Changes in guanosine tetraphosphate (ppGpp) level during nitrogenase expression in the phototrophic bacterium *Rhodopseudomonas palustris*

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Guanosine tetraphosphate (ppGpp) levels were followed in the non-sulfur purple bacterium *Rhodopseudomonas palustris* during derepression and repression of nitrogenase, and during nitrogen starvation which leads to overproduction of this enzyme. Conditions of *nif* gene activation were characterized by a rise in the ppGpp pool size ~90 min prior to commencing nitrogenase activity, whereas the opposite conditions caused a rapid depletion of ppGpp. The kinetic behavior of this nucleotide was, thus, in accordance with a possible regulatory role in *nif* gene expression. The correlation of ppGpp synthesis and degradation with the nitrogen-nutritional status indicated existence of stringent control in this photosynthetic bacterium.

Nitrogen fixation *Nitrogenase regulation* *Photosynthetic bacteria* *Guanosine tetraphosphate* Rhodopseudomonas *Stringent control*

1. INTRODUCTION

Nitrogenase [reduced ferredoxin:dinitrogen oxidoreductase (ATP-hydrolyzing), EC 1.18.2.1] is the key enzyme in supplying a diazotrophically growing prokaryote with reduced nitrogen as source for nitrogenous cell constituents. The cellular level of this enzyme may be gradually adjusted through an antagonistic control system of genes *nifA* and *nifL*, ranging from universal repression in cells provided with sufficient ammonia, to overproduction or 'hyperinduction' in cells deprived of nitrogen [1–3]. Nitrogen starvation elicits in stringent bacterial strains a composite response which involves, among other things, increased formation of ppGpp, changes in RNA synthesis and protein turnover, and stimulation or inhibition of the synthesis of many proteins [4]. Since nitrogenase expression is closely dependent on the nitrogen-nutritional status of the cell, we have studied in *Rhodopseudomonas palustris* (for which we had demonstrated overproduction of nitrogenase under N-starvation [3]), whether the level of ppGpp in this bacterium would undergo changes during nutritional shifts and parallel those leading to *nif* gene activation or shut-off, respectively.

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Abbreviation: ppGpp, guanosine 3′-diphosphate 5′-diphosphate

2. MATERIALS AND METHODS

2.1. *Organism and growth conditions*

Rhodopseudomonas palustris (ATCC 17001) was grown under described conditions as diazotrophic or N-limited culture in a basal

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medium for Rhodospirillaceae [3]. Tris (0.6 g/l) substituted phosphate as buffering component to achieve a better partitioning of $^{32}P_i$ during nucleotide labeling.

2.2. *Labeling procedure*

Samples of cell suspension were usually incubated for 6 h in the light (10000 lux) under N_2 or Ar, with 1.87 mBq carrier-free $^{32}P_i$/ml (Amersham, Braunschweig) to allow sufficient labeling of the cellular nucleotide pool. To assay for nitrogenase activity, unlabeled samples were prepared in parallel and subjected to identical treatments.

For derepression of nitrogenase, 500 ml of a 3-day-old ammonia culture (0.5 g NH_4Cl/l) was centrifuged (20 min, 10800 × *g*), and the cell pellet resuspended under N_2 in 5 ml yeast extract-free medium. A sample from this cell suspension was further diluted 10-fold in the above medium and was used with the additions described in section 3 for experiments.

For overproduction of nitrogenase, a sample of a 2-day-old N_2-grown culture was divided after ^{32}P-labeling into two parts that were kept either under N_2 (control) or under Ar (for N-starvation).

Nitrogenase repression was followed in 2-day-old N_2-grown and in N-limited cultures [3]. In each case, 5 ml cell suspension was directly withdrawn from the growth flask, labeled for 6 h, and nitrogenase repression initiated by making the sample 7 mM in NH_4Cl. The atmosphere during the incubation period was N_2 for the N_2-grown cell sample and Ar for the N-limited cell sample.

2.3. *Extraction and separation of nucleotides*

Samples of 100 μl cell suspension were injected into 50 μl ice-cold HCOOH (98%) and flash-frozen in liquid N_2 for storage. After thawing, they were extracted in the cold for 15 min and the cells were pelleted by centrifugation (2 min at 13000 × *g*). Supernatant (10 μl) was spotted onto polyethyleneimine (PEI) cellulose plastic sheets (Macherey and Nagel, Düren) and developed in one dimension with 1.5 M KH_2PO_4 (pH 3.4) [5]. Centrifugation of the cell suspension prior to formic acid extraction was avoided, because of possible light-dependent changes in ppGpp pool size [6]. The lysozyme/desoxycholate procedure of [7] was not superior to formic acid extraction [8,9] for this organism. For two-dimensional chromatography the PEI-cellulose sheet was developed in the first dimension with 1.5 M LiCl in 2 M HCOOH [10], dried with warm air and washed for 60 min in methanol [11]. After drying again, the sheet was developed as for one-dimensional chromatography. Nucleotides were localized by their fluorescence, or by 2–4-day exposure on X-ray film (Kodak X-AR). Radioactive ppGpp spots were cut from the foil and counted with ready-solve (Beckman, München) in a liquid scintillation counter.

3. RESULTS AND DISCUSSION

3.1. *Identification of ppGpp*

The presence of ppGpp in photosynthetic prokaryotes and its possible role as messenger signal of nutritional or energy deprivation, has been demonstrated unequivocally only in the cyanobacteria (e.g., [9,12]). Light-dependent changes of the level of an oligo-phosphorylated nucleoside that migrated like ppGpp in one-dimensional thin-layer chromatography, had been reported for *Rhodopseudomonas sphaeroides* [6]. However, the presumptive ppGpp was chromatographically poorly separated from material remaining at the starting point and reliance on only one-dimensional chromatography for the identification of a nucleotide had been repeatedly criticized [4,13,14]. This caution appears justified for phototrophic bacteria when one considers the detection of an unidentified highly phosphorylated nucleoside in *Rhodospirillum rubrum* (possibly a modified adenosine dinucleotide), that co-migrated with ppGpp [15].

Fig.1 shows the resolution of ppGpp in extracts from *R. palustris* by two-dimensional chromatography. The nucleotide, tentatively identified as ppGpp by one-dimensional separation in the solvent of [5], migrated as a single spot also in the second dimension and co-chromatographed with authentic ppGpp (PL-Biochemicals, St Goar). The unidentified nucleotide described for *R. rubrum* was not observed, possibly due to its high instability, as suggested [15]. Therefore, we were able to rely on one-dimensional separation, and will show changes of the level of ppGpp during transitions of the nutritional status of *R. palustris* with respect to nitrogen.

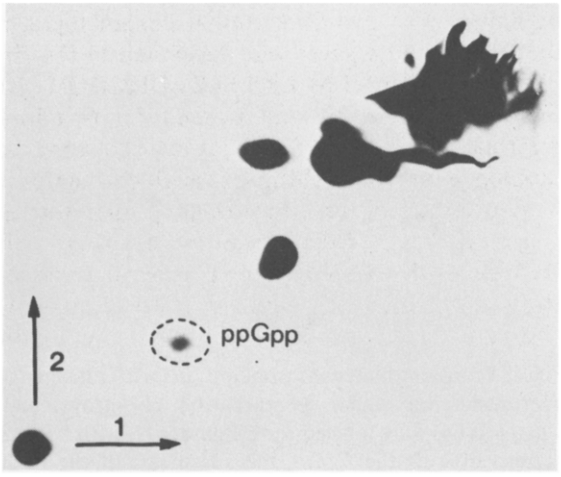

Fig.1. Resolution and identification of ppGpp in *R. palustris* by two-dimensional thin-layer chromatography. A formic acid extract of N_2-grown and $^{32}P_i$-labeled cells was spotted together with authentic ppGpp onto PEI-cellulose sheets and was co-chromatographed as in section 2. The location of authentic ppGpp was determined by its fluorescence in ultraviolet light (dotted circle); radioactive nucleotides were localized by autoradiography (exposure time 69 h).

3.2. *Levels of ppGpp during nitrogenase derepression*

Cultures supplied with a limiting amount of ammonia deplete this nitrogen source during growth and become starved for reduced nitrogen. This situation is comparable to a nutritional shift-down by depriving a heterotrophic bacterium of amino acids, which elicits the stringent response [4]. Fig.2 shows the time-resolved increase in the ppGpp level when ammonia-limited cells were shifted to N_2 as nitrogen source. The quantitative relation in the level of ppGpp and nitrogenase expression is represented in fig.3. About 5.6 h after commencement of the experiment, ppGpp started to increase and stabilized within 80 min at a 2.5-fold higher level. The ppGpp concentration in a control sample with 5 mM ammonia remained unchanged. Nitrogenase was detected 80–90 min after the ppGpp pool began to increase, and its level continued to rise linearly over the time of observation (fig.3). Resuspension of the cell sample under N_2 with 0.75 mM NH_4Cl and addition of $^{32}P_i$ was taken as zero time in this experiment. In that way the small amount of ammonia prevented *nif* ex-

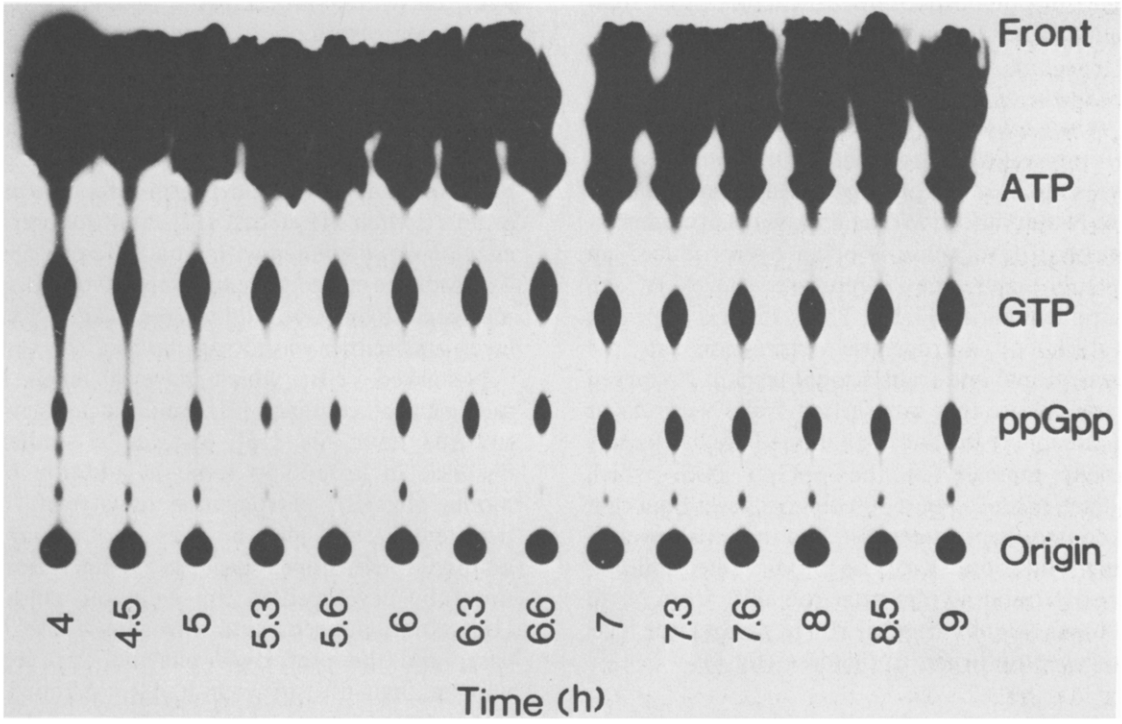

Fig.2. Changes in and resolution of ppGpp by one-dimensional thin-layer chromatography during derepression of nitrogenase. Experimental conditions as in section 2. The time scale denotes the time elapsed after start with $^{32}P_i$-labeling (see section 3 and fig.3).

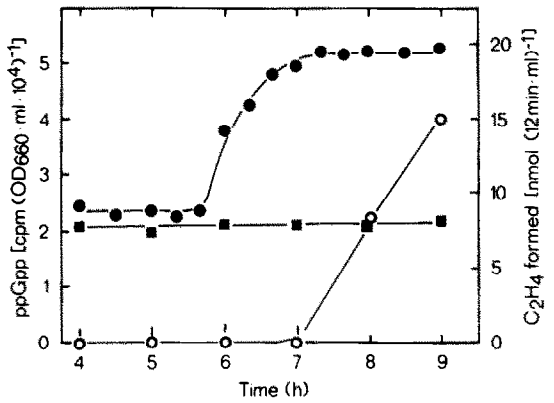

Fig.3. Kinetics of ppGpp pool changes and of nitrogenase derepression in *R. palustris* shifted from ammonia to N_2. Experimental conditions as in sections 2 and 3: (●) kinetics of ppGpp under conditions of nitrogenase derepression; (○) kinetics of development of nitrogenase activity under the same conditions; (■) level of ppGpp in a control sample with 5 mM NH_4Cl.

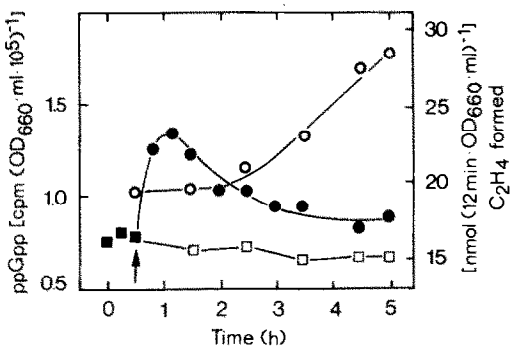

Fig.4. Transient increase in ppGpp, prior to nitrogenase overproduction under N-starvation. N_2-grown cells (A_{660} = 0.64) were labeled for 6 h under N_2 with $^{32}P_i$ and at zero time of the figure, measurements of the initial level of ppGpp (■) were started. After 30 min (→), samples of 5 ml were injected into an Ar- (●) or N_2-filled (□) vial, and measurements of the ppGpp level were continued at the indicated time intervals. Nitrogenase activity (○) was assayed in a parallel, unlabeled sample kept under Ar.

pression for ~6 h. During that time the internal nucleotide pool was sufficiently equilibrated with $^{32}P_i$ and further manipulation of the cell sample was avoided, which could have superimposed changes in ppGpp pool due to factors other than the shift from ammonia to N_2.

3.3. *Levels of ppGpp during overproduction of nitrogenase*

Withdrawal of N_2 from a culture without alternative source of nitrogen produced conditions of severe N-starvation. We had shown that under N-starvation, nitrogenase is being overproduced up to 8-fold over the derepressed level of an N_2-supplied culture [3,16]. Thus, there is a specific activation of nitrogenase expression at the transcriptional and translational level in N-starved cells. A culture that was shifted from N_2 to Ar as gas atmosphere produced an immediate but largely transient increase in the ppGpp pool which stabilized after 2 h at a 30% higher level than that of a control kept under N_2. The maximal level of ppGpp was about twice the initial value, and was reached around 40 min after the shift from N_2 to Ar. About 90 min after the rise in ppGpp, the level of nitrogenase began to increase (fig.4).

3.4. *Levels of ppGpp during nitrogenase repression*

Since activation of the *nif* genes under conditions of derepression and overproduction were both accompanied by increases in the ppGpp pool size, one would expect for a regulatory involvement a decrease in pool size under conditions of *nif* genes deactivation. Fig.5A shows the fast decrease of the ppGpp level on ammonia addition to a N_2-fixing (C_2H_2-reducing) culture to about half its level. Nitrogenase activity was inhibited immediately due to the short-term inhibition by ammonia (switch-off effect) [17], and the preformed enzyme was degraded with a half-life of ~58 h [3]. Towards the end of the experiment the ppGpp level increased slightly, but did not reach again its initial level, and neither was nitrogenase activity restored.

N-starved cells, which have a much higher nitrogenase content [3], and lack sensitivity towards ammonia [18], showed a similar fast decrease in its ppGpp level on addition of ammonia (fig.5B). Nitrogenase activity of the experimental cell batch was not completely ammonia-insensitive, because N-starvation was not fully developed in the 2-day-old culture yet [18]. Ammonia repressed nitrogenase also in this case, and the preformed enzyme was degraded with a half-life of ~98 h [3]. Although the qualitative response of the ppGpp level showed a variable pattern, above all at the onset of nitrogenase overproduction, the initial rates of its

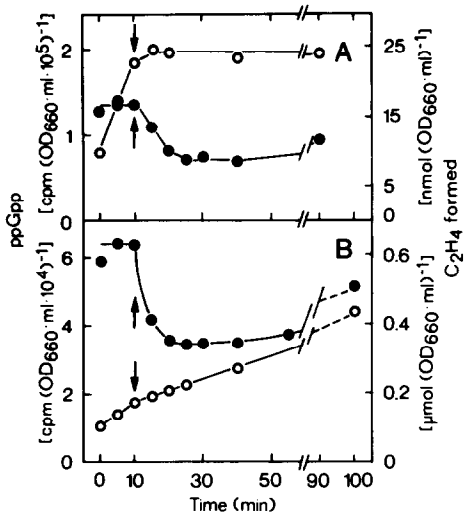

Fig.5. Kinetics of ppGpp pool changes and of nitrogenase activity during repression of enzyme synthesis by ammonia. Batches of 2-day-old N_2-grown (A) and N-limited cultures (B) were labeled for 6 h and at zero time of the figure determinations of the level of ppGpp (●) and of nitrogenase activity (○) were started. After 10 min (→), both samples were made 7 mM in NH_4Cl. N_2-grown cells showed the regular switch-off effect for nitrogenase activity, and N-limited cells were, expectedly, largely ammonia-insensitive as a consequence of N-starvation [3,18].

increase and decrease were comparable under the three conditions studied.

4. CONCLUSIONS

Guanosine 3′-diphosphate 5′-diphosphate was identified in the phototrophic bacterium *R. palustris*, and showed changes in pool size in response to the nutritional state of the cell similar to that of heterotrophic bacteria with stringent response. Addition of ammonia to N_2-fixing bacteria lowers the ppGpp level [19]. In diazotrophic bacteria, including *R. palustris*, the nitrogen-nutritional status is intimately related to expression of the *nif* genes, the detailed mechanism of which is still the subject of intensive investigation in several laboratories. Here, we have shown that the ppGpp level responded to the general nitrogen-nutritional status of the cell, and, more specifically, paralleled *nif* expression in a time frame adequate to indicate a regulatory role for this nucleotide.

While this work was in preparation, the characteristics of *nif* derepression of wild-type *Klebsiella pneumoniae* were compared with those of a *relA* mutant that was defective in stringent control [20]. The *relA* mutant was also defective in *nif* derepression, showing a 4–5-fold lower rate of nitrogenase synthesis. Wild-type *nif* derepression was regained after introducing into the mutant an F′-factor carrying the *relA*$^+$ allele from *Escherichia coli* [20]. The product of the *relA* gene (stringent factor) synthesizes ppGpp from its precursor [21]. Although at present it remains open by precisely which mechanism ppGpp would interact with nitrogenase regulation, one hypothesis envisages it as corepressor of the *nifL* gene product [22]. However, that the role of ppGpp is not conserved in all diazotrophic bacteria is illustrated by *Rhizobium* [23].

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