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Glutamine signalling in bacteria

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TABLE OF CONTENTS

1. ABSTRACT

Glutamine is a metabolite of central importance in bacterial physiology. In addition to its function as one of the 20 standard amino acids in protein synthesis, glutamine is required for the biosynthesis of a variety of nitrogen-containing compounds. Of particular importance is glutamine synthesis as primary reaction of ammonium assimilation. Because of this versatile role, glutamine metabolism is tightly controlled in response to the cellular nitrogen status in bacteria. Recent progress in elucidating the molecular basis of nitrogen signalling has shed light on the role of glutamine as a signalling molecule. Bacteria belonging to the phylogenetic domains of proteobacteria and low G+C Gram-positives (firmicutes) have evolved different mechanisms to monitor glutamine as an indicator of the state of nitrogen metabolism, which then regulates nitrogen metabolism at the transcriptional and post-transcriptional levels. Using the conserved P_{II} signal transduction system, major groups of prokaryotes, including the cyanobacteria, have evolved yet another strategy to monitor the cellular nitrogen status, which relies on 2-oxoglutarate instead of glutamine as the signalling molecule. In addition to monitoring the intracellular glutamine level, bacteria may respond to extracellular glutamine, which is used as a nutrient. This overview details our current knowledge of glutamine-regulated processes in bacteria.

2. INTRODUCTION

To maintain the dynamic equilibrium of nutrient uptake and consumption, bacteria have evolved sophisticated strategies of metabolic control. These strategies operate to ensure that energy-generating catabolic reactions are adjusted to energy-consuming anabolic reactions, that nutrients consumed in catabolic and anabolic pathways are metabolised in a balanced manner and that nutrients are acquired from the environment according to the metabolic demand. Microorganisms are exposed to continuously changing nutrient availability and due to their small cell size and high surface to volume ratio, any environmental changes almost instantaneously impacts upon the metabolic state. To cope with these changes, microorganisms have evolved elaborate molecular mechanisms that allow restoration of metabolic homeostasis. Prerequisites for these adaptation processes are sensitive systems to monitor the metabolic state and signal-controlled output responses, which counterbalance the initial perturbation.

Bacteria have evolved different mechanisms to monitor the state of central nitrogen metabolism. Despite this diversity, one regulatory molecule, the P_{II} signal transduction protein, has been conserved as the central control unit in nitrogen metabolism. However, the signal input – output interactions of the P_{II} signal transducer have

adapted to the various metabolic routes bacteria have evolved. In the large and diverse phylogenetic group of proteobacteria, glutamine is a central signal molecule, which is transduced by P_{II}. Bacteria belonging to the Bacillus group have evolved a different strategy to monitor the cellular glutamine level. These various mechanisms will be presented in the following sections.

3. THE ROLE OF GLUTAMINE IN BACTERIAL METABOLISM

3.1. General features of glutamine metabolism

Due to the tight coupling of metabolism and signalling, I shall first briefly consider the functions of glutamine in bacterial metabolism. From a global perspective, three major metabolic functions can be attributed to glutamine,

- as primary product of ammonium assimilation
- as nitrogen donor in various biosynthetic reactions
- as one of the 20 standard amino acids needed for protein synthesis.

Because of the central importance of glutamine metabolism, synthesis of glutamine itself will be considered in some detail below. Glutamine, either synthesized by the cells or taken up from the environment, is required as a nitrogen donor for a range of biosynthetic reactions, including synthesis of nucleic acids and various transamidation reactions. A striking feature in bacterial glutamine metabolism is the fact, that glutamine which is incorporated into proteins is not directly derived from the free cellular glutamine pool via a specific glutaminyl-tRNA synthetase. Instead, in most bacteria, the glutamine-specific $tRNA^{Gln}$ is first aminoacylated with glutamate, and Gln-$tRNA^{Gln}$ is then formed from Glu-$tRNA^{Gln}$ by a tRNA-dependent amidotransferase reaction requiring ATP and an amido donor such as glutamine, asparagine or occasionally other compounds (1). In contrast, in eukaryotes, glutamine is directly aminoacylated to $tRNA^{Gln}$. The rationale for the bacterial pathway of Gln-$tRNA^{Gln}$ formation is not known, but it may be a relic of the evolution of the genetic code (e.g. through tRNA-dependent amino acid biosynthesis), or it may provide so-far unrecognized regulatory possibilities to uncouple glutamine metabolism from translation.

3.2 Glutamine synthesis, the primary reaction in ammonia assimilation

The enzyme glutamine synthetase (GS) catalyzes the synthesis of glutamine from glutamate and ammonium. GS incorporates ammonia into the γ-carboxyl group of glutamate in a reaction that involves initial phosphorylation of the γ-carboxyl of glutamate by ATP, followed by ammonium incorporation (amidation) and release of inorganic phosphate. ATP hydrolysis drives this reaction in the direction of glutamine synthesis and allows efficient glutamine synthesis even at low ammonium concentrations.

Although various pathways exist in which ammonium can be assimilated into organic compounds, the most efficient pathway is the assimilation via the GS reaction coupled to glutamate synthase activity. Glutamate synthase, also called glutamine-2-oxoglutarate-amido transferase (GOGAT) uses the amide group of glutamine to reductively aminate 2-oxoglutarate, generating two molecules of glutamate. The overall reaction of the so called GS/GOGAT pathway is thus the synthesis of glutamate from 2-oxoglutarate and ammonium, consuming one ATP and two electrons (mostly derived from NADPH), as shown below:

GS: Glutamate + NH_3 + ATP → Glutamine + ADP + P_i
GOGAT: Glutamine + 2-oxoglutarate + 2[H] → 2 Glutamate
GS/GOGAT:2-oxoglutarate + NH_3 + ATP + 2[H] → Glutamate + ADP + P_i

The GS/GOGAT pathway is the dominant and universally conserved pathway of ammonium assimilation in all organisms that are able to utilize ammonium as nitrogen source. Most free-living bacteria are capable of this prototrophic life-style. In bacteria, three classes of glutamine synthetases have been defined, and in numerous bacteria, multiple GS isoenzymes have been found, which may belong to any of the three classes, depending on the organism. The most widely distributed form of GS in prokaryotes is GSI, encoded by the *glnA* gene (for reviews, see e.g. 2, 3, 4). This class of GS is not present in eukaryotic organisms. GSI is a homododecameric enzyme composed of approx. 55 kDa subunits, forming a double hexameric ring structure. On the basis of phylogenetic analysis, GSI enzymes were further subdivided into a GSI-alpha and a GSI-beta group (5). Genes encoding GSI-alpha homologues are found in the low G+C Gram-positive endospore-forming bacteria (e.g. Bacillus and Clostridia) and in the Euryarchaeota (to which the methanogenic and halophilic archaea belong). Enzymes of the GSI-beta group are found in most other bacteria. The GS enzymes that are regulated by adenylylation (see below) belong to this group, whereas GSI-alpha enzymes appear not to be regulated by covalent modification (3). A phylogenetically and structurally rather different type of GS is the form GSII, encoded by *glnII* genes. This enzyme was found in some soil bacteria like *Rhizobium*, *Agrobacterium* and *Streptomyces* and relatives (6, 7, 8) and has similarity to plant GS. In *Rhizobia*, a further type of GS was described, which was termed GSIII, and is encoded by *glnT* (9). However, phylogenetic analysis revealed that this GS is distantly related to GSI (10). Yet another type of GS was discovered in anaerobic bacteria of the genus *Bacteroides* (11) and *Butyrivibrio* (12). This type of GS was also termed GSIII, although it is not related to the *glnT*-encoded GS from *Rhizobium*. The *Bacteroides* /*Butyrivibrio* GS enzymes are rather different from GSI and GSII; with an approximate size of 70 - 75 kDa, the subunits are much larger, and they form a hexameric structure. Therefore, and in accord with phylogenetic analysis, the designation GSIII is appropriate for this type of GS (10, 13) A homologue of the *Bacteroides* GSIII was found in the cyanobacterium *Synechocystis* PCC 6803 (encoded by *glnN*) (14), and GSIII homologues are present (in addition to GSI) in various other non-nitrogen-fixing cyanobacteria (15). This enzyme appears to increase viability of cyanobacteria under conditions of prolonged nitrogen starvation (16). The wide distribution and deep phylogenetic branching of the various glutamine synthetases reflects the fact that it is believed to be one of the most ancient enzymes in nature (17).

Summing up, glutamine synthesis has two different functions: it has to provide enough glutamine to satisfy the demand for glutamine in various anabolic reactions and under conditions requiring the assimilation of ammonia (which may be derived from various organic or inorganic nitrogen sources), glutamine synthesis is the primary reaction for ammonia assimilation. Due to this central role in nitrogen metabolism, the activity of glutamine synthetase is tightly regulated by the nitrogen status in bacteria. High activities are required under conditions where the GS/GOGAT pathway is engaged in nitrogen assimilation, whereas lower activities are sufficient when under otherwise nitrogen replete conditions (e.g. in the presence of amino acids), since only the need for glutamine in other biosynthetic reactions, including protein synthesis, has to be satisfied. Bacteria have evolved sophisticated mechanisms to monitor the nitrogen state of the cells, in order to adjust the activities of the nitrogen assimilatory enzymes to their requirements. Various mechanisms of monitoring the cellular nitrogen state have been developed in the course of evolution and it is not surprising that in a large variety of bacteria, glutamine plays an important role as a signalling molecule. However, monitoring the glutamine level is not a general feature of nitrogen control in all bacteria. A more ancient signal transduction system, however, is conserved in all bacteria that maintain nitrogen control, and this system will be detailed in the following section.

4. THE P_{II} SIGNAL TRANSDUCTION PROTEIN, A COMMON MODULE IN BACTERIAL NITROGEN CONTROL

Despite the high degree of diversity in mechanisms of bacterial nitrogen control, one common regulatory module is present in almost all bacteria capable of ammonium assimilation and that is the P_{II} signal transduction protein. Although P_{II} is not directly a sensor of glutamine, P_{II} regulation is tightly coupled to glutamine signalling in a variety of bacteria.

4.1 P_{II} signalling proteins

Several aspects of P_{II} signal transduction have been reviewed recently and the reader is referred to these articles for a more comprehensive description (18, 19, 20). Briefly, the P_{II}-like signalling proteins constitute one of the most widely distributed families of signal transduction proteins, whose members are found in bacteria, archaea and plants. They are trimeric proteins of 12 – 13 kDa subunits, whose structure is highly conserved in the different lines of descent. The three subunits are organized to form a short cylindrical structure composed of a central core of anti-parallel beta-sheets. The lateral surface is composed of alpha-helices and three loop structures per subunit of which a large surface-exposed loop (termed T-loop) is of particular importance. The P_{II}-like signalling proteins can be further subdivided into three subfamilies, encoded by the *glnB*, *glnK* and *nifI* genes. According to a classification proposed by Arcondeguy et al (19), those genes encoding P_{II}-proteins, which are genetically linked to the ammonium-tranporter gene *amtB,* are termed *glnK*, while the *nifI* genes are linked to nitrogen fixation genes in archaea and some strictly anaerobic bacteria (19).

Functionally, P_{II} proteins can be regarded as signal integration molecules. Mechanistically, there are two modes of signal perception by P_{II}, as detailed below. In all cases investigated so far, the P_{II}-like signalling proteins bind the effector molecules ATP and 2-oxoglutarate (21-24). The second mode of signal perception occurs by covalent modification of the P_{II} protein at the apex of the solvent-exposed T-loop. Covalent modification is not a general property of P_{II} signalling, but in cases where it occurs, it can integrate additional signals into the P_{II} signalling process, in particular the glutamine signal. Effector molecule-binding and covalent modification result in different states of signal input. Depending on these modifications, the P_{II} binds to different P_{II} signal-receptors and by means of protein-protein interaction, modifies the activity of these target molecules (see Figure 1). In most cases, these targets are key regulatory enzymes, metabolic enzymes or are proteins involved in nutrient transport.

4.2. General properties of signal transduction by P_{II}-like proteins

A feature common to all P_{II} signal transduction proteins is the presence of binding sites for ATP. Binding of ADP to the ATP binding site has also been demonstrated (24). The adenine nucleotide binding sites of P_{II} proteins from *E. coli*, *Herbaspirillum seropediae* and *Thermus thermophilus* have been resolved by crystallographic analysis (25-28). The ATP (ADP) binding sites are located in the cleft between the subunits of the trimers, where the ligand is embedded in a cavity of two adjacent loop structures (B- and C-loop) of opposing subunits. This results in three identical binding sites in the trimeric protein. In *E. coli*, all three ATP binding sites of P_{II} are saturated at physiological ATP concentrations (29). By contrast, the ATP binding sites of the P_{II} protein from the cyanobacterium *Synechococccus* exhibit negative cooperativity: high affinity binding to one binding site reduces affinity to the other sites (22), and this may enable sensing of the cellular energy status.

In addition to binding adenine nucleotides, P_{II} proteins also bind 2-oxoglutarate. ATP and 2-oxoglutarate mutually stimulate each other (21-24). In the cyanobacterial and plant P_{II} proteins, 2-oxoglutarate binding can only be detected in the presence of ATP (22, 24). Although the 2-oxoglutarate binding site has not yet been resolved by crystallographic analysis, several lines of evidence suggest that 2-oxoglutarate binding occurs in the vicinity of the ATP binding sites (27). The three 2-oxoglutarate binding sites exhibit negative-cooperativity, allowing the P_{II} proteins to detect 2-oxoglutarate in a wide range of concentrations (reviewed in 30).

A second mode of signal perception occurs in those cases where P_{II} proteins are subject to covalent modification. In proteobacteria, modification of P_{II} proteins occurs by reversible uridylylation of a conserved tyrosyl-residue (Y51). Uridylylation and deuridylylation reactions are catalyzed by the same enzyme, a bifunctional uridylyltransferase/ uridylylremovase (UTase/UR) in response to the cellular glutamine level (29, 31-33) (see below). UTase/UR is the product of the *glnD* gene (34). In

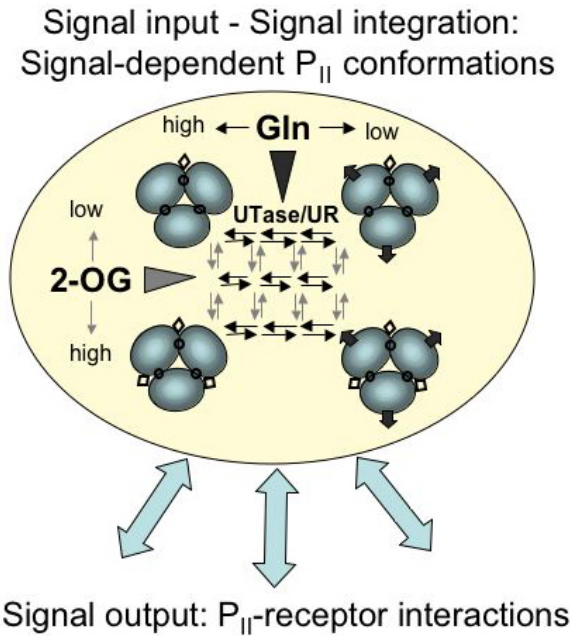

Figure 1. Schematic representation of the signal integration and transduction mechanism by proteobacterial P_{II} signalling protein. Under physiological conditions, all three ATP-binding sites of P_{II} protein ought to be occupied (symbolized by circles). In the presence of low cellular 2-oxoglutarate concentrations, one 2-oxoglutarate molecule is proposed to bind to P_{II} (symbolized by the diamond). Increasing cellular 2-oxoglutarate levels will lead to occupation of the second and third binding site. A second signal input comes from covalent modification (uridylylation of Y51, symbolized by the bold arrow on each P_{II} monomer) through the bifunctional enzyme UTase/UR (see text for details). At low glutamine levels, UTase/UR will uridylylate each P_{II} subunit, resulting in three successive uridylylation reactions, indicated by the central reaction arrows. Fully uridylylated P_{II} occurs under very nitrogen-poor conditions (low glutamine). Increasing glutamine leads to deuridylylation of P_{II}-UMP by UTase/UR. This "two dimensional" signal integration mode by P_{II} results in an array of different states of modification and ligand binding, as indicated by the central network of arrows. The black horizontal arrows indicate the successive uridylylation/deuridylylation reactions, the grey vertical arrows indicate binding/dissociation of 2-oxoglutarate to the second and third site. Depending on these states, P_{II} proteins interact with various signal receptors, thereby affecting their catalytic activity (for details, see text).

actinobacteria such as corynebacteria, mycobacteria and streptomycetes, P_{II} homologues of the GlnK subfamily are modified at the conserved tyrosyl 51 residue by adenylylation instead of uridylylation (35, 36). Adenylylation/deadenylylation responds to the nitrogen status of the cell in a similar manner than P_{II} uridylylaltion in proteobacteria, and the modifying enzyme, termed GlnD, is homologous to UTase/UR. Nevertheless, the signal leading to P_{II} adenylylation is not yet fully understood. Studies in *Corynebacterium glutamicum* challenge the view that glutamine is the signalling molecule in these organisms (37), although this issue still awaits clarification. Yet another type of P_{II} modification has been demonstrated in cyanobacteria. These oxygenic phototrophic bacteria phosphorylate and dephosphorylate a seryl residue (S49) located in the vicinity of the conserved Y51 (38). Kinase and phosphatase activities reside on different polypeptides, and glutamine was shown not to be involved in regulation of this process (39). Instead, phosphorylation and dephosphorylation are the responses elicited depending on the ligand binding status of P_{II}, (20,40). In this case, no additional signal is apparently integrated by P_{II}, and phosphorylation/dephosphorylation appears to increase the sensitivity or fine-tune the ATP/2-oxoglutarate signal (20). However, it should be noted that PII proteins in plants, in some cyanobacteria and presumably in various other bacteria seem not to be covalently modified.

In most cases reported so far, it appears that the T-loop structure is critical for the interaction of P_{II} with its receptor protein. This interaction responds to the state of ligand-binding and may be of covalent modification, as will be outlined in the following chapter in greater detail. Figure 1 illustrates schematically the principle of P_{II} signal integration – signal transduction in the *E. coli* system.

5. GLUTAMINE SIGNALLING IN PROTEOBACTERIA

5.1. GS regulation and the Ntr system in enteric bacteria

Assimilation of external nitrogen sources such as ammonium is tightly regulated by the intracellular nitrogen status. In enteric bacteria (such as *E. coli*, *Salmonella typhimurium* or *Klebsiella* sp.), as in many other proteobacteria, internal nitrogen limitation leads to an increase in GS specific activity as well as to the activation of gene expression specific for nitrogen assimilatory functions. This nitrogen regulatory (Ntr) system of transcription consists of the histidine kinase NtrB and its cognate response regulator NtrC and is controlled by the P_{II} signal transduction system (for reviews, see 2, 3, 18, 41). Already a low level of activated (phosphorylated) NtrC is sufficient to promote the expression of the *glnAntrBC* operon (encoding GS as well as the NtrB and NtrC proteins). Higher concentrations of activated NtrC are required for expression of other Ntr-dependent genes, such as *nac*, *glnK amtB* and others (42). The Nac protein is a transcription factor of various genes of nitrogen metabolism, which are, therefore, indirectly subjected to Ntr-dependent nitrogen control (43). Autoregulation of the NtrBC regulators and the differential promoter affinities of the various NtrC-regulated genes results in a sequential activation of genes subject to nitrogen control, depending on the strength and duration of nitrogen limitation. Initially, the *glnAntrBC* promoter, which contains a high-affinity-binding site for NtrC is activated, and only when higher concentration of activated (phosphorylated) NtrC is attained, is expression of genes with low affinity promoters such as *glnK* activated.

Taking together, GS, the key enzyme of ammonium assimilation, responds in a dual manner to nitrogen limitation: (1) transcription of its gene is subject to Ntr control and responds in a highly sensitive manner to Ntr activation; and (2) the specific activity of the enzyme is controlled by covalent modification. Biochemical and genetic analyses revealed that both of these responses are coordinated by the P_{II} signal transduction protein and ultimately respond to the cellular glutamine level. Two targets of glutamine perception have been identified: UTase/UR and the GS-modifying enzyme GS adenylyltransferase, briefly termed ATase (encoded by *glnE*).

5.2. UTase/UR as glutamine sensor

The primary sensor of the cellular glutamine level is the P_{II}-modifying enzyme UTase/UR. This enzyme was already described in the 70ies, and it could be shown that P_{II} uridylylation and deuridylylation respond to the effector molecules glutamine and 2-oxoglutarate (4, 31). At that stage, it was proposed that the ratio of glutamine to 2-oxoglutarate controls P_{II} uridylylation/deuridylylation, with a low glutamine/2-oxoglutarate ratio favouring uridylylation of P_{II} and high glutamine/2-oxoglutarate favouring deuridylylation (32). This view became text-book knowledge and was maintained until the mid 1990ies. Only after the individual components of the P_{II} uridylylation reaction had been investigated separately, was this model modified and updated.

5.2.1. Biochemistry of P_{II} uridylylation/deuridylylation

The UTase/UR enzyme was purified and a first biochemical characterization of this 95 kDa monomeric protein was published in 1983 (33). Ten years later, the sequence of the gene encoding UTase/UR, termed *glnD*, was published (34). It encodes a protein of 890 amino acids and the N-terminal portion of the enzyme exhibits homology to an ancient family of nucleotidyltransferase enzymes, including DNA polymerase β from the eukaryotic DNA polymerase family X,, kanamycin nucleotidyltransferase and ATase (see below). A detailed enzymological characterization of the UTase/UR enzyme and its interaction with the P_{II} signalling protein was reported in 1998 (29). At that time, it was already established that the sensor of 2-oxoglutarate and ATP is not UTase/UR but the P_{II} protein itself (21). Only P_{II} bound by ATP and 2-oxoglutarate is capable of interacting with UTase/UR. However, the extent of 2-oxoglutarate binding (one, two or three sites occupied) had no effect on the uridylylation reaction in the presence of Mg^{2+}. Kinetic analysis of the UTase/UT reaction indicated that uridylylation and deuridylylation of P_{II} occurs at a single catalytic site. The uridylyl-transferase reaction follows a sequential ordered bi-bi kinetic mechanism. First, P_{II} is bound by the enzyme, forming a transient complex, to which UTP binds with transfer of the UMP moiety to Y51 of P_{II}. Subsequently, the reaction products pyrophosphate and then P_{II}-UMP are released from the enzyme. The reaction is non-cooperative with respect to the single P_{II} subunits, that is, uridylylation of one subunit is not affected by the uridylylation status of the other subunits. Uridylyl-removing activity of the enzyme involves binding of P_{II}-UMP to the active site, with subsequent random release of products UMP and P_{II} (29).

5.2.2. Regulation of UTase/UR by glutamine

The UTase/UR reaction is regulated by the glutamine concentration. Glutamine appears to have one single binding site on the enzyme and binding affects the two antagonistic activities in an opposite manner. Whereas glutamine binding activates P_{II}-UMP hydrolysis with a K_{act} (Gln) of approx. 80 μM, the same concentration leads to inhibition of the uridyl-transferase reaction (K_i (gln) = 70 – 80 μM). Inhibition of the UTase reaction is probably due to inhibition of the catalytic step of the transferase reaction, whereas uridylyl-removing activity is stimulated by lowering the K_m and increasing the k_{cat} . This enzymological study implied that the P_{II} uridylylation monocycle catalyzed by UTase/UR serves mainly as a highly sensitive measuring system for the cellular glutamine level, whereas 2-oxoglutarate concentrations in the physiological range had little effect on P_{II} uridylylation. In reconstituted systems containing purified P_{II}, UTase/UR, UTP, ATP, 2-oxoglutarate and Mg^{2+}, the steady state uridylylation level of P_{II} responded strongly to changes in glutamine levels between 0.1 and 4 mM, which is well within the physiological range of cellular glutamine concentrations (29).

5.3. Transduction of the glutamine signal trough P_{II} receptor interactions

The glutamine signal, through to the uridylylation status of P_{II}, is transmitted to the P_{II} receptors. In *E. coli*, the main receptors are NtrB, the ammonium transporter AmtB and ATase. NifL or NifA are additional targets present in nitrogen-fixing proteobacteria.

5.3.1. P_{II} signalling to the Ntr system in enteric bacteria

Under conditions of nitrogen-excess, P_{II} is present in the non-modified state and forms a complex with NtrB to switch the catalytic activity of the sensor kinase/phosphatase NtrB (44). The NtrB-P_{II} complex has phosphatase activity for phosphorylated (activated) NtrC. Thus, the signal “high glutamine” is transmitted via non-modified P_{II} and NtrB to shut down transcription of genes required under nitrogen-poor conditions. By contrast, under nitrogen-poor conditions, uridylylated P_{II} does not productively interact with NtrB. In this state, NtrB has kinase activity for NtrC, leading to activation of this transcription factor. Thus, the signal “low glutamine”, via P_{II}-uridylylation and NtrB-mediated NtrC-phosphorylation, results in transcription of Ntr-dependent genes. Binding of P_{II} to NtrB was studied in some detail and it appears that the non-uridylylated T-loop from one P_{II} subunit interacts with the C-terminal part of NtrB (44, 45). Partial uridylylation of the P_{II} trimers lowers the number of NtrB-interacting P_{II} species, resulting in a gradual activation of NtrC as P_{II} becomes more and more uridylylated with decreasing cellular glutamine levels.

5.3.2. P_{II} signalling in nitrogen-fixing proteobacteria

In nitrogen-fixing proteobacteria, the genes encoding enzymes of nitrogen fixation (*nif*-genes) are controlled by the transcription factor NifA (reviewed in

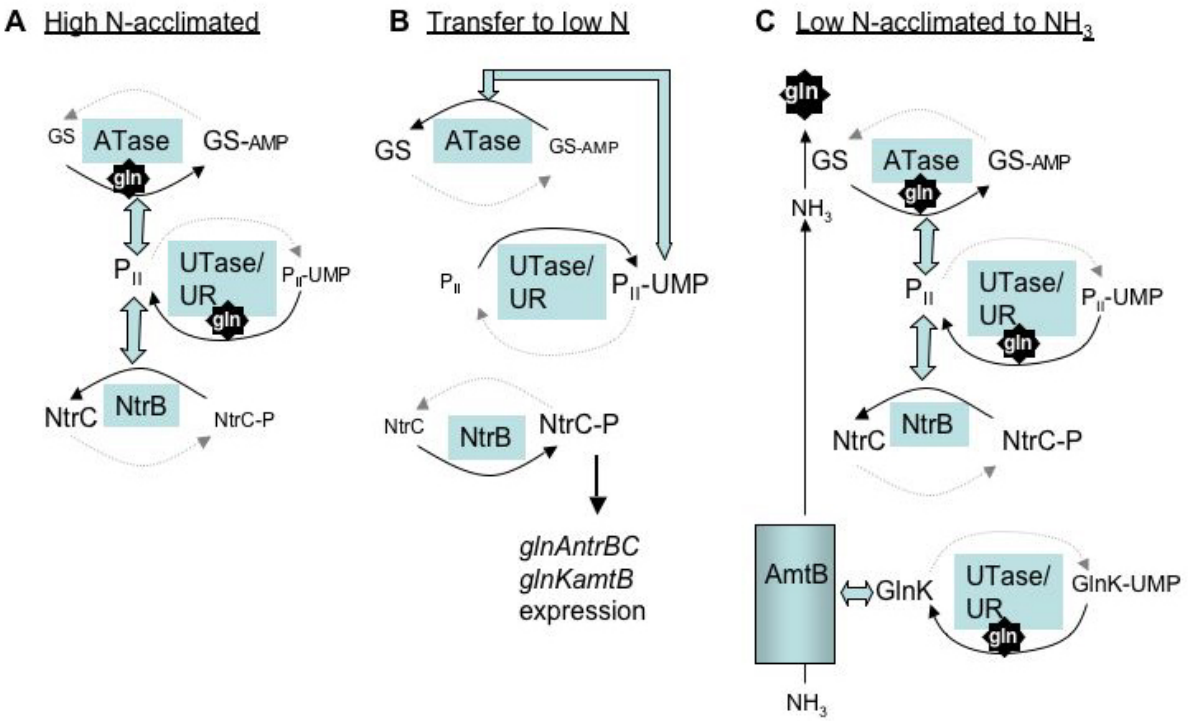

Figure 2. Schematic representation of the enterobacterial nitrogen control system. The P_{II} interacting proteins are shown in blue boxes. Protein-protein interactions are symbolized by double-headed bold arrows. The reactions catalyzed by the converter enzymes UTase/UR and ATase are indicated by curved arrowhead lines, the dotted lines indicate the inhibited reverse reaction. The signal glutamine is highlighted by an asterisk. (A) In cells acclimated to nitrogen excess conditions (high internal glutamine), P_{II} accumulates in the non-modified state (through UTase/UR sensing glutamine). P_{II} productively interacts with NtrB to keep the response regulator NtrC in the inactive, non-phosphorylated state. Ntr-dependent genes are not expressed under these conditions. Furthermore, P_{II}, in concert with glutamine, interacts with ATase to adenylylate GS, thereby inhibiting its activity. (B) Following transfer to nitrogen-poor conditions, the cellular glutamine level drops, P_{II} becomes uridylylated by UTase/UR and NtrB now acts as a kinase to phosphorylate NtrC, which leads to the expression of the Ntr-regulon. First, the *glnAntrBC* operon is activated, followed by expression of *glnKamtB* when NtrC-P accumulates. The adenylylated form of GS is now deadenylylated by ATase in concert with P_{II}-UMP, leading to activation of GS. **(C)** Prolonged growth under these conditions leads to accumulation of GlnK-UMP (uridylylated by UTase/UR) and AmtB proteins in the cell. When those nitrogen-poor acclimated cells are exposed to ammonium, the highly active GS will rapidly assimilate ammonium, entering through the AmtB channel, producing glutamine. Increased glutamine causes P_{II} as well as GlnK deuridylylation. Non-modified P_{II} causes NtrC dephosphorylation and GS adenylylation, and GlnK inhibits AmtB, preventing an excess uptake of ammonium.

46). Expression of the *nifA* gene itself is controlled by NtrC. This results in a first level of nitrogen control on *nif*-gene expression. In addition, NifA activity is subject to nitrogen control by P_{II}-dependent processes. Three different mechanisms have evolved in the various proteobacteria, to link nitrogen signalling of P_{II} proteins to ammonium control of NifA activity. In the first case, found in the gamma-proteobacteria *Azotobacter vinelandii* and *Klebsiella pneumoniae,* the P_{II}-like GlnK protein is required for regulation of NifL-mediated inhibition of NifA activity. In *Azotobacter vinelandii*, GlnK favours NifL-NifA interaction under [+N] conditions, whereas in *Klebsiella pneumoniae*, GlnK inhibits NifL-NifA interaction under [–N] conditions. Second, in bacteria like *Azospirillum brasilense, Herbaspirillum seropedicae* and *Rhodospirillum rubrum,* NifA activity (under [–N] conditions) depends on the presence of GlnB. Third, in *Azorhizobium caulinodans* and *Rhodobacter capsulatus,* neither GlnB nor GlnK is required for NifA activity, but both GlnB and GlnK can inhibit NifA activity in the presence of ammonium. For a comprehensive description of the complexity of interactions and signal transduction pathways involved in regulation of NifA activity, the reader is referred to recent reviews covering this topic (46-48).

5.3.3. GlnK-AmtB interaction

Another target of P_{II} control is the ammonium transporter AmtB. The bacterial and archaeal *amtB* genes are almost invariably associated with the *glnK* gene, encoding a P_{II} paralogue, and are highly expressed under nitrogen-poor conditions (49). Therefore, during prolonged growth under nitrogen-poor conditions, AmtB and GlnK proteins accumulate. Under these low glutamine conditions, GlnK is uridylylated by UTase/UR and AmtB is fully active to scavenge traces of ammonium in the medium. Upon addition of ammonium to the medium, ammonium enters the cells and is rapidly assimilated by the GS enzyme, leading to a sudden increase in cellular glutamine levels (see Figure 2). In turn, this leads to deuridylylation of the P_{II} proteins GlnK and GlnB and non-modified GlnK

now forms a complex with membrane-bound AmtB to inhibit further uptake of ammonium (50). Membrane sequestration of the P_{II} proteins by AmtB was suggested to have additional regulatory functions, for example by removing excess non-uridylylated P_{II} proteins from other recepors. From these findings, it was proposed that AmtB is part of an ammonium-sensing system, in which glutamine is a "second messenger" of increased external ammonium (49). In agreement with this proposal, GS activity seems to be functionally coupled to AmtB (51).

5.3.4. Regulation of GS activity through P_{II}/glutamine-controlled adenylylation/deadenylylation

P_{II} control over GS activity operates via a bifunctional adenylylytransferase (ATase) enzyme that regulates the activity of GS through reversible adenylylation/deadenylylation of tyrosyl-residue 397 (52, 53). Activity of GS is low in the adenylylated state and high in the absence of adenylylation. Adenylylation can occur on each of the twelve subunits, resulting in a gradual inactivation of GS with increasing adenylylation. In *E. coli*, GS is further subject to cumulative feedback inhibition: several amino acids (Ala, Gly, His, Trp) AMP, CTP and AMP allosterically inhibit GS activity (54, 55). The adenylylated form of GS is more sensitive to cumulative feedback inhibition than the non-adenylylated form. Interestingly, glutamine is not an allosteric inhibitor of GS in *E. coli* but exerts regulation over GS activity *via* ATase and the P_{II} signal transduction system, as will be outlined in the following.

Regulation of GS activity by reversible adenylylation/deadenylylation was already recognized in the late 1960ies (56) and was subsequently subject of intensive research (reviewed in ref. 4). Today it is one of the paradigms of enzyme regulation by cascade control. The sequence of the gene encoding ATase (*glnE*) was reported in 1993 (34) and recently, the crystal structure of the N-terminal domain of ATase was published (57). ATase is a large enzyme of 945 amino acid residues and is composed of two duplicated homologous domains. The N-terminal half (residues 1 – 411; AT-N) shares approx. 25 % sequence identity with the C-terminal part (residues 523 – 945; AT-C). Adenylylation of GS is catalyzed by the AT-C domain, while deadenylylyation of GS-AMP is catalysed by the AT-N domain (58). Isolated AT-C or AT-N retains catalytic activity, however, their regulation by P_{II} and effector molecules is adversely affected. The reaction of full-length ATase with GS was characterized in detail (52). In the absence of any effector molecules and P_{II}, ATase has a bias to slowly adenylylate GS (52). Therefore, in mutant cells devoid of P_{II} signalling proteins, GS accumulates in a highly adenylylated state (59). Non-modified P_{II} strongly enhances the adenylylation reaction; however, only when glutamine is present at moderate concentrations (1 mM). In the absence of glutamine, P_{II} only slightly stimulates GS adenylylation. On the other hand, glutamine in the absence of P_{II}, also has only weak effects on the activity of ATase. In contrast to the full-length ATase, the adenylylation activity of the isolated AT-C domain could be stimulated by glutamine alone (58), implying that glutamine binds allosterically to a site in AT-C. In the full-length ATase, the two domains are proposed to interact such that binding of glutamine to the AT-C domain is a prerequisite for P_{II}-mediated stimulation of adenylylation activity. Elevated levels of 2-oxoglutarate (10 mM) could inhibit the stimulatory effect of P_{II} on the adenylylation reaction. Apparently, when the second and third 2-oxoglutarate-binding sites of P_{II} are occupied (recall that 2-oxoglutarate binding to P_{II} exhibits negative cooperativity), interaction of P_{II} with its receptors is impaired (52). This means that the stimulatory effect of P_{II} on GS adenylylation can be counteracted by elevated 2-oxoglutarate concentrations. By contrast, P_{II}-UMP (signalling low cellular glutamine levels) favours the deadenylylation reaction and the 2-oxoglutarate status of P_{II}-UMP does not appear to affect this activity. Interestingly, the isolated AT-N is not regulated by P_{II}-UMP. From these results, it was suggested that the AT-N and AT-C domains interact such that when non-modified P_{II} binds to full-length ATase (enhanced by binding of glutamine to AT-C), the T-loop of P_{II} blocks the AT-N active site, whereas when P_{II}-UMP binds, the uridylylated T-loop blocks the AT-C active site (57).

Taking these studies together, it appears that glutamine exerts dual control over GS activity, via P_{II} uridylylation and by directly binding to ATase. The detailed enzymological characterization of the bicyclic GS regulatory cascade allowed system theoretical modelling of the regulatory cascade and calculation of the signal input – signal output ratios at various concentrations of converter enzymes (UTase/UR and ATase), P_{II} signalling protein and effector molecules (60). It turned out that the system is designed for ultrasensitive and robust detection of fluctuating glutamine levels. "The steepness of the dose/response curves obtained from glutamine as input and GS adenylylation as output was invariant at various component enzyme concentrations and 2-oxoglutarate concentrations" (60). This conclusion from theoretical modelling is in perfect agreement with physiological studies performed in *S. typhimurium*, which revealed through measuring internal metabolite pools, that external nitrogen limitation is primarily perceived by internal glutamine limitation (61). A summary of the P_{II}-dependent signal transduction schemes in *E. coli* under various cellular nitrogen states is provided in Figure 2.

5.4. Cross-talk between glutamine and glucose signalling in *E. coli*

The importance and dominant role of glutamine as the signal transduced by the P_{II} signalling system was also revealed in studies, which investigated the carbon-source effect on P_{II} signalling, Ntr and GS control in *E. coli*. Previously, it was known that *E. coli* grown with glucose as carbon source and glutamine as sole nitrogen source has low internal glutamine levels due to slow glutamine uptake and thus, senses nitrogen-limitation. The Ntr system is activated and GS is deadenylylated under these conditions. Replacement of glucose by poor carbon sources led to repression of NtrC-dependent *glnA* expression. This effect could be attributed to elevated intracellular cAMP levels, which correspond to the conditions of catabolite derepression, when the cells utilize poor carbon sources. It was suggested that the CRP-cAMP

activator protein complex, in addition to directly affecting the *glnA* promoter, negatively interacts with activated NtrC-P (62). However, examination of the P_{II}-signalling system under these conditions revealed that in the presence of poor carbon sources and glutamine as nitrogen source, P_{II} was deuridylylated and signalled nitrogen sufficient conditions, whereas in the presence of glucose/glutamine, P_{II} was uridylylated and signalled nitrogen depletion (63). These findings were explained by a glucose-repressed, low-affinity glutamine transport system. Through the process of catabolite repression, glucose inhibits the uptake of alternative carbon sources. Glutamine can be utilized both as C and N source and catabolite repressible glutamine transport had been described. Therefore, when catabolite repression is relieved by poor carbon sources, glutamine uptake is greatly enhanced, reaching high intracellular glutamine levels which promote deuridylylation of P_{II}. In turn, deuridylylated P_{II} inhibits NtrC-dependent gene expression and mediates GS adenylylation (63).

6. GLUTAMINE SIGNALLING OUTSIDE THE PROTEOBACTERIA GROUP

6.1. P_{II} signal transduction

In the high G+C Gram-positive bacteria of the actinobacteria group (corynebacteria, mycobacteria, streptomycetes), several components of the P_{II} signalling system and GS regulatory system are conserved, such as the GlnK-AmtB couple, GlnD (UTase/UR homologue) and GlnE (ATase homologue). However, an NtrB/NtrC two-component system is absent (37, 64). Covalent P_{II} modification catalyzed by the GlnD-like converter enzyme adenylylates P_{II} on Y51 instead of uridylylating it (35, 36). Actually, it is not clearly established to which signal P_{II} adenylylation responds in this group of bacteria. Measurement of internal metabolite levels challenges the assumption that adenylylation is a response to the cellular glutamine level, since the pool size of this metabolite remains high even under conditions of elevated intracellular GS activity (37 and A. Burkovski, personal communication). Organisms in this group of bacteria contain an unusually large arsenal of GS enzymes, including several GSI paralogues and eukaryotic-like GSII enzymes. The GSI enzymes are regulated by ATase-dependent adenylylation/deadenylylation in response to the nitrogen status (65-67), in a physiologically similar manner to that in *E. coli*. However, it is not clearly established whether glutamine has a direct regulatory role in this reaction. The situation in the low G+C Gram-positives will be outlined in the following section.

Even more divergent is the situation in cyanobacteria: here, only the P_{II} signal transduction protein of the GlnB subfamily is conserved, whereas neither GlnD, GlnE nor NtrBC homologues have been detected. All investigations of nitrogen-signalling in cyanobacteria come to the conclusion that these organisms sense the cellular N-status by perceiving the intracellular 2-oxoglutarate level, with the PII protein being the central sensor (20, 38-40, 68, 69). P_{II}-signalling proteins have also been found in the thermophilic bacterium *Thermus thermophilus* (28) and in methanogenic Archaea (70). No glutamine responses have been reported in these studies and it seems that in these cases 2-oxoglutarate is the major signal, as in cyanobacteria.

6.2. Regulation of GS and glutamine signalling in *Bacillus subtilis*

In *Bacillus subtilis*, ammonium assimilation and its response to changing nitrogen availability phenotypically proceeds in a similar manner to that described for enteric bacteria (71). Ammonium is assimilated through the GS-GOGAT pathway and during nitrogen-limited growth, the activity of GS increases. Despite the presence of a gene encoding a glutamate dehydrogenase (*rocG*), this enzyme is not used for glutamate synthesis, but rather represents a glutamate catabolic enzyme (72). *B. subtilis* contains one type of GS enzyme, belonging to the GSI-alpha family. The enzyme is not subjected to regulation by covalent modification, but in contrast to GS enzymes from proteobacteria, is sensitive to feedback-inhibition by glutamine. As will be detailed below, GS feedback inhibition by glutamine represents the primary mechanism of sensing the nitrogen status and represents a novel mechanism of glutamine sensing compared to proteobacteria.

The only conserved proteins of the nitrogen signalling apparatus are homologues of AmtB and GlnK, encoded in an operon previously termed *nrgA-nrgB* (73). Physiological studies implied that the AmtB homologue functions as an ammonium transporter and the GlnK homologue is co-localized with AmtB. Furthermore, the P_{II}-like GlnK protein does not seem to be modified by covalent modification. Both sites of known P_{II} modification, Y51, as well as S49, are not conserved. So far, no clear function could be attributed to GlnK. *glnK* mutants do not show a general loss of nitrogen control, although nitrogen-controlled gene expression was impaired under low pH conditions (73).

In the past decade, a complex regulatory system operating nitrogen control in *Bacillus subtilis* could be elucidated. Two transcription factors, termed GlnR and TnrA, and the GS enzyme are the major players in global nitrogen control (74, 75). GlnR and TnrA, which are both members of the MerR family of transcriptional regulators, control the expression of nitrogen-regulated genes (74). They bind to similar DNA sequences, and therefore, regulate an overlapping set of genes and cross-regulate each other's expression. GlnR is a transcriptional repressor that is active during growth under nitrogen-excess conditions, where it represses, among others, the *glnRA* operon and the *tnrA* gene. When nitrogen becomes limiting, repression by GlnR is relieved, increasing the synthesis of GS and TnrA. The precise mechanism, by which the activity of GlnR responds to the nitrogen status, is not yet elucidated; however, the GS protein appears to be directly involved (Figure 3) (75). Under nitrogen-limited conditions, the TnrA factor is the major transcription factor of nitrogen-regulated genes (74). TnrA can act both as transcriptional activator and repressor, depending on the position of its binding site in target promoters. TnrA up-regulates genes required for utilization of various nitrogen

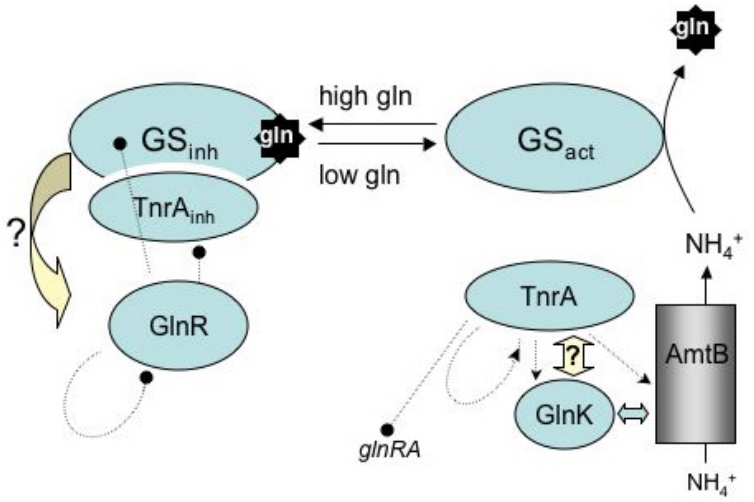

Figure 3. Glutamine sensing in *Bacillus subtilis*. Under nitrogen excess conditions (high cellular glutamine levels), GS is feedback inhibited by glutamine (GS_{inh}). In this state, GS binds and inactivates transcription factor TnrA ($TnrA_{inh}$) and activates the repressor GlnR by an unknown mechanism. GlnR represses, among others, transcription of *glnRA* and *tnrA* (gene repression is indicated by dotted lines with an endpoint). When cellular glutamine levels drop, feedback-inhibition of GS disappears (active GS: GS_{act}) and GlnR no longer represses *tnrA*. The TnrA factor is now produced and activates transcription of its own gene, and several other genes of nitrogen assimilation (not indicated) including the *amtBglnK* operon (gene activation is indicated by dotted lines with an arrowhead). Potential regulatory interactions of GlnK with TnrA are not yet elucidated. AmtB promotes high affinity ammonium uptake, and is possibly regulated by GlnK, which is not modified by covalent modification.

sources such as urea or nitrate, as well as the *amtBglnK* operon and its own synthesis. On the other hand, it represses *glnRA* and *gltAB* (encoding glutamate synthase) expression. TnrA activity is directly regulated through protein-protein interaction with the feedback-inhibited form of GS (76-78). *In vitro*, it was shown that complex formation with the feedback-inhibited GS abolishes the DNA-binding activity of TnrA. Glutamine and AMP are the strongest feedback inhibitors of GS. Glutamine seems to bind directly to the active site of the enzyme rather than to bind to an allosteric regulatory site (76). Binding of glutamine or AMP induces a conformational change, which enables high affinity binding of TnrA (78). In addition to regulation by GS, the P_{II}-like GlnK homologue appears to impact TnrA activity under certain physiological conditions (73). GlnK might relieve TnrA from GS control by removing TnrA into a membrane-localized complex (K. Forchhammer, unpublished data).

Taken together, it appears that GS has a dual role in *B. subtilis*: first, as a biosynthetic enzyme for assimilation of ammonium and synthesis of glutamine, and second, as a glutamine sensor, transducing the glutamine signal to TnrA by means of inactivating the transcription factor through protein-protein interaction (and regulating GlnR by a so far unknown mechanism). In the GS sequestered state, TrnA loses control over its regulon, and transcriptional control is taken over by GlnR. The various regulatory interactions of TnrA are schematically depicted in Figure 3.

7. GLUTAMINE AS EXTRACELLULAR SIGNAL

Glutamine can serve as carbon and nitrogen source for a large variety of bacteria. *E. coli* appears to employ two different glutamine uptake mechanisms. The first is a poorly characterized low-affinity transporter, presumably through a low specificity L-asparate-L-glutamate transport system (79), which is catabolite-repressed (see above), and the second is a high-affinity ABC-transporter, encoded by *glnHPQ*. The latter is under Ntr-control and is expressed under nitrogen-limited conditions and its role is to scavenge traces of glutamine from the environment. Despite the potential value of glutamine as a bacterial nutrient, there are very few reports documenting glutamine as a chemo-attractant in bacterial physiology. One publication reports that the differentiation of swarming cells in *Proteus mirabilis* is initiated by exogenous glutamine, acting as chemo-attractant (80). However, signal perception of the exogenous glutamine was not elucidated and no subsequent studies addressed this issue (81). In bacteria of the group B streptococci, a link between glutamine transport and virulence-associated fibronectin adhesion was reported (82). A mutant in the glutamine transport gene *glnQ* showed decreased virulence *in vivo* and reduced adherence to, and invasion of, respiratory epithelial cells *in vitro*, implying a specific, but unidentified role of glutamine transport or intracellular glutamine in the virulence of group B streptococci. Further analysis is required to clarify this issue.

Recently, a two-component regulatory system was identified in *Bacillus subtilis*, which is involved in the regulation of glutamine uptake (83). The potential sensor kinase-response regulator pair was unfortunately named GlnK-GlnL, a nomenclature, which leads to confusion with the P_{II}-like GlnK protein (previously designated NrgB in *B. subtilis,* see above). Originally, this two-component system

was identified by sequencing the *B. subtilis* genome, and the genes were designated YcbA/YcbB. This original nomenclature should be maintained until an unambiguous terminology is proposed. Mutation in the *ycbA/ycbB* ("*glnK/glnL"*) genes affects the expression of the *glsA* and *glnT* genes, encoding a glutaminase and a glutamine transporter, respectively. Expression of *glsA* and *glnT* is stimulated by extracellular glutamine in a YcbA-dependent manner. YcbA is a potential histidine kinase with four potential membrane-spanning domains and a cytoplasmatic histidine kinase domain. It is suggested that YcbA senses glutamine in the medium and phosphorylates YcbB in the cytoplasm upon stimulation (83). However, the biochemical mechanism of glutamine sensing by YcbA awaits elucidation, since no domain with similarities to a known glutamine-binding protein could be identified.

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Abbreviations: G+C: guanosine and cytosine; GOGAT: glutamine-2-oxoglutarate-amido transferase (glutamate synthase); GS: glutamine synthetase

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