THE SYNTHESIS OF HIGHLY PHOSPHORYLATED NUCLEOTIDES, RNA AND PROTEIN BY *STREPTOMYCES AUREOFACIENS*

JOZEF ŠIMÚTH, JOZEF HUDEC, HOANG THANH CHAU*, ONDREJ DÁNYI and JÁN ZELINKA

Institute of Molecular Biology, Slovak Academy of Sciences, Department of Enzymology, Mlynské Nivy 59, 885 34 Bratislava, Czechoslovakia
*Faculty of Pharmacology, University of Hanoi, Vietnam

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During the sudden decrease in RNA synthesis in *Streptomyces aureofaciens, i.e.* around the 6th hour of cultivation, synthesis of adenosine and guanosine tetraphosphates and pentaphosphates begins. The synthesis of these nucleotides is highest during the onset of chlortetracycline production, around the 20th hour of cultivation and continues. During this phase of growth of *S. aureofaciens*, RNA and protein synthesis are reduced by about one order of magnitude as compared to the rate which can be observed at the beginning of cultivation, but the synthesis is not inhibited by exogenous CTC.

In the production of chlortetracycline (CTC) a pronounced decrease in the level of RNA's[1], especially of rRNA[2], takes place during the synthesis of the antibiotic. CTC also causes an accumulation of RNA degradation products in the cultivating medium[3]. During the phase of CTC synthesis the activity of nucleolytic enzymes increases[4] and the activity of RNA polymerase[5] and of polynucleotide phosphorylase[6] decreases. The last mentioned enzyme is competitively inhibited by CTC[7]. The decrease in RNA levels in antibiotic-producing streptomycetes could be caused by the direct effect of the antibiotic in a similar way as found for *Escherichia coli*[9]. An decrease in RNA level was also observed during the onset of streptomycin and actinomycin production[10,11]. Tetracycline (TC) is known to inhibit protein synthesis[12,13] as well as rRNA synthesis[9] and in the absence of charged t-RNA it can also inhibit the synthesis of ppGpp in *E. coli*[14]. Thus *Streptomyces aureofaciens* could be a useful natural model for the comparison of CTC and ppGpp effects on RNA and protein synthesis.

It is the aim of this work to clarify some questions pertaining to RNA and protein synthesis in relationship to the synthesis of higher phosphorylated nucleotides by 2 mutants of *S. aureofaciens* producing different quantities CTC.

Materials and Methods

Chemicals

Chlortetracycline (CTC) was obtained from Biotika, Slovenská Ľupča, Czechoslovakia; 2-^{14}C-uracil spec. activity 53 mCi/mmol, ^{14}C-leucine, spec. activity 210 mCi/mmol and ^{32}P-KH_2PO_4 (carrier free) were from the Institute for the Research, Production and Application of Radioisotopes, Prague, Czechoslovakia. The ppGpp standard was kindly supplied by Dr. M. CASHEL, NIH, Bethesda, Maryland, U.S.A.

Strains, media and growth conditions

The high-yielding strain of *S. aureofaciens*, BM-K, producing about 2,000 μg of CTC/ml and the low-yielding strain, B 96 XVI-130367, producing about 100 μg CTC/ml were used. The growth medium and cultivation conditions were the same as published previously[3].

Synthesis of highly phosphorylated nucleotides

The cells were cultivated in 60 ml of growth medium; 2 ml were withdrawn at different times of cultivation and labelled with ^{32}P-KH_2PO_4 250 μCi/ml. After 15 minutes of cultivation the medium was centrifuged and to the sediment 0.3 ml of 2 M formic acid was added. After 10 minutes at 0°C the mixture was centrifuged and the supernatant was removed, quickly frozen and stored at −20°C until used for thin-layer chromatography. Two μl of the supernatant were spotted on PEI-cellulose sheets and chromatographed with 1.5 M KH_2PO_4 (pH 3.4) as solvent. After drying, X-ray film was superimposed on the sheets for 24 hours. All radioactive spots were cut out and counted in a toluene based scintilation fluid in a Beckman model LS-230 scintilator.

Determination of RNA and protein synthesis

From the growing culture of *S. aureofaciens* at different times one ml sample was withdrawn and incubated with 0.2 μCi of ^{14}C-uracil or with 0.2 μCi of ^{14}C-leucine. For the experiments following the effect of CTC on RNA synthesis one ml sample from the growing culture of *S. aureofaciens* (in 6 or 48 hours of cultivation) was withdrawn and incubated for 10 minutes with CTC (from 10 to 500 μg CTC per ml). The control sample was incubated without CTC. Thereafter 0.2 μCi ^{14}C-uracil was added to each sample.

The rate of total RNA synthesis was determined by measuring ^{14}C-uracil incorporation into RNA (cold TCA-insoluble fraction) as previously described[15].

The rate of total protein synthesis was determined by measuring ^{14}C-leucine incorporation into protein (hot TCA-insoluble fraction).

The samples were precipitated with 2 ml 10% trichloroacetic acid (TCA) after 20 minutes at 90°C; samples were chilled, filtered and washed on membrane filters (Synpor 6, 0.4 μm pore size). The radioactivity was counted as mentioned above.

Results

The rate of RNA synthesis was estimated by pulse labelled ^{14}C-uracil in both, the low- and high-yielding strains of *S. aureofaciens*. We detected intense RNA synthesis during the first 6 hours of cultivation; after this period of time a pronounced decrease in the RNA synthesis rate was observed (Fig. 1). In both strains the protein synthesis rate is highest around the 12th hour of cultivation.

Fig. 1. RNA, protein and CTC synthesis and growth of the high-yielding strain of *S. aureofaciens*.

Experimental details are given in the text. These results and others mentioned were reproducible observed throughout 4 replicate experiments.

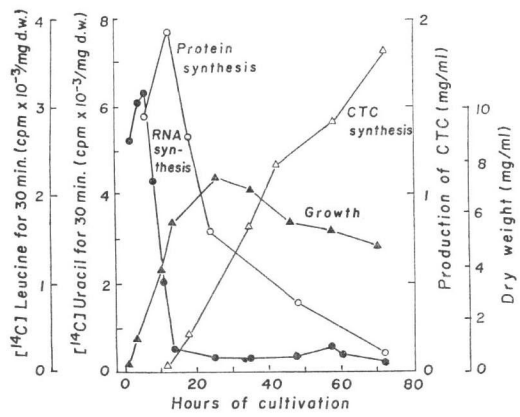
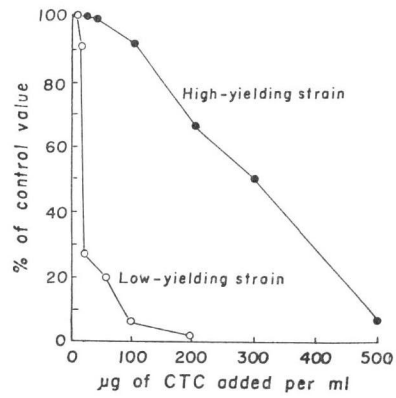


Fig. 2. Effect of CTC on RNA synthesis of *S. aureofaciens*.

Results given as % of control which represent the value measured in the absence of exogenous CTC. In all cases, addition of CTC was followed 10 minutes later by the addition of ^{14}C-uracil. The other experimental details are given in the text.



RNA and protein synthesis does not cease even during intense CTC formation. For the clarification of the relationship of CTC to RNA synthesis in *S. aureofaciens* we have studied the effect of exogenous CTC on the RNA synthesis rate. We have found that addition of 50 μg of CTC to the low-yielding strain in the logarithmic phase of growth (6th hour of cultivation) causes the RNA synthesis to decrease by about one order of magnitude. In the high-yielding strain this degree of RNA synthesis reduction can be reached only by an addition of 500 μg of CTC per ml (Fig. 2).

In the logarithmic phase of growth, RNA synthesis in the low-yielding strain is much more affected by CTC than in the high-yielding strain. On the other hand, during CTC biosynthesis, exogenously added CTC even at concentrations of up to 500 μg of CTC per ml does not affect RNA synthesis in either of the studied strains. We have also studied whether *S. aureofaciens* synthesizes higher phosphorylated nucleotides. Cells were pulse labelled with ^{32}P-KH_2PO_4 and in the acid-soluble fraction the presence of higher phosphorylated nucleotides was detected after chromatography on PEI cellulose. We have found radioactivity in fractions I and II containing 4 higher phosphorylated nucleotides (HPN) and in HPN_5 (Fig. 3). Comparison with ppGpp, GTP, GDP and ATP standards as well as with known Rf values of some higher phosphorylated nucleotides[16,17] has shown that fraction I corresponds to guanosine and adenosine tetraphosphates, fraction II to guanosine and adenosine pentaphosphates and HPN_5 probably to guanosine or adenosine hexaphosphate. Using a modification of the method of CASHEL and

Fig. 4. The rate of synthesis of some higher phosphorylated nucleotides during cultivation of *S. aureofaciens*.

Experimental details are given in the text.

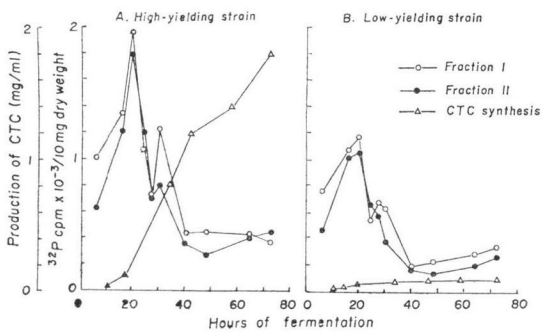


Fig. 3. Autoradiogram of thin-layer chromatography on PEI-cellulose sheets (Merck) of ^{32}P-KH_2PO_4 labelled cells of *S. aureofaciens* (from the 20th hour of cultivation) extracted with 2 M formic acid.

Experimental details are given in the text. A: low-yielding strain. B: high-yielding strain. HPN: higher phosphorylated nucleotide.

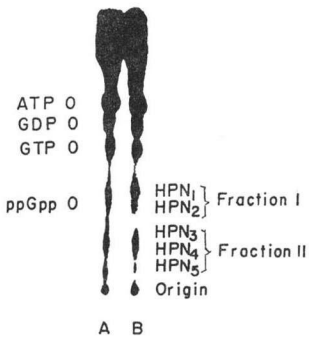
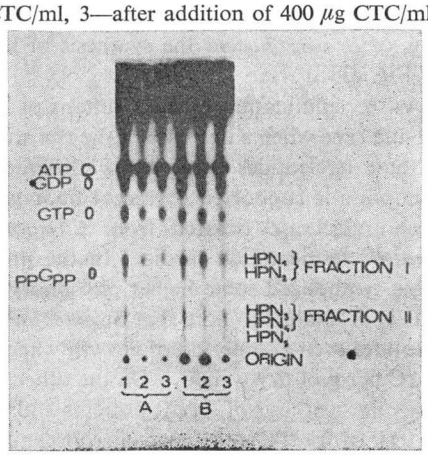


Fig. 5. Effect of CTC on the synthesis of highly phosphorylated nucleotides of *S. aureofaciens*.

In all cases, addition of CTC was followed 10 minutes later by the addition of ^{32}P-KH_2PO_4.

The other experimental details are given in the text and in the legend to the Fig. 3.

A: low-yielding strain. B: high-yielding strain. HPN: higher phosphorylated nucleotide, 1—control (without CTC), 2—after addition of 200 μg CTC/ml, 3—after addition of 400 μg CTC/ml



KALBACHER[18] the presence of guanosine and adenosine tetraphosphates and guanosine and adenosine pentaphosphates was confirmed by their isolation from mycelium of the high-yielding strain of *S. aureofaciens*, but hexaphosphates of these nucleotides were not found.

We have found in both strains that the synthesis rate of nucleotides contained in fractions I and II is highest around the 20th hour of cultivation (Fig. 4). We have studied as well the effect of exogenous CTC on the synthesis of these nucleotides in both strains of *S. aureofaciens*. The cells were preincubated for 10 minutes with 200 and 400 μg of CTC per ml and the nucleotide synthesis rate was determined in the presence of ^{32}P-KH_2PO_4 in an identical manner to that in the experiments described above. We have found that the addition of exogenous CTC causes a decrease in the rate of synthesis of the some highly phosphorylated nucleotides first of all guanosine tetraphosphate (Fig. 5).

Discussion

The growth of *S. aureofaciens* can be characterised by 2 different phases of the rate of RNA synthesis. The phase of intense RNA synthesis occurs around the 6th hour of cultivation, *i.e.* prior to the onset of CTC production. This is followed by a phase of reduced RNA synthesis overlapping CTC production. The highest rate of protein synthesis is reached around the 12th hour of cultivation, *i.e.*, about 6 hours later than the maximum rate of RNA synthesis (Fig. 1).

On the other hand, neither RNA nor protein synthesis is completely stopped during CTC production. Exogenous CTC significantly decreases RNA synthesis only in the logarithmic phase of growth and we have found that the low-yielding strain of *S. aureofaciens* is much more sensitive to the presence of CTC than the high-yielding strain (Fig. 2). In the phase of CTC production the exogenously added antibiotic does not affect RNA synthesis in any of the studied strains. KAPLAN *et al.*[14] have published that TC causes a stimulation of RNA synthesis in *E. coli* but we have not found any increase in RNA synthesis in *S. aureofaciens* caused by exogenous CTC (Fig. 2). Exogenous CTC was also found to inhibit protein synthesis in the logarithmic phase of growth but not in the phase of CTC production[19].

Our results further support the idea of the effect of CTC on the metabolism of the producing organism[8] by indicating the possibility of the existence of 2 different mechanisms participating on the regulation of gene expression and translation. These 2 mechanisms are probably affected by CTC in a different way, possibly even in cooperation with other low molecular weight substances or proteins. This is supported by our finding that *S. aureofaciens* also synthesizes higher phosphorylated nucleotides, first of all adenosine and guanosine tetra- and pentaphosphates, which could take part in the regulation of RNA synthesis in a way postulated for other microorganisms[16,20]. In *S. aureofaciens* the culmination of the synthesis of higher phosphorylated nucleotides was observed during the drastic decrease in the level of rRNA,[2] that is between the 12~20th hour of cultivation. In both strains of *S. aureofaciens* the synthesis of these nucleotides continues even during antibiotic production (Fig. 4).

As the culmination of the synthesis of higher phosphorylated nucleotides in *S. aureofaciens* takes place at a time when a decrease of the ribosomal level by one order[2] was observed it could be presumed that these nucleotides could be synthesized enzymatically even in the absence of ribosomes. This presumption is supported by recent findings on the presence of extracellular purine nucleotide pyrophosphotransferase isolated from *Streptomyces adephospholyticus*[21,22,23] and *Streptomyces morookaensis*[24] fermentation media. In the presence of ATP, donating the pyrophosphate moiety, this enzyme synthesized some higher phosphorylated guanine and adenine nucleotides[24,25,26].

It is of interest to note that *S. aureofaciens* synthesizes RNA, proteins and higher phosphorylated nucleotides even at a period of growth when the mycelium has already accumulated more than 200 mg of CTC per g of dry weight. On the other hand exogenous CTC added in the initial phase of growth inhibits the synthesis of RNA, proteins and higher phosphorylated nucleotides (Fig. 5) in a similar way as described for TC and other microorganisms[9,14,19,27,28].

Taking into account the specific inhibition of rRNA synthesis in *E. coli* by tetracyclines[9] or by ppGpp[29] and considering our results, there is reason to propose that certain genes expressed during the logarithmic phase of *S. aureofaciens* growth are turned off in the period of CTC production. At present, however, it cannot be excluded that the maintenance of postlogarithmic RNA and protein synthesis in *S. aureofaciens* is probably the result of the derepression of some genes by protein(s)[30] in cooperation with CTC and highly phosphorylated nucleotides.

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