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Title: Guanosine Tetrophosphate Accumulation in Rhizobium meliloti

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The effect of carbon source, nitrogen source, and amino acid starvation on the accumulation of guanosine tetraphosphate, and RNA and protein synthesis in Rhizobium meliloti are described in this report.

An accumulation of ppGpp and a decrease in the rate of RNA and protein synthesis were observed in response to nitrogen source starvation. This nucleotide also accumulated when cells were starved for carbon or essential amino acids. RNA synthesis was inhibited during serine starvation, but upon methionine starvation, even though high levels of ppGpp were induced, no inhibition of RNA synthesis was observed.

Based on these results, it was concluded that guanosine tetraphosphate is in fact synthesized in R. meliloti, and is probably involved in the regulatory response of this bacterium to carbon or nitrogen source limitation. Furthermore, it was apparent that ppGpp is not directly responsible for the inhibition of RNA synthesis during amino acid starvation.

Guanosine Tetrophosphate Accumulation in *Rhizobium meliloti*

by

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Typed by Mary Ann (Sadie) Airth for Mohammad Saeed Saedi

To my parents
and brother Vahid

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INTRODUCTION

Prevention of aminoacyl-tRNA complex formation in bacteria elicits a regulatory response termed the stringent response (15, 2, 8, 32). This effect is usually exerted on the bacteria by amino acid deprivation (4) or by the use of amino acid analogues (39, 34, 41).

During this response in *Escherichia coli* and *Bacillus subtilis*, two highly phosphorylated guanosine nucleotides accumulate; guanosine-5'-diphosphate-3'-disphosphate (ppGpp) and guanosine-5'-triphosphate-3'-disphosphate (pppGpp) (15). Concurrently, stable RNA (ribosomal and transfer RNA) and protein synthesis are reduced, and the rate of protein turnover is increased (15, 17, 33). Messenger RNA, however, responds heterogenously. The transcription of some messages like the messages transcribed from the *his* and *trp* operons is simulated (44, 30) while others like those for ribosomal proteins is inhibited (24).

Several mutants defective in (p)ppGpp production have been isolated. Known as relaxed mutants, most of these map in the *relA* locus of the genome (45, 41, 13). The product of this gene is the enzyme mediating (p)ppGpp synthesis (5). In addition, mutations mapping within the *relC* (13) and *relB* (31) loci also result in the relaxed phenotype. The former gene codes for the ribosomal protein L11, a protein needed for (p)ppGpp production of the ribosome (35), and mutations in

the latter locus result in the production of an *in vitro* protein synthesis inhibitor during amino acid deprivation (31).

Guanosine tetraphosphate (ppGpp) has a regulatory role in the transcription/translation machinery (15, 2, 32). *In vitro* studies show that ppGpp stimulates transcription of the *his* and *lac* operons (44, 1), and inhibits the transcription of ribosomal proteins (24). However, there are controversial reports about its *in vivo* effects on RNA synthesis (19, 16). Normally as the *in vivo* level of ppGpp increases, RNA synthesis ceases (32, 15). However, as Hansen *et al.* reported (15), after a carbon source downshift reversal in some strains of *E. coli*, RNA synthesis resumed well before any reduction in the ppGpp level was observed. Furthermore, Gallant (16) noticed that certain strains accumulate high levels of ppGpp after a temperature upshift without any reduction in the rate of rRNA or tRNA synthesis. These results show that ppGpp is not the sole element involved in regulating RNA synthesis.

The role of ppGpp in translation has been reviewed by Gallant (15), and he notes that the rate of protein synthesis in *relA*$^-$ strains of *E. coli* under stringent condition is much faster than the *relA*$^+$ parental strains. However, some of the proteins synthesized by the former strains are defective and have some amino acid substitutions. Furthermore, he rationalizes this result by a model which speculates that ppGpp slows down the translation process, preventing the wrong amino acid substitutions, due to codon similarities, in *relA*$^+$ strains. This hypothesis can be further substantiated by

the fact that elongation factor Tu, which is needed for peptide chain elongation, is complexed with ppGpp in *relA*$^+$ cells undergoing the stringent response (37). Moreover, this complex, EF-Tu-ppGpp, is ineffective in forming a ternary complex with aminoacyl-tRNA (22), a process known to be necessary for protein synthesis. It could be postulated that ppGpp slows down the translational event by decreasing the relative amount of EF-Tu available for translation.

Synthesis of ppGpp can also be stimulated by carbon and nitrogen source starvation in *E. coli* (19), and various other prokaryotes (41, 27). This nucleotide still accumulates in *relA*$^-$ strains of *E. coli* in response to carbon source starvation (14, 3); but, as yet, no unusual nucleotide accumulation in *relA*$^-$ mutants of *B. subtilis* or *K. pneumoniae* has been reported (39, 41). This indicates that the stringent response during carbon source limitation in *E. coli* is independent of the *relA* gene. Two genes have recently been reported to be involved in the basal level of ppGpp production during this downshift in *E. coli*: *relX*, which maps close to *relA* (25), and *relS*, which maps near the *lac* operon (39). In *B. subtilis*,however, a locus called *relG*, which maps independent of *relA*, seems to be responsible for these effects (39).

Nitrogen starvation can be accomplished by either shifting the culture to a medium lacking a nitrogen source (20, 9, 27), or by using substrate analogues (41). Reisenberg and Csaba (41) and Gordon and Brill (18) have used methionine sulfoximine (MSX), an analogue of glutamine, to block the activity of glutamine synthetase. The

inhibition of this enzyme induces nitrogen starvation in Klebsiella, which results in ppGpp accumulation (41).

The accumulation of guanosine tetraphosphate has been shown to occur during life cycle transition (9, 27, 28) and induction of nitrogenase (the enzyme mediating nitrogen fixation) biosynthesis in bacteria (42, 22). The former function has been reported in two organisms; Myxococcus xanthus (28) and Caulobacter crescentus (9). Experiments on the former organism show that ppGpp is accumulated and multicellular fruiting body formation is induced in response to amino acid starvation. Nitrogen source starvation in C. crescentus also leads to ppGpp accumulation, and a transition from swarm cell to stalked cell (9). The putative role of guanosine tetraphosphate in regulating nitrogen fixation was first suggested by Kleiner and Phillips (22) when they observed that the addition of ammonia to nitrogen-fixing strains of Azotobacter vinelandii, Klebsiella pneumoniae, and Clostridium pasteurianum resulted in both repression of nitrogenase and reduction of ppGpp within the cells. Furthermore, it was also noticed that if nitrogen was exhausted, both accumulation of ppGpp and nitrogenase derepression occurred in the wild type strains of K. pneumoniae but not in the relA$^-$ counterparts (42). However, minor levels of nitrogenase activity in the latter strains were detected when aspartate and adenine were added during nitrogen starvation.

Rhizobium encounters nutritional downshifts in both soil, its natural habitat, and during plant inoculation, when bacteria grown in rich media are added to soil or to nitrogen free slants. The

regulatory adjustment of the cells to the nutritional limitations is therefore vital to their survival. To determine if ppGpp is involved in this process, the level of this nucleotide was measured during various nutritional downshifts. The other reason for the interest in monitoring the level of ppGpp, was its possible involvement in nitrogenase biosynthesis and life cycle transition. Rhizobium is capable of fixing nitrogen, and the genes responsible for this function show sequence homology to the nitrogenase structural genes of K. pneumoniae (10). In addition, nitrogen fixation can only be induced after R. meliloti has gone through a transition from a bacterium, a free living form, to the bacteroid, a symbiotic form associated with nodules (4).

In this study, the level of ppGpp, and the ability to synthesize RNA were monitored during carbon source, nitrogen source, and amino acid starvation in Rhizobium meliloti. Results showed that the level of ppGpp is increased in response to nitrogen or carbon source deprivation. In amino acid starvation experiments, serine limitation resulted in the inhibition of RNA and protein synthesis and an accumulation of ppGpp. Methionine starvation, however, resulted in an increase in the level of this nucleotide with no immediate effect on RNA synthesis.

MATERIALS AND METHODS

Media

An enriched medium was used, called yeast extract-mannitol (YM), which contained per liter: 0.5 g K_2HPO_4, 3 H_2O, 0.2 g $MgSO_4$, 7 H_2O, 0.1 g NaCl, 3 g yeast extract, and 10.0 g mannitol.

The minimal medium (MN) was composed of per liter: 0.1 g NaCl, 0.2 g $MgSO_4$, 7 H_2O, 0.5 g NH_4Cl, 10 mis vitamin mix (explained below), 10 mls trace elements (explained below), 1.1 g Na glutamate (except for the experiments involving carbon or nitrogen source starvation), 2.0 g mannitol (5.0 g in experiments involving carbon or nitrogen source starvation), 0.026 g K_2HPO_4, and 12.1 g Tris. The trace element solution contained, per liter: 5.0 g $CaCl_2$, 145 mg H_3BO_3, 125 mg $FeSO_4$, 7 H_2O, 70 mg $CoSO_4$, 7 H_2O, 5.0 mg $CuSO_4$, 5 H_2O, 4.3 mg $MnCl_2$, 4 H_2O, 108 mg Zn SO_4, 7 H_2O, 146.25 mg Na_2MoO_4, 2 H_2O, and 239 mg FeEDDHA. The vitamin mix contained 20 mg of: Riboflavin, PABA, nicotinic acid, biotin, thiamine HCl, Pyridoxine HCl, Ca pantothenate, and inositol per one liter of 0.01 M Na_2HPO_4.

Nitrogen free nodulation media (NT) contained per liter: 1.0 g $CaHPO_4$, 0.2g K_2HPO_4, 0.2 g $MgSO_4$, 7 H_2O, 0.2 g NaCL, 0.1 g $FeCl_3$, 15.0 g Bacto Agar, and 1 ml trace elements (explained below). Trace elements for NT media: had per liter 0.28 of H_3BO_3, 0.18 g $MnCl_2$, 0.022 g $ZnSO_4$, 7 H_2O, 0.013 g Na_2MoO_4, 2 H_2O and 0.008 g $CaSO_4$.

Strains

The bacterial strains used in this study are listed in Table I.

Materials

Serine hydroxamate (SHX) and methionine sulfoximine (MSX) were purchased from Sigma, ^{32}P orthophosphate from New England Nuclear (NEN), ^{3}H-leucine and 5-^{3}H-uridine from Amersham, polyethyleneimine impregnated plates (PEI-cellulose) from Brinkmann Inc., and O-succinylhomoserine from Calbiochem. O-acetylhomoserine was obtained from Dr. Leo Parks.

Mutagenesis and Mutant Selection

Cells were grown in MN media to a density of 1×10^{8} cells/ml. Mutagenesis was accomplished by exposing the culture to U.V. light at an intensity of 60 $\mu w/cm^{2}$ and counter selecting for auxotrophs by carbenicillin treatment at 1 mg/ml, using the method described by Miller (29).

Isolated colonies were obtained, spotted on MN + methionine master plates, and the Met^{-} mutants were detected by their ability to grow on MN+ methionine but not on MN.

Infectivity, Effectivity and the Reisolation of the Cells

Infectivity of strains was established by the cells' ability to form nodules on alfalfa sprouts. Alfalfa seeds were first sterilized

Table I

Rhizobium meliloti Strains and Phenotypes

Strain	Genotype	*Phenotype	Source
C15	W.T.	Nod^+, Nif^+	O.S.U. culture collection
104A13	W.T.	Nod^+, Nif^+	O.S.U. culture collection
C15M1	*met*$^-$	Nod^+, Nif^+	This study
C15M5	*met*$^-$	Nod^+, Nif^+	This study
RM41	W.T.	Nod^+, Nif^+	Dr. O'Gara
RM41H1	*his*$^-$ *leu*$^-$	Nod^+, Nif^+	Dr. O'Gara

*Nodulation (Nod) and nitrogen fixation (Nif) capability

by washing for one minute with 95% ethanol and for 3 minutes with 1.2% mercuric chloride. Seeds were then washed with sterile water, and allowed to sprout on sterile agar plates (15 g Bacto Agar per liter of water) for 24 hours. Each sprout was then transferred to NT slants and incubated with one ml of an overnight culture (ca. 5×10^8 cells in YM media) of the desired strain. Infective (Nod^+) strains caused visible root nodules 3 weeks after inoculation.

Nitrogen fixation effectivity was established by comparing the size of the inoculated and uninoculated alfalfa plants, 5 weeks after inoculation. Plants inoculated with effective strains (Nif^+) were consistently larger and greener than the non-inoculated plants.

To re-isolate the bacterial cells, nodules were first separated from the plant roots and sterilized with 95% ethanol for one minute and 1.2% mercuric chloride for 3 minutes. These nodules were then washed with sterile water and opened in the YM media which was supplemented with 10% mannitol. Individual colonies were finally isolated by plating this suspension on YM media.

Extraction and Quantitation of Nucleotides

For measurements of nucleotides, cells were grown in the presence of 300-400 μCi/ml of ^{32}p-orthophosphate for 2.5 generations. After imposing various treatments, samples were removed and extracts were prepared by adding formic acid to a final concentration of 0.1 N.

After 45 minutes on ice, the extracts were neutralized (pH 7.0) by adding Tris, pH 8.0, to a final concentration of 0.2 M.

Nucleotides were resolved by one dimensional thin layer chromatography (TLC) on PEI-cellulose plates using 1.5 M K_2HPO_4, pH 3.4, or two dimensional TLC using 4M HCOOH and 1M LiCl, in the first dimension, and 1.5 M KH_2PO_4, pH 3.4, in the second dimension (40).

Labeled nucleotides were located by autoradiography using a Kodak no-screen X-ray film and identified by co-migration with standards. Appropriate spots were cut out, and radioactivity was measured by a liquid scintillation counter (Beckmann L5800) using 5 mls of a toluene-base scintillation fluor. Specific activity of the medium was measured by counting a small portion of the sample along with each set of experiments, and adjustment for background was made by cutting out an adjacent equal-sized spot from the chromatogram.

RNA and Protein Synthesis

RNA synthesis was monitored by labeling logarithmically growing cultures with 5-^{3}H-uridine (at a final concentration of 3 µCi/ml with 25 µg/ml non-radioactive uridine as carrier) and allowing the pools to equilibrate for 30 minutes. Selected treatments were then employed by the addition of 2 mg/ml of various analogues (in case of analogue treatments), or centrifuging and resuspending the cells in media without the appropriate nutrient (in case of nutrient starvations). Samples (0.1 ml) were then removed at the times indicated, added to an equal volume of 10% TCA (trichloroacetic acid), and left

on ice for one hour. TCA precipitable material was then isolated by filtering each sample on a glass fiber prefilter (Millipore). The radioactivity (cpm) of each sample was measured by a Beckmann scintillation counter using 5 mls of a toluene-base scintillation fluor.

Protein synthesis was measured by labeling the cells with ^{3}H-leucine (at a final concentration of 0.5 μCi/ml with 30 μg/ml non-radioactive leucine as carrier) and monitoring the radioactivity of TCA precipitable material as described above.

RESULTS

Isolation and Characterization of Methionine Auxotrophs

To monitor the physiological response of the cells to amino acid starvation, methionine auxotrophs were isolated. Strain C15 was chosen because of its relatively short generation time of 4 hours in MN medium. This strain was grown in MN, mutagenized, enriched for the auxotrophic marker, colonies spotted on master plates, and the colonies replica plated to screen for the Met^- phenotype. Among the 400 colonies tested, two methionine auxotrophs, C15M1 and C15M5, were isolated and chosen for further studies.

To characterize these mutants, their reversion frequency and generation time in MN + methionine were measured. Both auxotrophs exhibited a reversion frequency of 1×10^{-9}, consistent with normal reversion rates of single site mutants. C15M1 had a generation time of 4 hours, which was the same as its parental counterpart (C15). Effectivity and infectivity tests indicated that both strains were Nod^+ and Fix^+ (Table I). Cells reisolated from nodules retained the Met^- phenotype. To further characterize these auxotrophs, the biochemical block in methionine synthesis was determined by monitoring the ability of the strains to grow in MN supplemented with precursors of the methionine biosynthetic pathway (Figure 1). The chemical intermediate between homoserine and cystathionine in methionine biosynthesis is different in various bacteria. In *E. coli*, O-succinylhomoserine is derived from a reaction between

homoserine and succinyl-CoA and catalzed by an acyl transferase (homoserine transsuccinylase) (42), while in B. subtilis, O-acetyl-CoA mediated by homoserine transacetylase (7). Since this intermediate has not yet been identified in Rhizobium, both O-succinyl-homoserine and O-acetylhomoserine were used as supplements. It was found that both strains could grow on 20 µg/ml of methionine, homocysteine or cystathionine, but not on homoserine, O-succinylhomoserine, or O-acetylhomoserine. This suggests that both strains are defective in cystathionine synthesis (step 2, Figure 11).

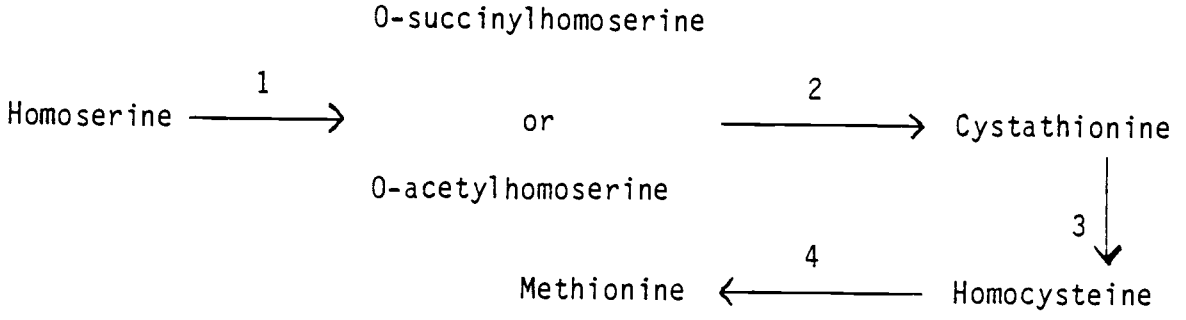

Figure 1. Methionine biosynthetic pathway

Response to Carbon and Nitrogen Source Starvation

In E. coli (19, 20) and K. pneumoniae (41), RNA and protein synthesis decrease and ppGpp accumulates in response to carbon or nitrogen source starvation. In both organisms carbon source starvation was accomplished by treating cultures grown on glucose as their sole carbon source with α-methyl glucoside (αMG), a non-metabolizable analogue of glucose. This system could not be used for Rhizobium

because all of the Rhizobium strains studied showed a very slow growth rate on glucose. Carbon source starvation was therefore exerted by growing C15 in MN and shifting the culture to MN without its sole carbon source, mannitol. Five minutes after this shift, a 30 fold increase in the level of ppGpp was observed (Table II). The level of this nucleotide decreased and finally stabilized at 160 picomoles/O.D. 20 minutes after this shift.

In E. coli, nitrogen starvation has been accomplished by depriving the cells of nitrogen. Nitrogen limitation in Klebsiella (41) has been exerted by treating the cells with methionine sulfoximine (MSX), a glutamine analogue which blocks the activity of glutamine synthetase. Both of these systems were used successfully in our studies. Strain 104A13, the only MSX sensitive strain mentioned in Table I, was grown in MN to mid-log phase and then starved of nitrogen by the addition of 2 mg/ml MSX. Within 5 minutes, a 16 fold increase in the level of ppGpp (Table II) was observed and an immediate decrease occurred in the rates of RNA and protein synthesis (Figure 2). The level of this nucleotide decreased and stabilized at 70 picomoles/O.D. 15 minutes after this treatment. On the other hand, strain C15 was starved of nitrogen by growing it in MN media and depriving the logarithmically growing culture of its sole nitrogen source, NH_4Cl. This shift resulted in a 200 fold increase in the ppGpp level after 10 minutes (Table II). Ten minutes after this increase, the concentration of this nucleotide decreased by half and stabilized at 430 picomoles/O.D.

Table II

Accumulation of Guanosine Tetraphosphate (ppGpp) in strains of Rhizobium meliloti

Strain	Condition	Picomole ppGpp/ O.D. 650 nm		*Time
		Basal	Max	
C15	Carbon source starvation	16	480	5
C15	Nitrogen source starvation	4	900	10
104A13	MSX treatment	7	110	5
C15	SHX treatment	9	30	5
RM41	SHX treatment	8	17	5
C15M1	Methionine starvation	5	150	10
RM41H1	Leucine starvation	7	250	15

*Minutes after the imposition of various treatments at which maximum level of ppGpp was detected.

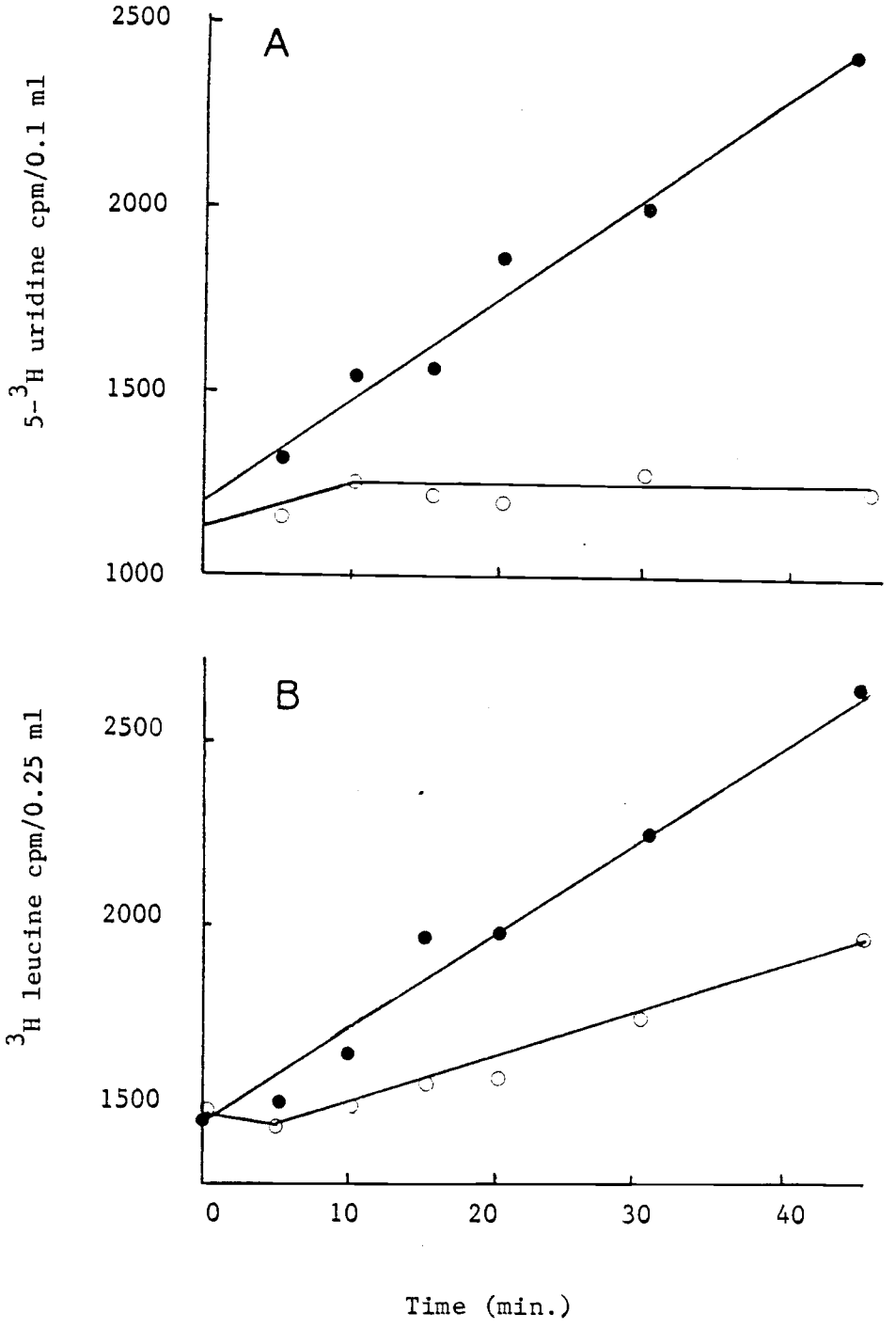

Fig. 2. Net RNA (A) and protein (B) accumulation in strain 104A13 upon treatment with methionine sulfoximine (MSX). The incorporation of labelled compounds were monitored as described in Materials and Methods. At time 0, a portion of an exponentially growing culture in MN was treated with 2 mg/ml MSX. Untreated sample (●), MSX treated sample (○).

Response to Amino Acid Starvation

The regulatory response of Rhizobium to amino acid limitation was monitored by starving the cells for serine, leucine and methionine.

Serine starvation was accomplished by treating two of the serine hydroxamate (SHX) susceptible strains (C15 and RM41) with 2 mg/ml SHX. This compound, a serine analogue, competitively inhibits the charging of $tRNA^{Ser}$ (38), which results in a decrease in RNA synthesis and an increase in the ppGpp level in K. pneumoniae (41). While this treatment caused a cessation in RNA and protein synthesis in C15 (Figure 3) and RM41 (data not shown), only a slight increase in the level of ppGpp was observed (Table II).

To investigate the effects of leucine and methionine starvation, auxotrophic strains of Rhizobium were deprived of the corresponding essential amino acid. Leucine starvation was accomplished by growing RM41H1 in MN supplemented with histidine and leucine to mid-log and then shifting it to medium lacking leucine. Fifteen minutes after this shift, a 30 fold increase in the level of ppGpp was observed (Table II). Upon methionine starvation, accomplished by depriving strain C15M1 of methionine, a 30 fold increase in the level of ppGpp (Table II) and a cessation in protein synthesis (Figure 4B) was observed. However, RNA synthesis continued at the same rate and stopped only after 20 minutes (Figure 4A). This result shows an

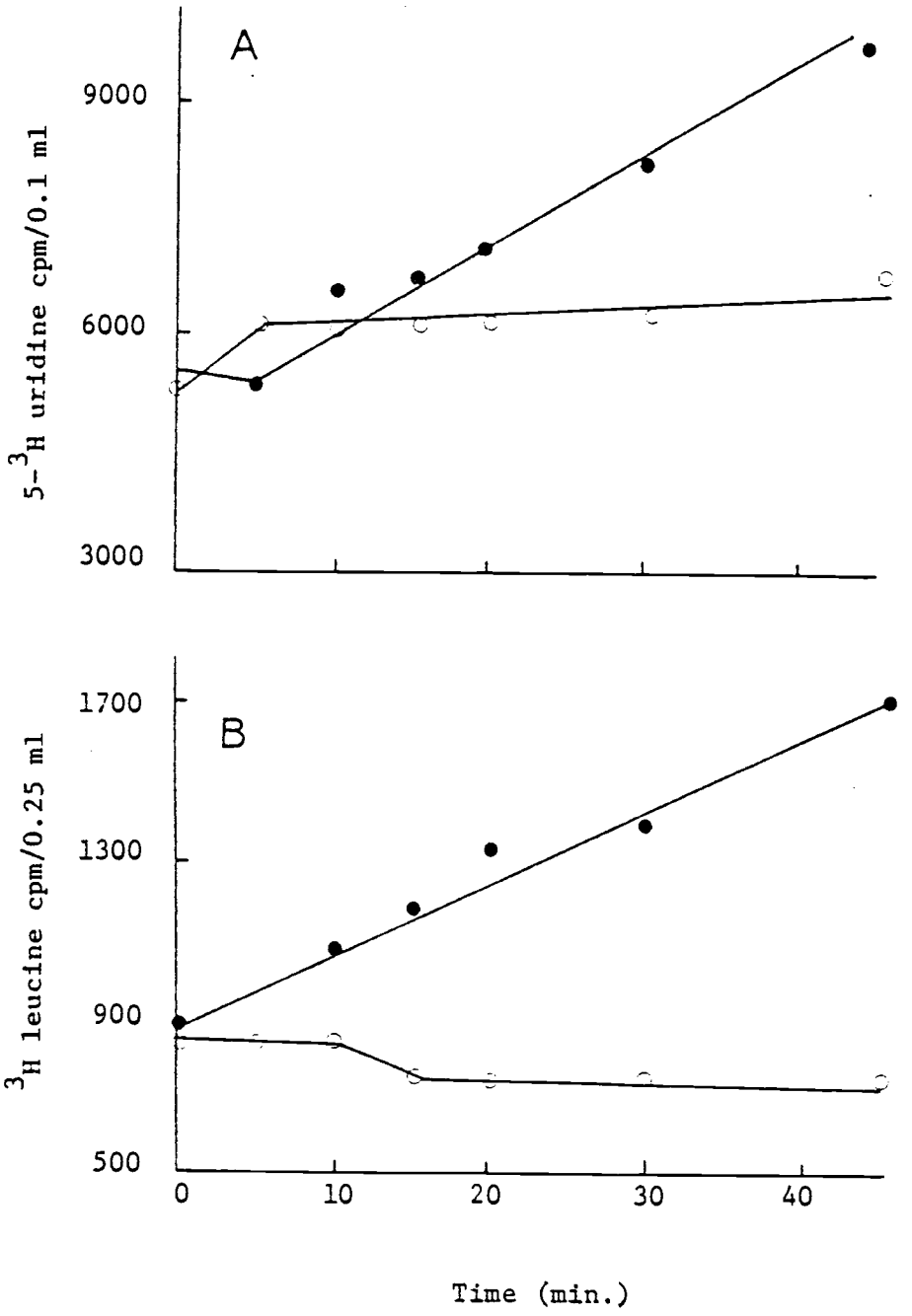

Fig. 3. Net RNA (A) and protein (B) accumulation in strain C15 upon treatment with serine hydroxamate (SHX). The incorporation of labelled compounds were monitored as described in Materials and Methods. At time 0, a portion of the exponentially growing culture in MN was treated with 2 mg/ml SHX. Untreated sample (●), SHX treated sample (○).

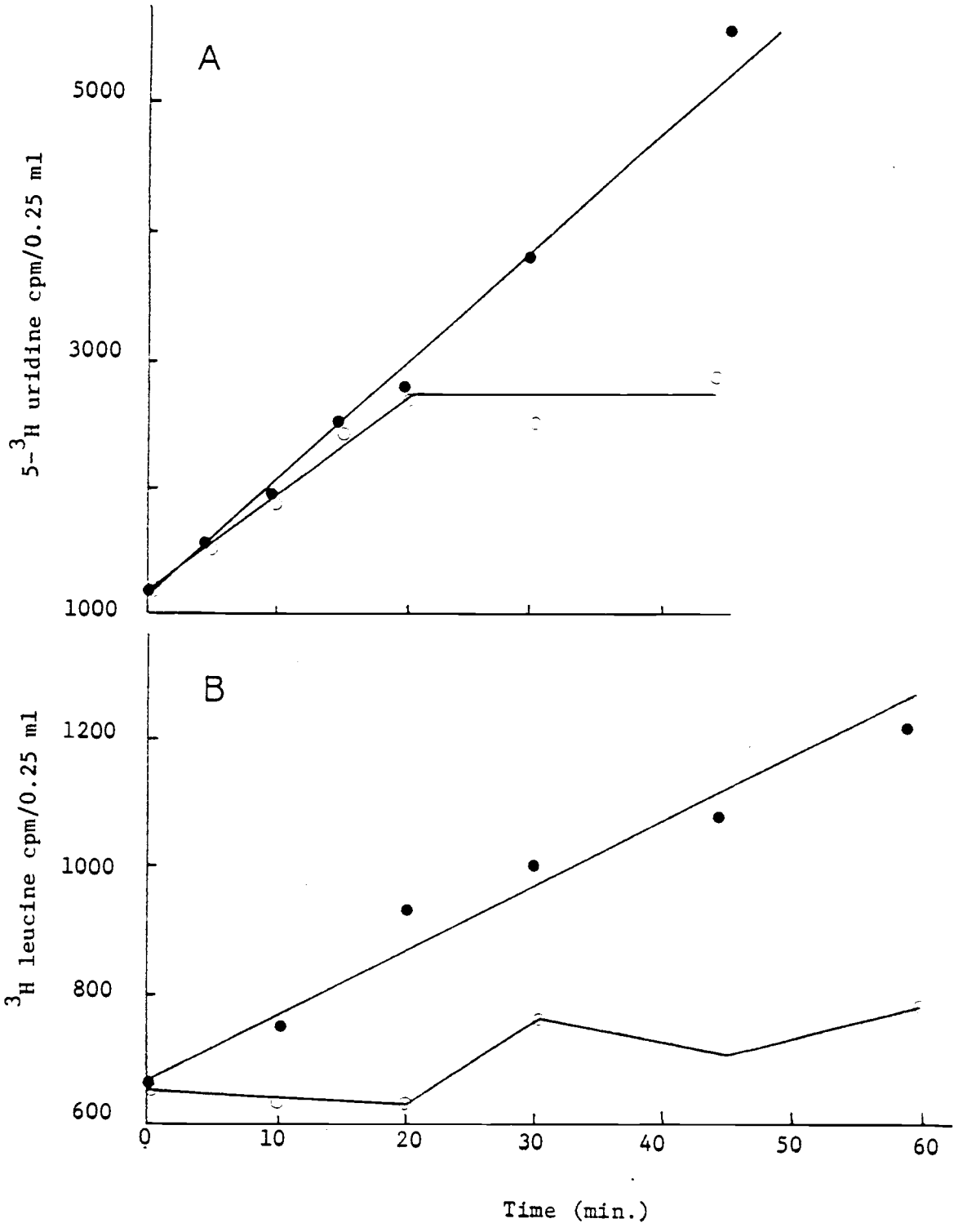

Fig. 4. Net RNA(A) and protein(B) accumulation in strain C15M1 upon methionine starvation. The incorporation of labelled compounds were monitored as described in Materials and Methods. At time 0, a portion of the exponentially growing culture in MN + methionine was centrifuged and resuspended in MN without methionine. Untreated sample(●), starved sample(○)

unusual lack of correlation between ppGpp accumulation and RNA synthesis in C15M1. In experiments with leucine and methionine starvation, the level of ppGpp decreased and finally stabilized. In the leucine limitation, it stabilized at 110 picomoles/O.D. 30 minutes after the shift. In methionine starvation, ppGpp stabilized at 100 picomoles/O.D. 20 minutes after the shift.

Discussion

Guanosine tetraphosphate accumulation and a decrease in RNA synthesis upon amino acid starvation is associated with the stringent response in E. coli and most other prokaryotes. Moreover, the curtailment of RNA synthesis during this response is shown to be simultaneous with ppGpp accumulation in these organisms (15, 2, 8). In the Rhizobium strains studied here, RNA synthesis was inhibited (Figure 4A) and low levels of ppGpp accumulated (30 pico moles/O.D.) (Table II), in response to SHX treatment. However, an internal concentration of 150 pico moles/O.D. of this nucleotide did not have any immediate effect on RNA synthesis (Figure 3A) when C15M1 was starved for methionine. Protein synthesis, on the other hand, was inhibited in both experiments, indicating that the cells did go through an amino acid starvation (Figure 3B and 4B). These results suggest that ppGpp does accumulate in Rhizobium in response to amino acid starvation, but it is not directly involved in the inhibition of RNA synthesis. The reason for the rapid decrease in RNA synthesis upon SHX treatment is not known, but it could be due to other factors produced

in response to the inhibition of $tRNA^{Ser}$ charging. The lack of correlation between ppGpp and the inhibition of RNA synthesis has also been reported in other bacteria by Gallant *et al.* (16) and Hansen *et al.* (19), and has been reviewed in the Introduction.

Ammonia assimilation in bacteria is accomplished through two enzymatic pathways, GDH and GS-GOGAT. GDH (glutamate dehydrogenase) is the enzyme, active in the presence of excess nitrogen, that mediates the conversion of one molecule of 2-ketoglutaric acid to glutamate. The GS-GOGAT system, on the other hand, consists of two enzymes, GS (glutamine synthetase), active only when nitrogen is limited, and GOGAT (glutamate synthase). The action of these two enzymes facilitates the conversion of one molecule of 2-ketoglutaric acid to two molecules of glutamate (25). It has been shown that GS-GOGAT is the only system used for ammonia assimilation in *R. meliloti* (23); therefore, it is possible to starve this bacterium for nitrogen by inhibiting the activity of GS. This task was accomplished by treating strain 104A13 with methionine sulfoximine (MSX). Upon this treatment, an increase in the level of ppGpp (Table II) and an immediate decrease in RNA and protein synthesis (Figure 2) were observed. A 200 fold increase in the level of this nucleotide was also apparent when the cells were deprived of their nitrogen source (Table II). In spite of the same end result (nitrogen source starvation), the deprivation of NH_4^+ entry into the ammonia assimilation pathway in these two treatments is quite different. During treatments with MSX, ammonia can still enter the cell, but is not assimilated; whereas, in depriving

the cell of NH_4^+ as a nitrogen source, no NH_4^+ can be transported inside the cell. The result, showing a ppGpp accumulation upon both treatments, is the same response exhibited in Klebsiella (41, 42), and may indicate that ppGpp is accumulated in Rhizobium in response to the lack of ability to assimilate ammonia.

The role of guanosine tetraphosphate in Rhizobium is not clear. However, concurrent accumulation of ppGpp and inhibition of RNA synthesis during nitrogen starvation, along with the fact that carbon source starvation also resulted in a 30 fold increase in the level of this nucleotide (Table II), imply that ppGpp is involved in the regulatory response of Rhizobium to these nutritional downshifts. This response is vital to the bacteria's survival, and probably involves the regulation of transcription and translation of specific operons. Furthermore, the accumulation of ppGpp in response to nitrogen source starvation in both Klebsiella and Rhizobium, the homology between the structural genes of nitrogenase in the two organisms (10), and the recent discovery of the positive control by ppGpp over the expression of nitrogenase in Klebsiella (42, 22), suggests that this nucleotide may be involved in the regulation of nitrogen fixation in Rhizobium. This conclusion is based on the assumption that the regulation of nitrogen fixation in Rhizobium is similar to that of Klebsiella. However, due to the following physiological differences, the validity of this assumption will rely on further research. There are two types of glutamine synthetase (GS I and GS II), in at least two species of Rhizobium, R. sp. 32H1 (25) and R. japonicum (11)

as opposed to only one type in Klebsiella (42). GSI is analogous to the GS of Klebsiella and it is needed in the unadenylated (active) form for nitrogenase derepression and ammonia assimilation (26, 25). GS II, however, is not adenylated and is involved in purine biosynthesis (25). Furthermore, ammonia produced by Rhizobium during nitrogen fixation is entirely exported to the host cytoplasm (6), whereas in Klebsiella the fixed nitrogen is used by the bacterium itself.

Although there are some slow growing species of Rhizobium that can fix nitrogen in the free living stage (21), studying the regulation of nitrogen fixation in R. meliloti is complicated by the fact that this species of Rhizobium can fix nitrogen only in the symbiotic stage. This stage is marked by the invasion of the alfalfa hair roots by the bacteria, the establishment of a nodule, and the transition of the bacteria to bacteroids (4). ppGpp has been shown to have a role in life-cycle transition in at least two organisms, Myxococcus (27) and Caulobacter (9). However, any speculation with regard to the involvement of ppGpp in the transition of bacteria to bacteroids in Rhizobium would be premature. Attempts to determine the role of ppGpp in bacteroids were not successful. Inadequate ^{32}P uptake by the bacteroids prevented the level of this nucleotide from being monitored (See Appendix).

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APPENDIX

BACTERIOLOGICAL METHODOLOGY

Isolation and Purification of Bacteroids

The legume nodules contain both the bacterium and the bacteroid forms of Rhizobium whose structure, composition, and sedimentation rate are different (2, 3, 4). Although two forms of R. japonicum are similar in size and shape (1), the bacteroids of R. meliloti are larger and easily distinguishable from the bacteria under the microscope (2).

The difference in the sedimentation rate of the bacteroids and the bacteria facilitated the use of sucrose gradient in separating these two forms of Rhizobium from soybean (1) and lupin (5) nodules. In this study, a RENOgrafin-76 gradient was used to separate the bacterium and the bacteroid forms of R. meliloti. Five week old nodules from 50 plants were crushed in one ml of SMM medium (0.5 M sucrose, 0.02 M maleate, pH 6.5, and 0.02 M $MgCl_2$). Upon microscopic examination, this crude bacteroid solution showed a mixture of bacteria, bacteroids, and plant debris. 0.2 ml of this solution was then applied to a 3 ml, 0 to 75% (v/v) RENOgrafin-76 (Squibb) gradient in 5-ml Corex tube. Two easily distinguishable bands were visible when the gradient was centrifuged for 10 minutes at 16,300g using the HB4 swinging bucket rotor of a Sorvall RC2-B centrifuge. Each band was removed from the top with a Pasteur pipette. Upon microscopic examination, the top band showed a mixture of plant

debris, bacteria, and bacteroids. The lower band, however, consisted of pure bacteroids. Thus we have developed a one-stop isolation method that allows for purification of bacteroids.

Viability of Bacteroids

Recent studies on the viability of R. japonicum (6) and R. lupini (4) bacteroids showed that in both cases they were viable and could form colonies on agar plates. Contrary to these studies, Paau et. al. (3) argue that the R. meliloti bacteroids are not viable, and the bacterium looses its viability after it differentiates into the bacteroid. In an attempt to explore the viability of bacteroids extracted from alfalfa nodules, a crude bacteroid solution in SMM was prepared (as described above). The number and the colony forming ability of the cells in this solution were examined by both plating and direct microscopic count. The direct microscopic count was done using a Petroff-Hausser counter. The crude bacteroid solution was diluted and plated on YM (see Materials and Methods) supplemented with 10% mannitol. Total cell count showed 1.5×10^9 cells/ml. Less than 0.1% bacterial contamination was found as judged by microscopic observation. The plate count, on the other hand, resulted in 2×10^8 viable cells/ml. Thus, approximately 10% of the bacteroids were viable. Furthermore, when viable cells/ml were measured, following a dilution of the bacteroids in a solution without additional mannitol, much lower number of colonies were observed, due to the osmotic sensitivity of the bacteroids (2). The above result further

substantiates the fact that the colonies in the initial experiments were due to viable bacteroids. High concentrations of mannitol has also been shown to be necessary for the colony forming ability of R. japonicum (2).

From these results we conclude that some of the bacteroids are viable and can revert to vegetative growth under appropriate conditions. This is contrary to the previous report (2). The significance of these results to survival in the natural environment is not clear.

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