Evidence for the presence of highly phosphorylated nucleotides in the cyanobacterium *Tolypothrix* sp.

Sambandam Shanmugasundaram* and Hans J. Rhaese

Institut für Mikrobiologie/Molekulare Genetik, Fachbereich Biologie, J.W. Goethe-Universität, Frankfurt am Main, FRG

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1. INTRODUCTION

Accumulation of guanosine-5'-diphosphate-3'-diphosphate (ppGpp) upon amino acid starvation [1] or chloramphenicol addition to vegetative cells of *Bacillus subtilis* [2] has been reported. In addition, carbon starvation causes accumulation of the highly phosphorylated nucleotide adenosine-5',3'(2')-bis-triphosphate (p_3Ap_3) in the sporogenous wild-type strain 60015 [3]. In sporulating cells of *B. subtilis* accumulation of additional highly phosphorylated nucleotides, namely adenosine-5'-3'(2')-bis-diphosphate (p_2Ap_2) and adenosine-5'-triphosphate–3'(2')-diphosphate (p_3Ap_2), have been observed after nutritional shift down [4]. Appearance of these substances has been correlated with sporulation [5].

Cyanobacteria such as *Anacystis nidulans* and *Anabena cylindrica* have been reported to synthesize ppGpp and pppGpp upon nitrogen starvation [6]. A similar physiological role of these unusual nucleotides as in *Escherichia coli* has been suggested also for the cyanobacteria.

Here, we report the accumulation of unusual, highly phosphorylated nucleotides upon nutritional shift down of *Tolypothrix* sp., which induces heterocyst formation. These substances co-chromatograph on polyethylene-imine thin-layer chromatograms (PEI) and in high-pressure liquid chromatography systems (HPLC) with chemically synthesized p_2Ap_2 and p_3Ap_3 (submitted).

* Visiting Scientist from the Department of Microbiology, MK-University, Madurai, India

2. MATERIALS AND METHODS

2.1. *Bacterial strain*

Tolypothrix sp. was obtained from the culture collection of MK-University, Madurai.

2.2. *Media and growth conditions*

The growth medium used was that in [7]. Nitrogen-free medium contained no source of combined nitrogen [8]. Phosphate-free medium contained all salts listed in [7] except phosphate. Cells were grown in Allen's medium as still culture at 28°C with 1000 lux light intensity at the surface of the culture.

Ten-day-old cultures that normally do not contain any heterocysts, were shifted to phosphate and nitrogen free medium by repeated (3 times) washing and resuspension in the same medium. After 24 h incubation, ¼ vol. of 5 mM potassium phosphate was added and incubated for 4 more days under nitrogen starvation conditions to allow completion of heterocyst formation.

2.3. *Labelling and extraction of highly phosphorylated nucleotides*

Cultures incubated for 24 h in nitrogen and phosphate starvation medium (see above) received $H_3{}^{32}PO_4$ at 500 μCi/ml and incubation continued for 4 days. Aliquots of 100 μl were removed at different times and added to an equal volume of 2 M ammonium formate. After incubation for 60 min at 0°C and subsequent centrifugation at 4°C (Eppendorf Microcentrifuge, 4 min or Sorvall SS 34, 10 000 rev./min, 5 min), the clear supernatant was

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spotted on PEI thin-layer plates and developed as in [3]. Chemically pure reference substances like ATP, GTP, ppGpp, pppGpp, p_2Ap_2, p_3Ap_2 and p_3Ap_3 were used to identify all spots. Autoradiograms were prepared as in [3].

2.4. *Large scale purification of highly phosphorylated nucleotides*

Either a 6 litre 10-day-old culture grown in Allen's medium (no heterocysts detectable) or a 6 litre culture starved for nitrogen and phosphate as in section 2.2 (large numbers of heterocysts present) were centrifuged and resuspended in 300 ml medium devoid of fixed nitrogen. This was immediately treated with 300 ml 2 M formic acid at 0°C for 60 min. The clear 40 000 × *g* supernatant was diluted 10-fold and passed over a column (30 × 3 cm) of 10 g DEAE–Sephadex A25 equilibrated with 0.1 M ammonium formate (pH 3.4). The column was washed with 500 ml each of 0.1, 0.2, 0.3, 0.4 and 1 M ammonium formate (pH 3.4).

Each wash was collected separately and flash evaporated to a minimal volume (just before the salt begins to precipitate). The remainder was treated with an equal volume of 100% ethanol and left for 24 h at −20°C. The pellet obtained after centrifugation at 40 000 × *g* (20 min) was dried in vacuo and then dissolved in a minimal volume of double distilled water.

2.5. *Identification of highly phosphorylated nucleotides by HPLC*

DEAE–Sephadex fractionated extracts of *Tolypothrix* sp. were analyzed using a Varian model 5060 instrument. A micro Pak SAX-10 anion-exchange column was injected with 10 μl extract and isocratically eluted with a phosphate–water mixture (1.5 M KH_2PO_4:H_2O, 25:75, v/v) at 2 ml/min and 100 atm. A guard column (Varian) was used to protect the SAX column from contaminating macromolecules.

2.6. *Characterization of degradation products of highly phosphorylated nucleotides*

Aliquots of substances identified as p_2Ap_2, p_3Ap_2 and p_3Ap_3 by comparison with authentic substances by thin-layer chromatography and HPLC were treated with either bacterial alkaline phosphatase or 1 M HCl at 100°C for 2 h. The hydrolysates were analyzed by HPLC on a Mikro Pak MCH-10 reversed-phase column using 0.15 M phosphate (pH 3.7):methanol (93:7) as eluant and 2 ml/min flow rate at 100 atm.

3. RESULTS

When 20 μl of an extract labelled with $H_3{}^{32}PO_4$ as above is spotted onto a PEI thin-layer chromatogram and developed with 1.5 M phosphate (pH 3.4) a spot near the origin is clearly visible, which comigrates with chemically synthesized p_3Ap_3 (fig.1). A similar spot can be detected under UV-light, when a sample of 0.5 $A_{260\,nm}$ of the 0.4 M ammonium formate fraction of a culture starved for fixed nitrogen (section 2.4) is chromatographed similarly (not shown). Other spots migrating below GTP and above p_3Ap_3 behave like authentic p_2Ap_2 and p_3Ap_2. Two-dimensional chromatograms of these extracts show after autoradiography the same pattern of spots as described for *B. subtilis* [4] and *Saccharomyces cerevisiae* [9].

These results indicate that *Tolypothrix* sp. synthesizes 3 highly phosphorylated nucleotides upon nitrogen and phosphate starvation, namely p_2Ap_2, p_3Ap_2 and p_3Ap_3.

To confirm this, a sample of the 0.4 M ammonium formate eluate from a 6 l culture starved for fixed nitrogen was precipitated with ethanol and subjected to HPLC analysis using an anion-exchange column. Three peaks were obtained at 2.1 min, 2.3 min and 2.9 min, respectively (see fig.2A). When compared with authentic substances, these peaks correspond to p_2Ap_2, p_3Ap_2 and p_3Ap_3, respectively.

The elution profile for ATP (fig.2B, left peak) at 1.8 min and that of authentic p_3Ap_3 (fig.2B, right peak) at 2.9 min obtained under identical conditions clearly shows that the 0.4 M formate fraction is free of ATP and definitely contains p_3Ap_3.

When purified p_3Ap_3 is added to the *Tolypothrix* extract, the profile shows an increase in the 2.9 min peak (fig.2C).

Additional evidence that the above substances found in formic acid extracts of *Tolypothrix* sp. are the highly phosphorylated nucleotides p_2Ap_2, p_3Ap_2 and p_3Ap_3 comes from enzymatic and acid hydrolysis. Fig.3a shows a mixture of p_2Ap_2 (2.1 min), p_3Ap_2 (2.3 min) and p_3Ap_3 (2.9 min) obtained from *Tolypothrix*. The same mixture was treated at times for 60 min at 37°C with DNase

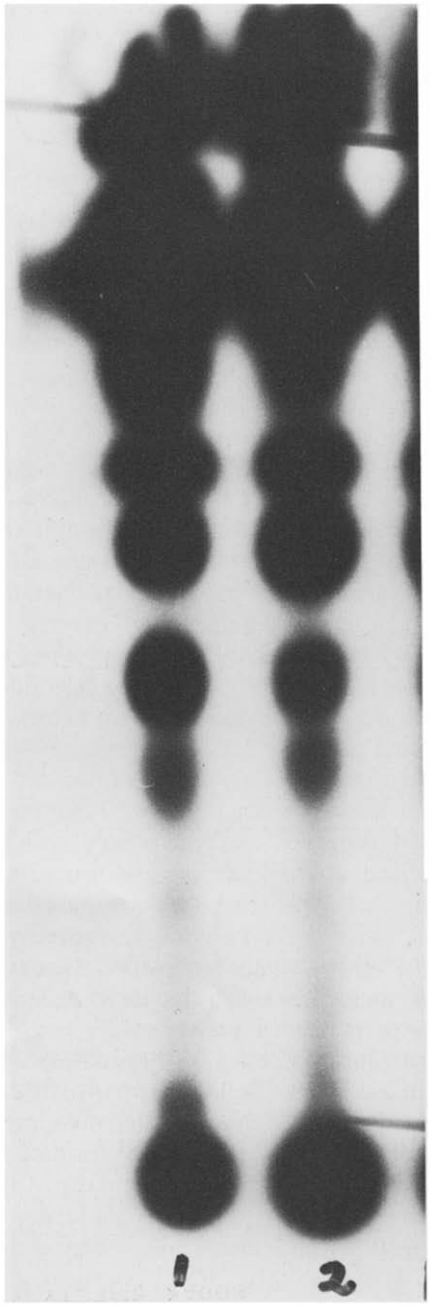

Fig.1. Autoradiogram of a one-dimensional, thin-layer chromatogram of a ^{32}P extract of heterocyst forming *Tolypothrix* sp. cells after 5 days of nitrogen starvation (1) and 24 h phosphate and nitrogen starvation (2). The spot near the origin cochromatographs with p_3Ap_3. The spot near GTP migrates with p_2Ap_2.

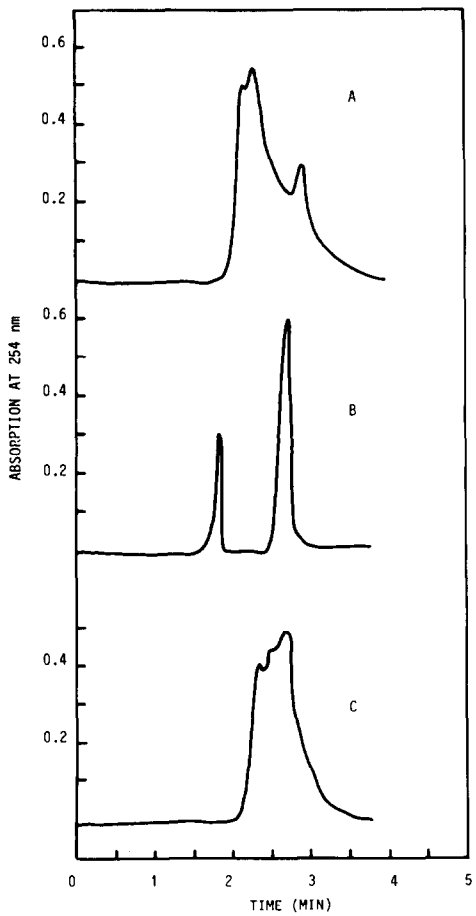

Fig.2. HPLC elution profile of a 0.4 M formate fraction of *Tolypothrix* (A). Comparison with authentic ATP (B, left peak) and p_3Ap_3 (B, right peak) shows that this preparation contains p_3Ap_3 and is free of ATP. Addition of pure synthetic p_3Ap_3 to the above extracts shows (C) that indeed the right peak in the extract (see A) is p_3Ap_3. Further comparison of the two other peaks with synthetic p_2Ap_2 and p_3Ap_2 (not shown) confirms the presence of these substances (p_2Ap_2 is the left peak in A) in the extract.

(fig.3b, 10 units/0.1 $A_{260\,nm}$), RNase (fig.3c, 2 units/0.1 $A_{260\,nm}$) and protease (fig.3d, 0.2 units/0.1 $A_{260\,nm}$). Obviously, these enzymes had no effect, since the pattern remained identical to that of a control experiment (fig.3a). However, when a mixture of p_2Ap_2, p_3Ap_2 and p_3Ap_3 (0.4 M formate fraction) was treated with phosphodiesterase (0.04 units/$A_{260\,nm}$) for 60 min at 37°C the pattern shown in fig.3e is obtained, indicating that phos-

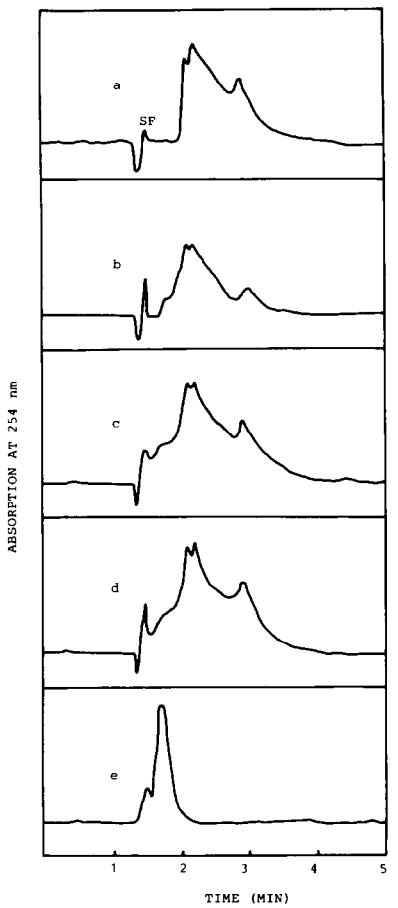

Fig.3. HPLC analysis of reaction products of an 0.4 M formate fraction treated with DNase (b), RNase (c), protease (d), and phosphodiesterase (e). An untreated control is shown in (a). The 3 peaks shown represent p_2Ap_2, p_3Ap_2 and p_3Ap_3, respectively (from left to right).

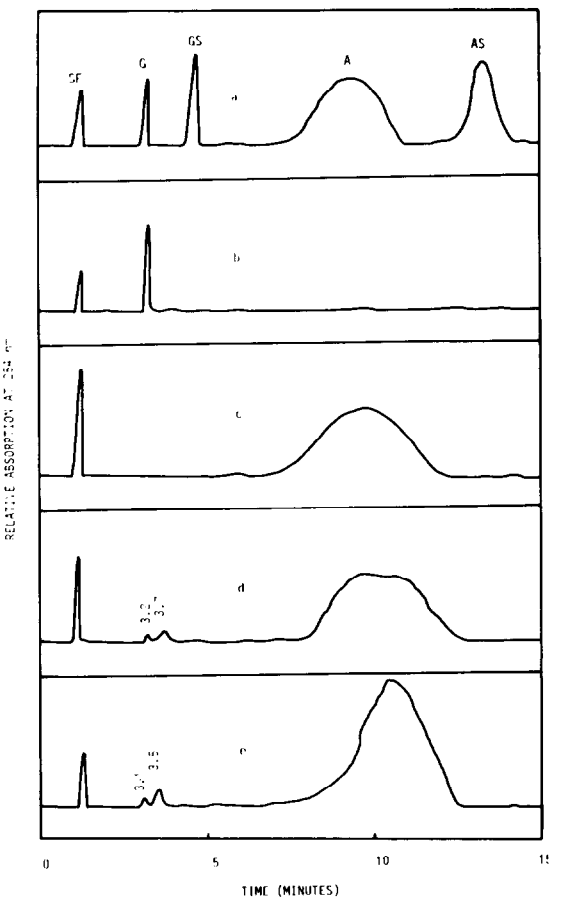

Fig.4. Identification of the acid hydrolysis products from a 0.4 M formate fraction. In (a) the elution profile of authentic substances of guanine (G), guanosine (GS), adenine (A) and adenosine (AS) are shown. SF is the solvent front. The acid hydrolysis profile of p_2Gp_2 and ATP are shown in (b) and (c), respectively. Acid hydrolysis profiles of synthetic p_3Ap_3 (d) and a 0.4 M formate fraction of *Tolypothrix* (e) clearly show that adenine is the only reaction product. There may be some slight contamination by a guanine containing compound in the synthetic preparation of p_3Ap_3. Likewise, the trace amount of guanine in the *Tolypothrix* extract may be due to some ppGpp.

phodiester bonds are cleaved by phosphodiesterase in mixtures of natural highly phosphorylated nucleotides. Similar results are obtained when synthetic highly-phosphorylated nucleotides were treated as above [10].

Further evidence that the 3 substances isolated from *Tolypothrix* sp. are p_2Ap_2, p_3Ap_2 and p_3Ap_3 comes from acid hydrolysis data. In fig. 4a, an elution profile of a mixture of commercially available guanine (G), guanosine (GS), adenine (A) and adenosine (AS) upon separation by HPLC using a reversed-phase chromatography column (section 2) is shown. Fig.4b and 4c show the position of elution of guanine and adenine alone, respectively. Fig.4c is indistinguishable from a 1 N HCl (100°C, 2 h) hydrolysis profile of synthetic p_2Ap_2, p_3Ap_2 or p_3Ap_3.

When a 0.4 M formate fraction was hydrolyzed by 1 N HCl (100°C, 2 h), a profile was obtained

(fig.4d) showing that adenine is released upon acid hydrolysis. This was confirmed, by adding authentic adenine to this reaction mixture and separating the products by HPLC (fig.4e). There were traces of a substance migrating like guanine (3.2 min). This was probably due to traces of ppGpp which have been shown to be produced transiently in cyanobacteria shortly after nitrogen starvation [6]. Since our 0.4 M formate extract was prepared from *Tolypothrix* starved for at least 24 h, ppGpp and/or pppGpp were obviously present only in trace amounts. The predominating unusual nucleotides are quite obviously highly phosphorylated nucleotides containing the adenine residue.

When the 0.4 M formate extract of a 6 litre culture of *Tolypothrix* sp. grown in Allen's medium for 10 days (section 2.2., no heterocysts visible) was analyzed as above, we were unable to detect the peak at 2.9 min shown in fig.2A, indicating that p_3Ap_3 is not present in *Tolypothrix* growing in a medium with phosphate and fixed nitrogen.

We conclude, therefore, that starvation as described here, causes synthesis of p_2Ap_2, p_3Ap_2 and p_3Ap_3.

4. DISCUSSION

Certain strains of cyanobacteria can form heterocysts upon nitrogen starvation, a process which may be regarded as a simple form of differentiation. Since the highly phosphorylated nucleotide p_3Ap_3 has been detected in differentiating cells of pro- (*B. subtilis*) and eukaryots (*S. cerevisiae*) and its synthesis correlated with sporulation, it was of interest to see, whether or not cyanobacteria may contain similar substances. This may be important in the investigation of the molecular mechanism of heterocyst formation.

Our finding that p_3Ap_3 can indeed be detected in heterocyst forming cells of *Tolypothrix* sp. is a first indication that p_3Ap_3 may play a role in the regulation of heterocyst formation and sporulation in cyanobacteria. However, more work is necessary to clarify this.

It was our aim to show unequivocally the presence of p_3Ap_3 in *Tolypothrix*. By means of isolating p_3Ap_3 by column chromatography from a 6 litre culture of *Tolypothrix*, we were able to identify p_3Ap_3 in this cyanobacterium by comparison with chemically synthesized p_3Ap_3 (submitted). HPLC and thin-layer chromatography are ideal tools for these investigations. Using different systems, like anion-exchange and reversed-phase columns, for comparing elution profiles of synthetic and natural p_3Ap_3 and for comparison of enzymatic and chemical hydrolysis products of p_3Ap_3 with commercially available adenosine and adenine, we were able to show that it is possible to isolate ~ 0.3 μg p_3Ap_3 from ~ 5 g (wet wt) of heterocyst forming *Tolypothrix*.

Since we were unable to find p_3Ap_3 in *Tolypothrix* growing in the presence of fixed nitrogen (which does not allow heterocyst formation) a correlation between p_3Ap_3 synthesis and heterocyst formation seems to exist.

An investigation to further correlate p_3Ap_3 formation with heterocyst formation is in progress.

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