A *rel* Mutation Abolishes the Enzyme Induction Needed for Actinomycin Synthesis by *Streptomyces antibioticus*

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A relaxed (*rel*) mutant was found among thirty spontaneous thiopeptin-resistant isolates of *Streptomyces antibioticus* strain 3720, an actinomycin-producing strain, which showed severely reduced ability to accumulate ppGpp during a nutritional shift-down. The pool size of GTP decreased markedly in the parental strain, but to a lesser extent in the *rel* mutant. The *rel* mutant did not show the induction of an enzyme, phenoxazinone synthase, which is involved in the biosynthesis of actinomycin. No negative effect of the *rel* mutation was observed on a constitutive enzyme, kynurenine formamidase, which also plays a role in actinomycin synthesis. The mutant also failed to produce melanin, but still retained the ability to form aerial mycelium and spores, although the onset of the formation of aerial mycelium was markedly delayed. Neither the phenoxazinone synthase activity nor the kynurenine formamidase activity was affected by ppGpp *in vitro*. It is suggested that the stringent response (ppGpp) may be generally essential for the induction of enzymes involved in secondary metabolism.

Members of the genus *Streptomyces* have distinct characteristics, such as the abilities to develop aerial mycelium and conidiospores, and to produce numerous antibiotics. Therefore, *Streptomyces* are excellent organisms in which to study both differentiation and secondary metabolism.¹⁾ Secondary metabolism is easier to study than morphological differentiation, especially in *Streptomyces*, because aerial mycelium is produced only on solid media, while antibiotics are produced both on solid media and in liquid culture. Thus, biochemical analysis of secondary metabolism can be performed rather easily. Taking secondary metabolism as "physiological differentiation," the initiation process through which secondary metabolism starts in cells was studied.^{2~5)}

When cells encounter adverse environmental conditions such as nutrient limitation, they show the stringent response, accumulating ppGpp (guanosine-5'-diphosphate-3'-diphosphate) and pppGpp (guanosine-5'-triphosphate-3'-diphosphate), which are believed to be responsible for the stringent response.

The stringent response can be avoided by the introduction of genes with the relaxed genotype (for review, see refs. 6 and 7). Since ppGpp and pppGpp are synthesized on ribosomes through the catalytic action of an enzyme (stringent factor), a certain disturbance of the ribosomal conformation by a mutation or by a drug which binds to ribosomes might result in the failure to produce ppGpp and pppGpp. This was the case for *Bacillus subtilis*; the accumulation of ppGpp and pppGpp was abolished by adding small amounts of chloramphenicol or fusidic acid,⁸⁾ or by introducing a thiostrepton-resistant genotype (*tsp-6 = relC*), which resulted in a lack of ribosomal protein BS-L11.^{9,10)} The most recent study indicated that the production of the antibiotic, formycin, by *Streptomyces* sp. MA406-A-1 is initiated by the stringent response (ppGpp) produced in response to nutrient limitation.⁵⁾

Investigation of a relaxed (*rel*) mutant of *Streptomyces antibioticus* with thiopeptin-resistance isolated in this study showed that the normal *rel* gene function is needed for the induction of an enzyme, phenoxazinone syn-

thase, which is believed to mediate actinomycin synthesis. To my knowledge, this is the first report of an essential role of the *rel* gene product (ppGpp) in the induction of an enzyme needed for a typical secondary metabolic process, antibiotic biosynthesis.

MATERIALS AND METHODS

Chemicals. *N*-Formylkynurenine was purchased from Calbiochem. 3-Hydroxyanthranilic acid and galactose (special grade) were from Nakarai Chemicals. [2-¹⁴C]-Uracil was from New England Nuclear. Thiopeptin (an analogue of thiostrepton) was a commercial product of our company.

Strains and growth conditions. *Streptomyces antibioticus* strain 3720 (= ATCC 14888), a prototrophic actinomycin producing strain, was used. Spontaneous thiopeptin-resistant mutants were obtained, as resistant colonies, which developed 24 days after the spreading of spores on sporulation agar (see below) supplemented with 0.5% polypeptone and 3 μ g/ml of thiopeptin.

The sporulation agar contained (per liter): soluble starch, 4 g; yeast extract, 4 g; malt extract, 10 g; and agar, 20 g (adjusted to pH 7.0 with NaOH). The synthetic medium (suitable for actinomycin production) was the same as the galactose/glucose/glutamate medium described by Gallo and Katz,¹¹⁾ except that 0.2% instead of 0.1% glucose was contained. [Glucose at concentrations greater than 0.2% reduced the actinomycin formation.] The maltose/polypeptone/yeast extract medium (MPY-medium; suitable as a complex medium for actinomycin production) was described previously.⁵⁾

The cells, grown in NZ-amine medium¹¹⁾ (50 ml in a 250 ml flask) at 30°C for 30 hr, were harvested by centrifugation, washed twice with 100 ml saline (0.9% NaCl) and then resuspended in the original volume of saline. The washed cells (1 ml) were inoculated into 50 ml of the synthetic medium or of the synthetic medium supplemented with vitamin-free Casamino acids (in a 250 ml flask), and then cultured at 30°C on a rotary shaker (230 rpm).

Assaying of nucleotide pools. The pool sizes of nucleotides, including ppGpp and pppGpp, were determined, after extraction with 1 M formic acid, by means of HPLC on a column of Particil PXS 10-25 SAX (Whatman), as described in detail previously.⁵⁾

Enzyme assays. Crude enzyme extracts were prepared by sonical disruption (1~2 min) of cells suspended in deionized water, followed by centrifugation (15,000 $\times$ g, 20 min at 3°C). The supernatant used as the crude enzyme preparation contained 6~10 mg protein per ml.

Phenoxazinone synthase was assayed by the method described by Katz and Weissbach,¹²⁾ using 3-hydroxyanthranilic acid as a substrate. The incubation mixture consisted of 0.3 ml of 6.6 mM 3-hydroxyanthranilic acid (neutralized with NaOH), 0.3 ml of 0.8 M potassium acetate buffer (pH 5.3), 5~20 μ l of the crude enzyme preparation and deionized water to give a final volume of 3 ml. The reaction was carried out at 37°C for 15 min. The enzyme activity was determined by measuring the increase in optical density at 452 nm (molar extinction coefficient of the product = 1.80×10^4). Kynurenine formamidase was assayed by the method described by Brown *et al.*,¹³⁾ using *N*-formyl-L-kynurenine as a substrate. The incubation mixture consisted of 0.3 ml of 10 mM *N*-formyl-L-kynurenine, 0.3 ml of 0.5 M potassium phosphate buffer (pH 7.4), 10~40 μ l of the crude enzyme preparation and deionized water to give a final volume of 3 ml. The reaction was carried out at 30°C for 10 min. The enzyme activity was determined by measuring the increase in optical density at 365 nm (molar extinction coefficient of the product = 4.53×10^3). Protein was determined by the method of Lowry *et al.* with serum albumin as a standard.

Assaying of actinomycin or RNA synthesis. Actinomycin was assayed spectrophotometrically¹⁴⁾ at 443 nm after extraction with ethyl acetate. If the amount of actinomycin produced in the medium was less than 10 μ g/ml, it was determined by a disk-plate method with *Bacillus subtilis* ATCC 6633 as a test organism (measurable down to 1 μ g/ml of actinomycin).

RNA synthesis was determined as described in detail previously.⁵⁾

RESULTS AND DISCUSSION

Accumulation of ppGpp by S. antibioticus

The ability of *Streptomyces antibioticus* to accumulate ppGpp and pppGpp was first examined. When cells are transferred from a nutritionally rich to a nutritionally poor one they have to adapt to the absence of amino acids. Such starvation conditions leading to amino acid deficiency might increase the fraction of uncharged tRNA, and thus the stringent response might be transiently effective; this was the case for *Streptomyces* sp. MA406-A-1.⁵⁾ A similar approach was used for *S. antibioticus*. Unlike *Streptomyces* sp. MA406-A-1, the cells of *S. antibioticus*, growing in synthetic medium supplemented with 1% Casamino acids, did not have the ability to accumulate ppGpp or pppGpp (Table I). The cells, however, were capable of accumulating

TABLE I. EFFECT OF THE GROWTH CONDITIONS ON THE ABILITY OF CELLS TO ACCUMULATE ppGpp AFTER A NUTRITIONAL SHIFT-DOWN

Grown with Casamino acids of	Shifted down after growth for:					
	8 hr	12 hr	16 hr ppGpp (pmol/mg dry wt)	20 hr	24 hr	28 hr
1%	5	4	3	5	ND	ND
2%	4	26	40	6	ND	ND
2.5%	18	42	78	58	23	20

Cells of strain 3720, grown in synthetic medium supplemented with various amounts of Casamino acids (vitamin-free) for the indicated times, were harvested and transferred to fresh synthetic medium without Casamino acids. After 10 min incubation with shaking, the cells were harvested and extracted for the assaying of ppGpp. The pool size of ppGpp just before the transfer of cells grown for 16 hr to conditions without Casamino acids was in the range of 3~6 pmol/mg dry wt.

ND, not determined.

marked amounts of ppGpp 10 min after a shift-down, if the cells had previously been grown with 2.5% instead of 1% Casamino acids; this ability was maximal for the cells grown for 16 hr (mid-exponential) and to a lesser extent for the cells grown for 8 hr (early exponential) or 24 hr (post exponential) (Table I). These results are in agreement with those for *Bacillus subtilis* and *Escherichia coli*, both of which showed the maximal capacity to form ppGpp during the mid-exponential growth phase.^{15,16} The size of the pppGpp pool was only one-fifth or less compared to that of ppGpp (not shown). Thus, one must take the growth conditions into consideration when examining the ability to accumulate ppGpp.

Isolation of a relaxed mutant

The addition of Casamino acids (1~3%) to the synthetic medium markedly enhanced the growth rate of cells, while actinomycin production was completely suppressed during 5 days incubation. This suppression might be due to interference with the intracellular accumulation of ppGpp, as was the case for the antibiotic production by *B. subtilis* Marburg³⁾ or the formycin production by *Streptomyces* sp. MA406-A-1.⁵⁾ To assess the contribution of the accumulation of ppGpp to the triggering of the formation of actinomycin, an attempt was made at the isolation of relaxed (*rel*) mutants, which might lack the ability to pro-

duce ppGpp. Since relaxed mutants (*relC*) were found among thiostrepton-resistant isolates of *B. subtilis*,^{9,10)} a search was made for *rel* mutants of *S. antibioticus* among spontaneous thiopeptin (an analogue of thiostrepton)-resistant isolates. A number of thiopeptin-resistant colonies developed on plates containing 3 µg/ml of thiopeptin after 24 days incubation. Among them, 30 strains selected randomly were tested as to their ability to produce ppGpp. Cells of each strain growing exponentially with 2.5% Casamino acids were transferred into fresh synthetic medium without Casamino acids, as described above. After 10 min incubation, the cells were collected and extracted, and the extracts were analyzed by HPLC. A strain, designated as No. 49, was found to accumulate a severely reduced amount of ppGpp, compared with the parental strain (Fig. 1). The other 29 strains examined accumulated as much ppGpp as the parental strain (not shown). Changes in the nucleotide pools of the parental strain during a shift-down were much more drastic than in the case of mutant No. 49; it was especially notable that the pool size of GTP had decreased by 62% 10 min after a shift-down of the parental strain, but to a lesser extent in mutant No. 49 (Table II).

The RNA synthesis of the parental strain was markedly reduced upon a nutritional shift-down, while mutant No. 49 showed continued

TABLE II. CHANGES IN THE INTRACELLULAR CONCENTRATIONS OF NUCLEOSIDE TRIPHOSPHATES AND ppGpp AFTER A SHIFT-DOWN FROM SYNTHETIC MEDIUM SUPPLEMENTED WITH 2.5% CASAMINO ACIDS TO SYNTHETIC MEDIUM LACKING CASAMINO ACIDS

Strain	Time of incubation	UTP	CTP	ATP (pmol/mg dry wt)	GTP	ppGpp
Parent (3720)	Zero-time	679	474	1850	652	5
	10 min	790	1180	4490	248	82
Mutant (No. 49)	Zero-time	995	878	2690	698	<4
	10 min	780	817	2590	403	12

FIG. 1. Elution Profiles of Nucleotides Extracted from Cells of the Parental Strain (3720) and Mutant No. 49 after Casamino Acids Deprivation.

The strains, grown to the mid-exponential phase (16 hr for the parent and 24 hr for the mutant) in the synthetic medium plus 2.5% Casamino acids, were harvested and transferred to fresh synthetic medium lacking Casamino acids. After 10 min incubation with shaking, the cells were collected and extracted, and the extracts were then analyzed by HPLC. The samples applied were equivalent to 9~11 mg cell dry wt for each profile. The scale for absorbance is 0~0.03.

RNA synthesis for at least 20 min at a rate rather higher than that in the presence of Casamino acids (Fig. 2). Thus, mutant No. 49 exhibited the relaxed phenotype with respect to RNA synthesis. The relaxed mutant thus obtained revealed no requirement for any nu-

FIG. 2. Effect of Casamino Acids Deprivation on RNA Synthesis by Cells of *Streptomyces antibioticus* Strain 3720 and Mutant Strain No. 49.

Cells, grown with 2.5% Casamino acids, as in Fig. 1, were transferred to the synthetic medium containing [2-¹⁴C] uracil with (●) or without (○) 2.5% Casamino acids, and then incubated with shaking for 40 min.

trients, as it grew normally in synthetic medium and was resistant to thiopeptin up to 10 μ g/ml, as determined after 20 days incubation on agar plates. [Growth of the parental strain was completely suppressed with 2 μ g/ml of thiopeptin.] The *rel* mutant No. 49 might be tentatively classified as a *relC* mutant, in analogy with the mutant of *B. subtilis* which lacks ribosomal protein BS-L11,^{9,10} but further work at the molecular level is required to

confirm this directly. The other 29 thiopeptin-resistant isolates may include mutants deficient in thiopeptin uptake and ones with altered ribosomal conformations, but these alterations in the membrane or the ribosomal conformation (if any) apparently did not affect the ability to produce ppGpp.

Characterization of the rel mutant

The *rel* mutation resulted in numerous pleiotropic effects: the most striking one was that the *rel* mutant lost the ability to produce actinomycin in both liquid and agar-plate cultures. Actinomycin amounted were less than detectable level (*i.e.*, 1 $\mu\text{g/ml}$) in both the synthetic medium and MPY-medium. The parental strain produced 110 $\mu\text{g/ml}$ (at 5 days) and 30 $\mu\text{g/ml}$ (at 2 days) of actinomycin in the synthetic medium and MPY-medium, respectively. The *rel* mutant also lost the ability to produce the black pigment, melanin, which is one of the distinctive characteristics of *S. antibioticus*. Although the *rel* mutant still retained the ability to form aerial mycelium and spores when cultured on sporulation agar, the amounts were less than those produced by the parental strain, and the onset of the formation of aerial mycelium was drastically delayed (2 days for the parent, but 8 days for the *rel* mutant). The aerial mycelium formation of several *Streptomyces* spp. examined was shown to be initiated by a partial decrease in GTP content.⁴⁾ The delayed appearance of aerial mycelium in the *S. antibioticus rel* mutant may be accounted for at least partially by the reduced decrease in GTP content upon amino acid deficiency (see Table II). The properties of the *S. antibioticus rel* mutant described above are well coincident with those found for *rel* mutants of *Streptomyces* sp. MA406-A-1.⁵⁾ To elucidate the properties of these *rel* mutations, a genetic study is clearly required.

Effect of the rel mutation on enzyme induction

Finally, whether or not the loss of the ability to produce actinomycin in the *rel* mutant resulted from a failure of expression of

enzyme(s) involved in actinomycin synthesis was examined. Two enzymes, kynurenine formamidase and phenoxazinone synthase, were investigated as suitable probes. [See ref. 17 for their mediation steps in the actinomycin biosynthetic pathway.] As reported by Katz and coworkers,^{11,18)} *S. antibioticus* strain 3720 showed phenoxazinone synthase induction prior to the beginning of actinomycin synthesis (a few data are presented in Table III). In contrast, *rel* mutant No. 49 did not show phenoxazinone synthase induction during 3 days cultivation (Table III), indicating that the normal *rel* gene function was needed for the induction of this enzyme activity. This is in contrast to the results obtained for actinomycin-nonproducing strains, which were found after curing treatment with acriflavin and still showed phenoxazinone synthase induction.¹⁷⁾

Unlike in the case of phenoxazinone synthase, the *rel* mutant produced formamidase constitutively: the activities were 2~3 folds higher than those produced by the *rel*⁺ parental strain (Table III). Apparently, the *rel* gene function (and thus ppGpp) was unnecessary for the expression of formamidase. This seems to be reasonable because formamidase is also utilized for a primary metabolic process, NAD biosynthesis. In another actinomycin producing organism, *Streptomyces parvulus*, two forms of formamidase have been found; one of them is a constitutive and the other an inducible (derepressible) isozyme, the activity of which appears just prior to actinomycin production.¹³⁾ Since *S. antibioticus* used here did not show increased formamidase activity during the onset of actinomycin production, only the constitutive form may exist in it.

The physiological concentrations of ppGpp (0.2~2 mM) produced *in vitro* neither inhibited nor stimulated the phenoxazinone synthase or kynurenine formamidase activity (Table IV). The reduced phenoxazinone synthase activity of the *rel* mutant remained at a low level in the presence of added ppGpp. Thus, it is highly likely that the reduced or increased enzyme activity of the *rel* mutant is due to an event at

TABLE III. KYNURENINE FORMAMIDASE AND PHENOXAZINONE SYNTHASE IN THE PARENTAL STRAIN (3720) AND RELAXED MUTANT (49) OF *S. antibioticus* GROWING IN SYNTHETIC MEDIUM

Strain	Specific activity (nmol of product/mg of protein/min)							
	Kynurenine formamidase				Phenoxazinone synthase			
	0 hr*	12 hr	48 hr	72 hr	0 hr*	12 hr	48 hr	72 hr
Parent (<i>rel</i> ⁺)	123	62	53	41	9	12	132	156
Mutant (<i>rel</i>)	378	213	173	87	3	14	15	19

* The enzyme preparation for 0 hr was obtained from cells grown in NZ-amine medium for 30 hr.

TABLE IV. *In Vitro* EFFECTS OF ppGpp ON KYNURENINE FORMAMIDASE AND PHENOXAZINONE SYNTHASE

ppGpp (mM)	Specific activity (nmol of product/mg of protein/min)			
	Kynurenine formamidase		Phenoxazinone synthase	
	Parent (<i>rel</i> ⁺)	Mutant (<i>rel</i>)	Parent (<i>rel</i> ⁺)	Mutant (<i>rel</i>)
None	49	181	124	12
0.2	47	174	118	12
2	49	169	125	12

The crude enzyme was prepared from cells grown in the synthetic medium for 48 hr. ppGpp was added to the assay mixture just before starting the reaction, to a final concentration of 0.2 or 2 mM.

the transcriptional (or translational) level. Phenoxazinone synthase expression has been well shown to be the subject of catabolite repression, and is controlled at both the transcriptional and post-transcriptional levels.^{11,19)} Unlike the systems in *E. coli*, cAMP does not appear to mediate the repression of actinomycin synthesis in *S. antibioticus*.^{11,20)} Since the *rel* mutation resulted in a failure of induction (derepression) of phenoxazinone synthase, it is of interest to determine whether or not effects elicited by the stringent response (ppGpp) were exerted at the transcriptional and/or translational level, and could be superimposed on the suppressing effect of catabolite regulation. As will be reported elsewhere, a *rel* mutation of *Streptomyces griseus* also abolished the induction of enzymes involved in streptomycin biosynthesis. The normal *rel* gene function, therefore, may be generally needed for the triggering of the synthesis of inducible enzymes necessary for secondary

metabolism.

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