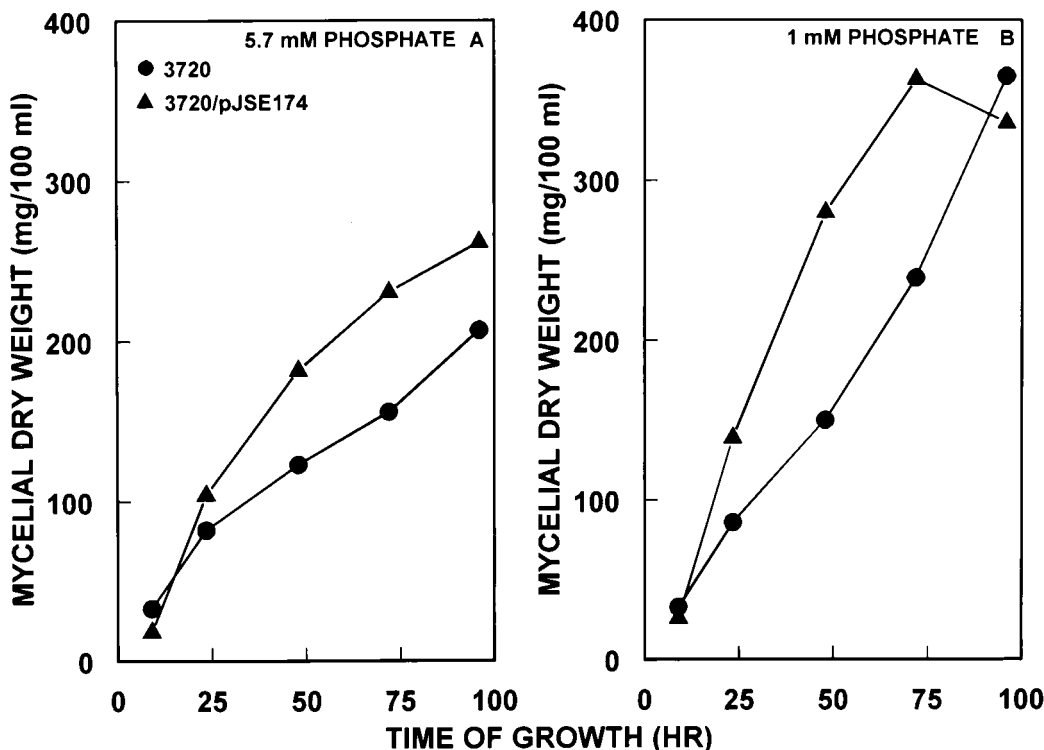
FIG. 5. Growth of *S. antibioticus* IMRU 3720, 3720/pJSE173, and 3720/pJSE174 on GGA medium at different phosphate concentrations. Growth and assay conditions were as described in Materials and Methods. Apramycin was included in NZ-amine medium, used to prepare inocula for growth on GGA medium, but the antibiotic was not included in the latter medium.

produce actinomycin. Therefore, subsequent experiments reported below were done with only strain 3720/pJSE174. Strain 3720/pJSE174 sporulated well on GGA agar and SFM agar; spores of this strain appeared normal to the unaided eye. The strain grew vigorously on NZ-amine medium, the medium used to prepare inocula for antibiotic production.

Strain 3720/pJSE174 lacks PHS. Because pJSE174 contains a truncated form of *phsA*, integration of this plasmid via homologous recombination into the *S. antibioticus* chromosome should inactivate the resident *phsA* gene. Strain 3720/pJSE174 was grown on actinomycin production medium (GGA), and mycelial samples were removed periodically to assay for PHS. Because previous studies suggested the possibility that both PHS activity and actinomycin production are regulated by phosphate (10; unpublished results), analyses were performed on cultures grown in either 1 or 5.7 mM phosphate. As shown in Fig. 4, extracts of the wild-type strain contained significant levels of PHS activity, as has been documented previously (18). The maximum specific activity in cultures grown in either 1 or 5.7 mM phosphate was achieved ca. 48 h postinoculation. It is interesting to note, however, that the maximum specific activity observed was about threefold lower in extracts of cultures grown in 1 mM phosphate (Fig. 4B) than in extracts of cultures grown in 5.7 mM phosphate (Fig. 4A). The data in Fig. 4 further indicate that no detectable PHS activity was present in strain 3720/pJSE174, containing a disrupted form of the *phsA* gene.

It seemed possible that, despite the absence of detectable levels of PHS activity in 3720/pJSE174, the disruptant strain might still produce PHS protein. To explore this possibility, the extracts used for the experiments shown in Fig. 4 were fractionated by SDS-PAGE and analyzed by Western blotting with

antibody to PHS. The results of such an analysis performed with two different concentrations of extract protein (20 and 50 µg) failed to reveal the presence of PHS protein in extracts of mycelia of strain 3720/pJSE174 (data not shown). Under the same conditions, PHS was easily detectable in the wild-type strain.

Growth characteristics of strains 3720/pJSE173 and 3720/pJSE174. The growth characteristics on antibiotic production medium (GGA) of the wild-type *S. antibioticus* strain and strain 3720/pJSE174 are shown in Fig. 5. Strain 3720/pJSE174 grew somewhat more vigorously than did the parental strain, *S. antibioticus* 3720, at both 1 mM and 5.7 mM phosphate.

Actinomycin production by the parental *S. antibioticus* strain and by strain 3720/pJSE174. Actinomycin production by the two strains of interest in this study was examined by two procedures. In the first, portions of the culture medium were extracted with ethyl acetate, a procedure shown previously to remove actinomycin preferentially from the growth medium (19). The amount of actinomycin present in the extracts was estimated spectrophotometrically. The results of this analysis are shown in Fig. 6. Levels of actinomycin produced by the parental *S. antibioticus* strain were low in medium containing 5.7 mM phosphate but significantly higher in medium containing 1 mM phosphate. Strain 3720/pJSE174 produced levels of actinomycin that were higher than or equal to those produced by the parental strain under both sets of conditions (Fig. 6).

The second method used to assess actinomycin production involved the incubation of mycelia (72 h postinoculation) with a radioactive precursor of actinomycin. Incubation mixtures were extracted with ethyl acetate, and the extracts were concentrated and analyzed by thin-layer chromatography. The chromatogram shown in Fig. 7 confirms that the parental *S.*
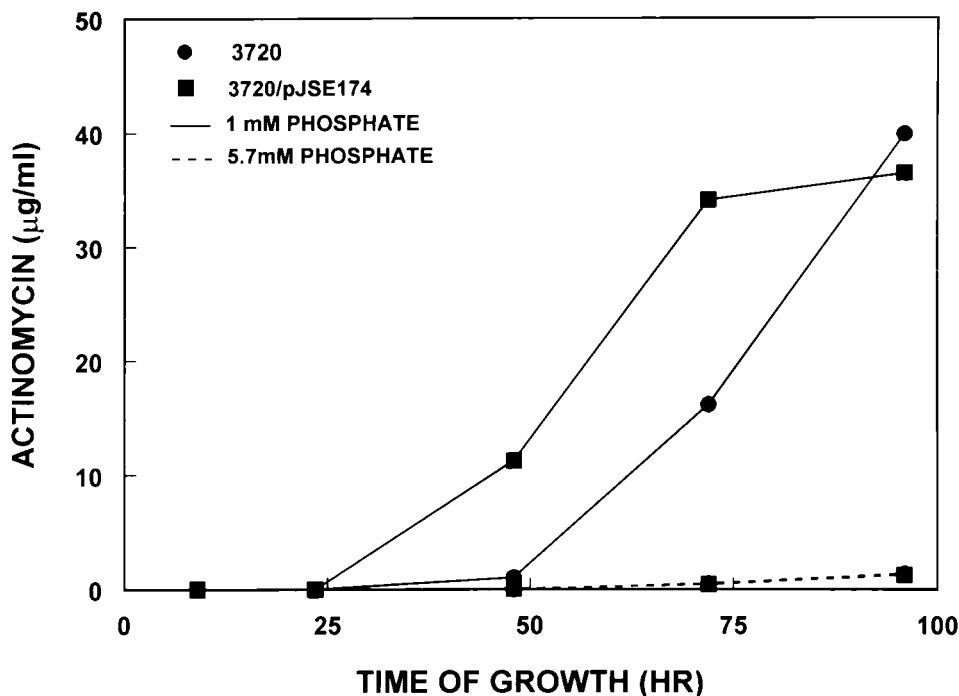
FIG. 6. Actinomycin production by strains IMRU 3720 and 3720/pJSE174. Actinomycin was assayed spectrophotometrically as described in Materials and Methods. Because of the scale used for this figure, the levels of actinomycin production by strains IMRU 3720 and 3720/pJSE174 in medium containing 5.7 mM phosphate appear identical, but strain 3720/pJSE174 produced slightly larger amounts of the antibiotic under these conditions.

antibioticus strain and strain 3720/pJSE174 produced authentic actinomycin.

DISCUSSION

The data presented above confirm the successful disruption of the PHS gene in *S. antibioticus*. While four plasmid constructs were prepared for mutational cloning, only two of these were successfully integrated into the *S. antibioticus* chromosome, presumably because the inserts in the other two recombinant constructs were too small to allow homologous recombination to occur. The disruption of the *phsA* gene and its consequences have implications for the mechanism of actinomycin production, discussed in greater detail below. It can also be concluded from the experiments described here that the *phsA* gene is not essential to the viability of *S. antibioticus*.

Strain 3720/pJSE174, produced by using a 1,325-bp truncated fragment of *phsA* (Fig. 2), sporulated well, grew well on NZ-amine and GGA media, and produced actinomycin in cultures containing either 1 or 5.7 mM phosphate. In general, strain 3720/pJSE174 was similar in its properties to the parental strain, except for the absence of detectable levels of PHS activity and PHS protein (Fig. 4 and data not shown). Because strain 3720/pJSE174 was capable of producing actinomycin in the absence of detectable PHS, it is reasonable to conclude that the *phsA* gene and the PHS enzyme are not required for actinomycin production in *S. antibioticus* IMRU 3720. Consistent with this conclusion is the observation, shown in Fig. 4 and 6, that conditions which produced the highest PHS specific activities in *S. antibioticus* mycelial extracts (growth at 5.7 mM phosphate) produced only low levels of actinomycin. By comparison, growth at 1 mM phosphate produced 3-fold lower PHS specific activities but up to 30-fold higher levels of actinomycin. That PHS is not required for actinomycin production does not mean that the wild-type strain does not use this

enzyme if it is available. It is known that PHS can utilize 4-methyl-3-hydroxyanthraniloyl peptides, suspected precursors of actinomycin in vivo, as substrates (20, 26).

At least two significant questions are raised by the results presented in this report. First, if PHS is not required for actinomycin production, what is it function in *S. antibioticus*? A possible answer to this question was suggested by comparisons of the amino acid sequence of the PHS subunit to those of proteins in the GenBank sequence database. PHS is quite similar to enzymes of the blue copper family (11), as it is a blue copper enzyme itself. Interestingly, PHS is also quite similar to the CotA protein of *Bacillus subtilis* (3). CotA is a component of *B. subtilis* spores and may play a role in the synthesis of a spore pigment (6). Because of this similarity between PHS and CotA, we examined *S. antibioticus* spores to determine whether they contain a PHS-like protein. These analyses showed that sonic extracts of purified spores contain a protein with the electrophoretic and immunological properties of the PHS subunit and significant amounts of a protein with a lower molecular weight. This protein, designated PHS*, is apparently produced by proteolysis of the PHS subunit (unpublished data). Thus, there is some evidence to suggest that PHS may be involved in the process of spore formation in *S. antibioticus*, and this may represent its major role in that organism. It is noteworthy that both PHS and PHS* are absent from the spores of strain 3720/pJSE174 (data not shown). This observation strongly suggests that PHS and PHS* are both products of the *phsA* gene. Studies are in progress to further elucidate the role of PHS in *S. antibioticus*.

The second question raised by the observations described above concerns the mechanism of actinomycin biosynthesis. If PHS is not responsible for the formation of the actinomycin chromophore, how is that moiety synthesized? There are at least two possibilities. There may be another enzyme elabo-
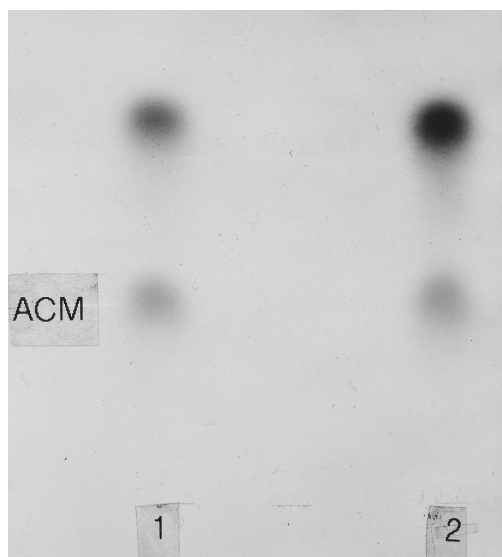
FIG. 7. Autoradiogram of a thin-layer chromatogram of neutral ethyl acetate-extracted products following incubation of strains IMRU 3720 (lane 1) and 3720/pJSE174 (lane 2) with [*methyl*-^{14}C]methionine. ACM, actinomycin.

rated by *S. antibioticus* and by other actinomycin producers that is capable of catalyzing the condensation of actinomycin half molecules to yield the antibiotic. Alternatively, the condensation may occur nonenzymatically, under the influence of pH and divalent cations. With regard to this possibility, it is perhaps noteworthy that the physical map of the actinomycin cluster from *S. chrysomallus* does not include a gene for the formation of the chromophore (27). Experiments are in progress to distinguish between the possibilities for the synthesis of the chromophore.

ACKNOWLEDGMENTS

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