Occurrence of Guanosine-5'-diphosphate-3'-diphosphate and Adenosine-5'-triphosphate-3'-diphosphate in *Streptomyces galilaeus*

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Synthetic activity and existence of ppGpp and pppApp in an anthracycline-producing strain *Streptomyces galilaeus* were determined by radioimmunoassay and ^{32}P -labeling method during cultivation under both the antibiotic productive and non-productive conditions. The cellular ppGpp(pppGpp)-synthesizing activity was highest at the end of exponential growth, and 3-fold higher in the antibiotic-productive cultivation than in non-productive cultivation. The intracellular level of ppGpp determined by radioimmunoassay was high at the end of exponential growth, and afterwards its level decreased by one fifth. The low level of cellular ppGpp during the period of intense antibiotic production suggests that ppGpp consumption may play an important role in antibiotic production of *S. galilaeus*. The concentration of pppApp was not determined intracellularly by radioimmunoassay.

ppGpp and pppGpp are important pleiotropic regulatory molecules in bacteria.¹⁾ Their synthesis in *Escherichia coli* is catalysed by the stringent factor protein which is coded for the *rel A* gene, and they are formed on ribosomes not actively engaged in protein synthesis.²⁾ On the other hand, the extracellular stringent factor-like enzyme, capable of synthesizing ppGpp and pppApp, was found to be present in *Streptomyces* cultures.^{3~5)} Furthermore, recently Simuth *et al.*⁶⁾ and An and Vining⁷⁾ identified ppGpp, pppGpp, ppApp and pppApp in the vegetative mycelia of streptomycin-producing *S. griseus* and chlorotetracycline-producing *S. aureofaciens*.

Therefore, we have been interested in possible roles of the enzyme and its products containing ppGpp and pppApp on the cell growth and cellular metabolism in *Streptomyces*.

Assay for intracellular highly phosphorylated nucleotides usually consists of culturing the organisms or cells with $\text{H}_3^{32}\text{PO}_4$, extraction with acid (usually formic acid), and PEI-cellulose thin-layer chromatographic separation of the products. However, ^{32}P -labeling method is complicated, and time-consuming with low accuracy. We, therefore, developed radioimmunoassay for ppGpp and pppApp.^{8,9)}

This report deals with the determination of intracellular ppGpp and pppApp levels in *Streptomyces galilaeus* MA144-M1 by radioimmunoassay, and the relationship between their changes in intracellular concentration and the onset on the production of, antitumor anthracycline antibiotic, aclacinomycin.

MATERIALS AND METHODS

Chemicals. $[2\text{-}^{14}\text{C}]\text{-uridine}$ (50 mCi/mmol), $[1\text{-}^{14}\text{C}]\text{-leucine}$ (50 mCi/mmol) and $[^{32}\text{P}]\text{-H}_3\text{PO}_4$ (carrier free) were

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Abbreviations: Guanosine-5'-triphosphate-3'-diphosphate = pppGpp; guanosine-5'-diphosphate-3'-diphosphate = ppGpp; guanosine-5'-monophosphate-3'-diphosphate = pGpp; guanosine-5'-diphosphate-3' (or 2')-monophosphate = ppGp; adenosine-5'-triphosphate-3'-diphosphate = ppApp; adenosine-5'-diphosphate-3'-diphosphate = ppApp; adenosine-5'-monophosphate-3'-diphosphate = pApp; guanosine-5'-tetraphosphate = ppppG; 100 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl and 0.1% $\text{Na}_2\text{S}_2\text{O}_3$ = TBS.

obtained from the Radiochemical Centre Amersham. The nucleoside polyphosphate lithium salts, pppApp, ppApp, pppGpp, ppGpp, pGpp and pApp, were our products (Sanraku-Ocean Co., Japan) prepared by ATP: nucleotide pyrophosphotransferase.⁴⁾ Other common nucleotides were purchased from Sigma Chemical Co.

Strains, media and growth conditions. *Streptomyces galilaeus* MA144-M1, strain No. 6U-21 capable of producing highly anthracycline antibiotic aclacinomycin A¹⁰⁾ was used. YS medium consisted of 0.3% yeast extract and 1% soluble starch, pH 7.2 and permitted the antibiotic production at a moderate yield. A modified YS medium with enhanced yeast extract by 1% was used as the antibiotic-non-productive condition. The cultivation was carried out in a 500-ml Erlenmeyer flask containing 50 ml of the medium at 28°C on a rotary shaker (220 rpm).

Cellular activity of ppGpp (pppGpp)-synthesis. At appropriate time intervals, 0.2 ml of cultured broth of *S. galilaeus* was withdrawn, added to a small tube (1.0 × 10 cm) containing 10 µl of [³²P]-H₃PO₄ (4 mCi/ml) and incubated at 30°C on a reciprocal shaking incubator for 30 min. It was then mixed with 20 µl concd. formic acid. After 20 min at 0°C, the mixture was centrifuged and the supernatant was removed, quickly frozen and stored at -40°C until used for determination. Five or ten µl of the supernatant were spotted on polyethyleneimine-cellulose (PEI-cellulose) sheets (Machery-Nagel MN-300 PEI-cellulose) with authentic ATP, GTP, pppGpp, ppGpp, pppApp and ppApp as markers. Nucleotides were resolved by either one- or two-dimensional ascending chromatography. For two-dimensional chromatograms a solution containing 3.3 M ammonium formate and 4.2% boric acid adjusted to pH 7 with ammonium hydroxide was used to develop in the first direction. The chromatogram was dried, soaked in methanol for 30 min, and dried again before the second development in 1.5 M potassium phosphate buffer, pH 3.4. For one-dimensional chromatography the latter solvent was used. ³²P-labeled compound were detected by exposing the chromatogram to X-ray film (Kodak no-screen medical X-ray film, Eastman Kodak Corporation, Rochester, New York). Identification of nucleotides solely on the basis of one-dimensional chromatography is very hazardous as pointed out by Gallant and Margasson.¹¹⁾ For example, in one-dimensional chromatography, the ³²P-labeled compounds that comigrated with ppGpp and pppGpp reference marker were present in the acid-cell extracts of *S. galilaeus* (Fig. 1-A). By two-dimensional chromatography these spots were clearly separated from ppGpp and pppGpp (Fig. 1-B, C, D). The radioactive spots comigrated with authentic ppGpp or pppGpp were cut out and counted in the Bray scintillator by a Aloka Scintillation Spectrometer Model LSC 653.

Radioimmunoassay for ppGpp and pppApp. Radioimmunoassay for ppGpp was performed by using

antibody for ppGpp-human serum albumin, and that for pppApp was performed by using antibody for 8-(6-aminohexyl)amino-pppApp-human serum albumin. Assay methods are described in details in the preceding papers.^{8,9)}

Pretreatment of radioimmunoassay samples. Five ml of the culture broth sampled at appropriate time intervals were separated into the supernatants and mycelia by centrifugation. The mycelial pellet was rinsed twice with 5 ml of cold 0.15 M NaCl and mixed with 5 ml of 0.2 N formic acid. After 20 min at 0°C, the mixture was centrifuged and the supernatant was removed and neutralized with 1 N NaOH in ice-water bath. The supernatant was diluted twice with distilled water and applied on the column of DEAE-Sephadex A-25 (1.0 × 3.0 cm, HCO₃⁻ form). After rinsing with 20 ml of the water, the column was eluted with 20 ml of 0.3 M NH₄HCO₃ and then with 20 ml of 0.6 M NH₄HCO₃; 0.3 M NH₄HCO₃ eluent was served to elute out most of all nucleoside (mono, di and tri)-phosphates including ppGp, pGpp and pApp, and subsequent 0.6 M NH₄HCO₃ eluate contained ppGpp, pppGpp, ppApp, pppApp and ppppG. The latter pools were freeze-dried. The residues were dissolved in 1 ml of TBS (100 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl and 0.1% NaN₃) and assayed for ppGpp and pppApp. When 1000 pmol ppGpp or pppApp was applied, recovery from column was quantitative.

Determination of RNA and protein synthesis. From the growing culture of *S. galilaeus* at different times 0.2 ml sample was withdrawn and incubated with 20 µl of [¹⁴C]-uridine (0.5 µCi/ml) or 20 µl of [¹⁴C]-leucine (4.5 µCi/ml) at 30°C for 5 min, and two drops of 1% bovine serum albumin and 0.2 ml of cold 10% trichloroacetic acid, the sediment was dissolved in 0.2 ml of concd. formic acid at 100°C for 10 min and measured for the radioactivity in Bray scintillator.

Aclacinomycin production and protein assay. At appropriate time intervals, aclacinomycin A and its analogs were extracted with 5 ml of chloroform from 5 ml of sample broth. The chloroform extract was concentrated *in vacuo*, and the residue was dissolved in 5 ml of methanol. Amount of aclacinomycins was determined by the measurement of absorbance at 430 nm.¹⁰⁾ Protein content was determined by the method of Lowry *et al.* using bovine serum albumin (Seikagaku Kogyo Co.) as a standard.¹²⁾

RESULTS AND DISCUSSION

Biosynthesis of highly phosphorylated nucleotides, RNA and protein

ppGpp (pppGpp)-synthesizing activity and cellular level of ppGpp were monitored during cultivation under both of the antibiotic productive and non-productive conditions. Figure

FIG. 1. Autoradiogram of a Thin-layer Chromatogram of the Acid-soluble Fractions from *S. galilaeus* Grown in YS Medium.

Experimental details were described in MATERIALS AND METHODS. Dotted circles indicated the positions of unlabeled reference nucleotides which were cochromatographed with the sample and located under ultraviolet light.

One-dimensional thin-layer chromatogram:

(A) Culturing time (hr): (1), 10; (2), 15; (3), 20; (4), 25; (5), 30; (6), 35; (7), 40; (8), 45; (9), 50; (10), 55; (11), 60; (12), 65; (13), 70.

Two-dimensional chromatogram:

(B) 20 hr; (C) 30 hr; (D) 60 hr.

2-A shows the time-course of the macromolecular synthesis, aclacinomycin formation and ppGpp (pppGpp)-synthesizing activity during cultivation in YS medium (antibiotic productive condition). The culture grew exponentially until about 25 hr and the cell mass reached to a plateau. A pulse-label with [^{14}C]-uridine and [^{14}C]-leucine showed that intense RNA synthesis already ended before the exponential growth while decrease in the protein synthesis occurred about 20 hr later than that in the RNA synthesis. The aclacinomycin production initiated at the end of exponential growth (about 30 hr). The cellular ppGpp

(pppGpp)-synthesizing activity of *S. galilaeus* was highest at the end of exponential growth in YS medium.

Similar RNA and protein levels in *S. galilaeus* were observed in a modified YS medium (antibiotic-non-productive condition), as shown in Fig. 2-B. However, the cellular ppGpp (pppGpp)-synthesizing activity was 3~10 fold lower than in productive cultivation. These results coincided with those obtained in the chlortetracycline-producing and non-producing strains as reported by Simuth *et al.*,⁶⁾ and suggest that the ppGpp (pppGpp) may have an important role in the

FIG. 2. Cellular ppGpp(pppGpp)-synthesizing Activity, Macromolecular Synthesis, and Aclacinomycin Production in *S. galilaeus* Grown in YS (A) and a Modified YS (B) Media.

Experimental details were described in MATERIALS AND METHODS.

Symbols: growth (—▲—); incorporation of [^{14}C]-uridine (—○—) and [^{14}C]-leucine (—●—); synthetic activities of ppGpp (—●—) and pppGpp (—○—); aclacinomycin (—△—).

exponential growing cells.

Determination of intracellular ppGpp and pppApp by radioimmunoassay

Concentration of ppGpp and pppApp in the mycelium of *S. galilaeus* grown in antibiotic-productive and non-productive media were determined by radioimmunoassay, as shown in Table I. The level of ppGpp was 1.5-fold higher in antibiotic-productive cultivation than in non-productive cultivation. This result agrees with that of ppGpp-synthesizing activity as described above, which was assayed by ^{32}P -labeling method. pppApp was not detected in the acid-cell extracts of *S. galilaeus* by radioimmunoassay. However, as shown in Fig. 1-C, autoradiogram of a two-dimensional chromatogram of the acid-extracts prepared from 30 hr-cultivated cells showed the presence of

^{32}P -labeled compounds comigrated with ppApp and pppApp, which were adsorbed by activated charcoal. These compounds could not be detected in the exponential (20 hr) and stationary (60 hr-cultivation) phases. These results suggest that pppApp and ppApp are

TABLE I. INTRACELLULAR LEVELS OF ppGpp AND pppApp in *S. galilaeus*

Cultivation time (hr)	ppGpp	pppApp
YS medium	(pmol/mg cell protein)	(pmol/mg cell protein)
20	88.6	< 2.8
30	8.0	< 2.8
Modified YS medium		
20	51.9	< 0.9
30	34.5	< 0.9

Experimental details were described in "MATERIALS AND METHODS."

FIG. 3. Cellular Level of ppGpp and Aclacinomycin Production during Cultivation of *S. galilaeus* in YS Medium.

Experimental details were described in MATERIALS AND METHODS.

Symbols: growth (○); ppGpp (●); aclacinomycin (△).

present in *S. galilaeus*, but that their intracellular levels are very low.

Figure 3 shows the cellular levels of ppGpp and aclacinomycin production in *S. galilaeus* in YS medium. The cellular concentration of ppGpp was high at the end of exponential growth and ranged about 90 pmol/mg cell protein, and afterwards its level decreased by one fifth. The low level of cellular ppGpp during intense antibiotic production suggests

that ppGpp consumption may play an important role on the onset of antibiotic-production in *S. galilaeus*.

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