Pleiotropic Effects of a *relC* Mutation in *Streptomyces antibioticus*

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Ochi (Agric. Biol. Chem. 51:829–835, 1987) has isolated a relaxed mutant of *Streptomyces antibioticus*, designated *relC49*. *relC49* accumulates significantly lower levels of ppGpp than the parent strain, IMRU3720. At its maximum, the ppGpp level in *relC49* was only one-fourth that observed in strain IMRU3720. Interestingly, a burst of ppGpp synthesis between 18 and 22 h of growth in IMRU3720 coincided with the onset of actinomycin production in that strain. As shown previously, the activity in protein synthesis of ribosomes from strain IMRU3720 decreases with the age of the culture. The decrease in activity was less pronounced in cultures of *relC49*. *relC49* mycelium contains reduced levels of phenoxazinone synthase, a key enzyme involved in actinomycin biosynthesis. The *rel* mutation prevents the normal increase in the activity of one of the other enzymes required for production of the antibiotic, 3-hydroxyanthranilate-4-methyltransferase, and a third enzyme, actinomycin synthetase I, appears to be completely absent from *relC49* mycelium. Levels of phenoxazinone synthase mRNA were examined by RNA dot blotting with the cloned phenoxazinone synthase gene as a probe. mRNA levels for phenoxazinone synthase were dramatically reduced in *relC49* compared with strain IMRU3720. These results are discussed in terms of the possible regulation of the onset of actinomycin production by ppGpp.

The stringent response to amino acid starvation in *Escherichia coli* results in the shutdown of stable RNA synthesis. The guanine derivative, ppGpp, has been shown to be an effector of the stringent response (reviewed in reference 2). Relaxed (*rel*) mutants of *E. coli* accumulate lower levels of ppGpp than wild-type cells and continue to synthesize stable RNAs under conditions of amino acid starvation. Ochi has isolated a relaxed mutant of *Streptomyces antibioticus* (16). That mutant displays a relaxed response under conditions of amino acid starvation and, most interestingly, is unable to make the antibiotic (actinomycin) normally produced by wild-type cultures. One goal of the study of mechanisms of antibiotic biosynthesis is to identify the biochemical effectors responsible for the onset of antibiotic production. The results from Ochi's laboratory suggest the interesting possibility that ppGpp may be one of the effector molecules which regulates the production of actinomycin. To further examine the characteristics of the relaxed mutant, we have assayed (i) the levels of ppGpp and other nucleotides in wild-type and mutant cultures grown in production medium, (ii) the activity in cell-free protein synthesis of ribosomes from the mutant and parent cultures, (iii) the levels of several enzymes involved in actinomycin biosynthesis in wild-type *S. antibioticus* and in the *rel* mutant, and (iv) the levels of mRNA for phenoxazinone synthase (PHS), an enzyme involved in actinomycin biosynthesis in *S. antibioticus*.

MATERIALS AND METHODS

Growth of organisms. *S. antibioticus* IMRU3720 and *relC49* were cultured from spores in NZ-amine and galactose-glutamic acid media as previously described (4). Mycelium was harvested 12, 24, 48, and 72 h postinoculation.

Measurement of mycelial weight and intracellular nucleotide concentrations.

At the times indicated in Fig. 1, samples (20 to 100 ml) were withdrawn from growing cultures of strain IMRU3720 or *relC49*. Samples were filtered and extracted as described previously (15). The intracellular concentrations of nucleotides were determined by high-pressure liquid chromatography (HPLC) after extraction with 1 N formic acid (15). Amounts are expressed relative to mycelium dry weight measured at the time of harvesting (i.e., picomoles/milligram [dry weight] of mycelium).

Preparation of ribosomes and activity assay. Ribosomes were prepared from 1 to 2 g of mycelium of the ages indicated above essentially as described previously (6). Conditions for cell-free protein synthesis were as previously described (6) by using poly(U) as the synthetic template and [³H]phenylalanine (Dupont NEN; 119 Ci/mmol). Soluble enzymes (6) in all cases were from 12-h mycelia of strain IMRU3720. The dependency of incorporation on ribosome concentration was determined, and reaction mixtures for the experiments were at concentrations of 12 A₂₆₀ units per ml of ribosomes (Fig. 1). Duplicate or triplicate reaction mixtures were incubated for 30 min at 37°C and processed as previously described (6).

Assays for actinomycin enzymes. After growth of mycelium for the periods indicated above in galactose-glutamic acid medium, mycelium was harvested, washed (4), and stored at –70°C until use. Usually about 1 g of mycelium was mixed with an equal weight of glass beads (Sigma; 150 to 212 μm) and suspended in 5 to 10 ml of 50 mM Tris-HCl (pH 7)–10% glycerol–1 mM phenylmethylsulfonyl fluoride. Mycelium was disrupted in an Omnimixer homogenizer at top speed for 3 min. The homogenizer cup was cooled in an ice-salt bath during the process. Homogenates were brought with Nonidet P-40 to 0.1% relative to the volume of buffer used in homogenization. After 20 min on ice, homogenates were centrifuged for 10 min at 12,000 × g.

To prepare extracts for actinomycin synthetase I (ASI) assays, 0.5 ml of a 70% suspension of DEAE-cellulose in homogenization buffer was sedimented in a 1.5-ml Eppendorf tube. The liquid was removed from the tube, and 450 μl

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FIG. 1. Changes in the intracellular concentrations of ppGpp and nucleoside triphosphates during the growth of *S. antibioticus* IMRU3720 (A) or *relC49* (B). Symbols: ●, GTP; ○, ATP; ■, UTP; □, CTP; Δ, cell dry weight. The pool sizes for each nucleotide at 17 h (3720) or 38 h (*relC49*), designated as 100%, were as follows (in picomoles per milligram): in 3720, GTP, 2,190; ATP, 13,200; UTP, 3,310; and CTP, 2,550; and in *relC49*, GTP, 810; ATP, 5,480; UTP, 1,700; and CTP, 1,640. The arrow indicates the time at which actinomycin production began. No pppGpp was detected in either 3720 or *relC49*.

of mycelial extract was added. The DEAE was suspended in the extract by gentle vortexing, and the suspension was left on ice for 15 min with occasional mixing. The DEAE was then sedimented by centrifugation for 1 min, and the supernatants were used in the ASI assays. DEAE treatment of the extracts was necessary in order to reduce the levels of 3-hydroxyanthranilate-independent ATP-PP_i exchange in the ASI assays.

The detergent-treated supernatants from the centrifugation at 12,000 × *g* were used without further treatment in PHS and 3-hydroxyanthranilate-4-methyltransferase (HAMT) assays. PHS and HAMT were assayed as described previously (4, 5), and ASI was assayed by measuring 3-hydroxyanthranilic acid (HAA)-dependent ATP-PP_i exchange essentially as described by Jones (7). ASI reaction mixtures contained 1.5 mM HAA and 5 μCi of ³²PP_iNa along with the components indicated by Jones (7). Specific activities were calculated as nanomoles per minute per milligram of protein (PHS and ASI) or picomoles per minute per milligram of protein (HAMT).

RNA preparation and dot blotting. Total RNA was prepared from 12- to 72-h mycelium (cultured as indicated above) as described previously (8). RNAs were spotted onto Nytran filters which were subsequently treated as previously described (8). The probe used in these experiments was the 2.45-kb *phs* gene, excised by *Sph*I digestion from pIJ2505, a pBR322 derivative containing that fragment (10). The nick-translated fragment was incubated for 48 h at 68°C, and filters were washed and exposed to X-ray film as described previously (8).

RESULTS

Effects of the *rel* mutation on growth, ppGpp, and other nucleotide levels in *S. antibioticus*. The *rel* mutant isolated by Ochi has been shown to contain an altered ribosomal protein (ST-L11 [19]), and by analogy with *E. coli* and *Bacillus*

subtilis mutants, has been designated as *relC*. Figure 1 shows the results of ppGpp and nucleotide pool assays on strains IMRU3720 and *relC49* over ca. 100 h of growth in antibiotic production medium. It is apparent that ppGpp levels in *relC49* were lower than those observed in IMRU3720 over the entire course of the experiment. Levels in *relC49* never exceeded 25% of the maximum ppGpp concentration in IMRU3720. Following the burst of ppGpp accumulation in strain IMRU3720 (see below), the GTP pool size decreased to 33 to 48% of the maximal value. Such a decrease was not observed in strain 49. Although the results obtained here are from experiments conducted using liquid cultures, it is possible that ppGpp could play a role in *S. antibioticus* in the initiation of aerial mycelium formation by effecting a reduction in the size of the intracellular GTP pool. Such a role for ppGpp has been suggested in *S. griseus* (17, 18, 20). *relC49* grows more slowly in galactose-glutamic acid medium than does strain IMRU3720 (Fig. 1).

Effects of the *rel* mutation on the activity of ribosomes from *S. antibioticus*. Previous studies on the biochemical changes which accompany actinomycin biosynthesis in *S. antibioticus* have demonstrated a decrease in the activity of ribosomes with increasing age of cultures grown on production medium (6). It was of interest to determine whether the *rel* mutation affected the activity of ribosomes in cell-free protein synthesis. To this end, ribosomes were prepared from 12-, 24-, 48-, and 72-h cultures of strains IMRU3720 and *relC49* grown on actinomycin production medium. Equal amounts of ribosomes (measured in A₂₆₀ units) were tested in poly(U)-dependent polyphenylalanine synthesis (Fig. 2). The data confirm the previous observations regarding the decrease in activity of ribosomes from the wild-type system. Interestingly, the decrease in activity was somewhat less pronounced when ribosomes from *relC49* were examined. This difference has been observed with three independent preparations of ribosomes. It is important to note that the only variable in these assays was the ribosome source.

FIG. 2. Activities of ribosomes from *S. antibioticus* IMRU3720 and *relC49*. Ribosomes and soluble enzymes were prepared and assays were performed as described in Materials and Methods. Results are expressed as counts per minute of [³H]phenylalanine incorporated per A₂₆₀ unit of ribosomes added to reaction mixtures. Assays were performed in duplicate, and numbers represent averages of duplicate samples.

Effects of the *rel* mutation on the activities of actinomycin enzymes. The activities of three enzymes involved in actinomycin biosynthesis in *S. antibioticus*, PHS, HAMT, and ASI, were assayed in extracts of *S. antibioticus* IMRU3720 and *relC49*. PHS and HAMT have been purified from *S. antibioticus* and characterized (4, 5). ASI has been characterized in *Streptomyces chrysomallus* (13) and has recently been purified from *S. antibioticus* in one of our laboratories (9). Enzyme assays were performed by using extracts of 12- to 72-h cultures of strains IMRU3720 and *relC49* (Fig. 3). There is an increase in HAMT activity between 12 and 72 h

FIG. 3. Activities of HAMT and ASI in extracts of *S. antibioticus* IMRU3720 and *relC49*. Extracts were prepared and assays were performed as described in Materials and Methods. Assays were performed in duplicate, and numbers represent averages of duplicate samples. It is important to note that data obtained in ASI assays in the presence of HAA were corrected by subtracting the level of ³²P incorporation in the absence of HAA.

TABLE 1. Specific activity of PHS and relative levels of PHS mRNA in *S. antibioticus* IMRU3720 and *relC49*

Age of culture (h)	Sp act ^a		mRNA ^b (%)	
	3720	<i>relC49</i>	3720	<i>relC49</i>
12	24.4	4.2	0.48	0.11
24	34.7	3.6	0.60	0.14
48	54.0	2.2	1.00	0.17
72	38.5	3.0	0.79	0.12

^a Nanomoles per minute per milligram of protein. Assays were performed in duplicate, and numbers represent averages of duplicate values.

^b Data in this column represent the levels of hybridization of the labeled *phs* gene to 25 µg of RNA in dot blot assays performed as described in Materials and Methods. The values are expressed relative to the corrected counts per minute detected for the 48-h RNA sample from strain IMRU3720 (489 cpm). Experimental values were corrected by subtracting a blank value (73 cpm) corresponding to a region of the filter which was not spotted with RNA.

in IMRU3720, as reported previously (5). In contrast, the level of HAMT in *relC49* remains essentially constant over the entire period analyzed in these experiments. Figure 3 also shows the time course of the change in ASI activity in extracts of IMRU3720. The pattern observed is similar to that reported for other enzymes involved in antibiotic biosynthesis (21). It is important to note here that the data presented for ASI represent levels of ATP-PP_i exchange in the presence of HAA minus the levels observed in its absence. When the data are corrected for HAA-independent ATP-PP_i exchange, it appears that no ASI activity is present in extracts of *relC49*.

As reported by Ochi (16), PHS levels are significantly reduced in the *rel* mutant compared with the wild-type parent (Table 1). At no point did the PHS level in strain *relC49* exceed 17% of the level detected in IMRU3720. Confirming other results reported by Ochi (16), *relC49* did not produce actinomycin in the experiments reported here.

Effects of the *rel* mutation on *phs* mRNA levels. In *E. coli*, ppGpp has been shown to differentially affect gene transcription, at least in vitro (11, 14). It was of interest, therefore, to determine whether the decreased levels of actinomycin biosynthetic enzymes observed in strain *relC49* (Table 1) were due to an effect at the level of transcription. It was possible to examine this question in the case of PHS by using the cloned *phs* gene as a probe in RNA dot blotting experiments. Quantitative estimates of *phs* mRNA levels were obtained by cutting out and counting the spots from a typical blotting experiment. The results shown in Table 1 demonstrate clearly that the levels of *phs* mRNA are significantly reduced in *relC49* compared with wild-type *S. antibioticus* cultures. mRNA levels in *relC49* never exceed 23% of those observed in IMRU3720.

DISCUSSION

The data presented in this communication demonstrate that ribosomes from strain *relC49* are slightly more active than ribosomes from mycelium of IMRU3720 of the same age. This observation may reflect the fact that one of the effects of the *rel* mutation is to slow the growth of the mutant strain. Thus, the physiological factors responsible for the decrease in ribosome activity may simply act more slowly in *relC49* than in the wild-type system. One enzyme, HAMT, which is involved in actinomycin synthesis, shows a significant difference in its pattern of activity over time in *relC49* compared with IMRU3720, whereas a second enzyme, ASI, which is easily detectable in IMRU3720, appears to be

absent from *relC49* (Fig. 3). The results of assays for these two enzymes and for PHS are consistent with other experiments which suggest that PHS and ASI may be regulated by similar mechanisms (i.e., both appear to be subject to catabolite control), whereas HAMT is regulated differently (4, 5; unpublished data).

In the case of PHS, it is clear that the *relC* mutation either decreases transcription of the *phs* gene or increases the rate of degradation of *phs* mRNA. Given the results of in vitro studies in *E. coli*, an effect of ppGpp on transcription of the *phs* gene is a most intriguing possibility. For example, Nagase et al. (14) have demonstrated a differential effect of ppGpp on transcription from the *metY* and *metZ* promoters. The guanine derivative inhibited transcription from the *metZ* promoter in vitro, whereas transcription from *metY* was relatively insensitive to it. In an earlier study, Kajitani and Ishihama (11) observed an inhibition of transcription from a rRNA promoter in a mixed in vitro transcription system from *E. coli*. The guanine derivative had essentially no effect on transcription from the *lacUV5* promoter and, interestingly, stimulated transcription from the *recA* promoter. While the mechanism of the stringent response in *E. coli* is almost certainly more complex than is suggested by the results of Kajitani and Ishihama (see reference 1, for example), it is possible to envision a model in which ppGpp would stimulate transcription of certain genes involved in actinomycin biosynthesis in *S. antibioticus*. The reduced levels of ppGpp in strain *relC49* may be insufficient to support the maximal rate of transcription of those genes. Consistent with this proposal is the observation that there is a burst of ppGpp production in IMRU3720 between ca. 17 and 22 h of growth in production medium (Fig. 1). It is interesting to note that this burst coincides with the time of appearance of actinomycin in the cultures. If the proposed model is correct, the data provided above would suggest that while transcription of the *phs* gene and perhaps the gene for ASI may be controlled by ppGpp, transcription of the gene for HAMT may not be. Levels of this enzyme are relatively constant over time in *relC49* cultures (Fig. 3). It will be interesting to examine the effects of the *rel* mutation on the activities of ASII and ASIII (12). Those enzymes are more difficult than ASI to assay in crude extracts and were not examined in the present study. Chater (3) has suggested a role for guanine nucleotides in the regulation of transcription during morphological differentiation in streptomycetes.

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