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Review article

Sensing of N-Status in *Gluconacetobacter diazotrophicus*: Biochemistry and Genetics of Nitrogen Fixation and Assimilation

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Abstract

Gluconacetobacter diazotrophicus (previously *Acetobacter diazotrophicus*) was originally isolated from sugarcane, but has subsequently been isolated from other plants of agricultural importance. *G. diazotrophicus*, which is an endophytic microorganism, has the capacity to fix molecular nitrogen and has been shown to contribute substantially to the N-requirement of sugarcane. This review covers present knowledge about the N-sensing mechanisms operating in this diazotroph, that allows it to supply its host with nitrogen for growth. In particular the components of the central N-sensing system, i.e. GlnD, the three P_{II} paralogs and the Ntr system are reviewed.

Keywords: *Gluconacetobacter diazotrophicus*, nitrogen metabolism, regulation, nitrogen fixation

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1. Introduction

Gluconacetobacter diazotrophicus (previously *Acetobacter diazotrophicus*) was originally isolated from sugarcane in Brazil (Cavalcante and Döbereiner, 1988), but has subsequently been isolated from sugarcane in Mexico (Fuentes-Ramirez et al., 1993), Australia (Li and Macrae, 1991) and Cuba (Dong et al., 1994), as well as other plants of agricultural importance, e.g. coffee (Jimenez-Salgado et al., 1997), sweet potato (Paula et al., 1991) and pineapple (Tapia-Hernandez et al., 2000). *G. diazotrophicus* which is an endophytic microorganism has the capacity to fix molecular nitrogen and has been shown to contribute substantially to the N-requirement of the plant host (Boddey et al., 1991; Urquiaga et al., 1992; Boddey et al., 1995). These initial studies have recently been confirmed by using Nif^- strains of *G. diazotrophicus* as inoculum (Sevilla et al., 1998; Sevilla et al., 2001) as well as by investigating the effect of inoculation of endophytic N_2 fixing bacteria on micropropagated sugarcane plants (Oliveira et al., 2002). In addition to the supply of fixed nitrogen, the production of plant growth promoting phytohormones such as indole acetic acid (IAA) may play an important role in this endophytic plant-microbe interaction (Sevilla et al., 2001).

To further explore and develop the capacity of *G. diazotrophicus* to supply the plant host with fixed nitrogen, a detailed understanding of the molecular processes of N_2 fixation and ammonium assimilation, and their regulation in *G. diazotrophicus* is required. The present knowledge at the molecular level of these issues will be the focus of this review.

2. Nitrogen Fixation in *G. diazotrophicus*

Biological nitrogen fixation is catalyzed by the enzyme complex nitrogenase, consisting of two proteins, dinitrogenase (the MoFe-protein) and dinitrogenase reductase (the Fe-protein) (Schindelin et al., 1997). Although no reports on *in vitro* studies of nitrogenase in *G. diazotrophicus* have been published, a number of studies on the physiology of N_2 fixation have been reported, with focus on the dependence on carbon substrate and the oxygen concentration required for optimal nitrogenase activity (Stephan et al., 1991; Burris, 1994; Reis and Döbereiner, 1998; Flores-Encarnacion et al., 1999; Luna et al., 2000; Pan and Vessey, 2001). An interesting feature is the tolerance to high oxygen concentrations, which requires carbon sources supporting high respiratory activity, and is partially due to expression of cytochrome *ba* under diazotrophic conditions (Stephan et al., 1991; Burris, 1994; Reis and Döbereiner, 1998; Flores-Encarnacion et al., 1999; Luna et al., 2000; Pan and Vessey, 2001). In addition, sudden increases in oxygen concentration do not lead

to irreversible inactivation of nitrogenase (Pan and Vessey, 2001), which has been attributed to conformational protection by a FeSII Shethna protein (Ureta and Nordlund, 2002), previously only found in species of *Azotobacter*.

Nitrogenase activity is partially inhibited by addition of ammonium ions, and less inhibition was seen at high concentrations of sucrose in the medium (Stephan et al., 1991; Reis and Döbereiner, 1998). This together with the fact that no hybridization to DNA from *G. diazotrophicus* using *draT* and *draG* genes as probes was found (Burris et al., 1991), makes it unlikely that nitrogenase activity in *G. diazotrophicus* is regulated by the same mechanism as that in *Rhodospirillum rubrum*, as well as in some other phototrophic bacteria and in some species of *Azospirillum* (Nordlund, 2000).

At the genetic level, 14 *nif* genes have been identified in *G. diazotrophicus* (Lee et al., 2000). These are organized in a 30.5 kb cluster also containing a *fixABCX* gene cluster. The *fix* genes were first identified in *Rhizobium meliloti* (Earl et al., 1987) and the gene products have been suggested to take part in electron transfer processes under diazotrophic conditions. In addition to the *nif* and *fix* genes, the 30.5 kb cluster also includes accessory genes coding for ferredoxins and proteins involved in high-affinity molybdate transport. The 30.5 kb cluster is the largest assembly of *nif-fix* and associated genes so far characterized in diazotrophic bacteria (Lee et al., 2000).

One of the *nif* genes identified is *nifA*, encoding the *nif* specific transcriptional activator. The deduced amino acid sequence of NifA shows that NifA of *G. diazotrophicus* contains a typical interdomain linker region between the C-terminus and the central domain carrying a motif which is believed to be involved in metal ion binding and redox sensing. This sequence is typical for NifA proteins that respond directly to oxygen (Fischer, 1994) and that are found in diazotrophs lacking NifL. It should be noted that no *nifL* gene has been identified in *G. diazotrophicus* (Lee et al., 2000).

The *nifA* gene in *G. diazotrophicus* is not preceded by a typical σ^{54}-dependent promoter, neither could a NtrC binding sequence be identified, both of which are components in the transcriptional regulation of N-metabolism in diazotrophic bacteria (Merrick, 1993; Merrick and Edwards, 1995). However, the expression of *nifA* is regulated in response to the N-status, as was shown using a *nifA::gusA* construct (Teixeira et al., 1999). Under 2% oxygen an 18-fold increase in expression, measured as GusA activity, was observed when the mutant strain was grown with 1 mM ammonium ions in the medium compared to growth under N-sufficient conditions (20 mM ammonium). Under 21% oxygen, the increase in *nifA::gusA* expression was 65-fold. These authors also studied the expression of *nifB*, using a *nifB::gusA* construct. *nifB* encodes a protein required for synthesis of the FeMo-cofactor of dinitrogenase (Allen et al., 1995) and the gene in *G. diazotrophicus* is preceded by both a σ^{54}-dependent promoter sequence and an upstream NifA binding sequence (Teixeira et al., 1999). With

low oxygen concentrations, the increase in expression was about 300-fold comparing low and high concentrations of ammonium ions. In the presence of 21% oxygen, there was essentially no expression with either of the two concentrations of ammonium ions used. This result indicates that the activity of NifA is sensitive to oxygen, most likely related to the deduced metal binding sequence. However, this result is in a sense contradictory to the ability of *G. diazotrophicus* to grow diazotrophically under aerobic conditions which does require transcription of *nif* genes, and could be due to the experimental conditions used. Nevertheless, from these studies it can be concluded that the transcription of *nifA* is regulated in response to the N-status of the cells and that this regulation is probably independent of σ^{54} and NtrC. It is thus possible that a novel system for transcriptional regulation of *nif* expression is operating in *G. diazotrophicus*.

3. Ammonium Assimilation in *G. diazotrophicus*

In all diazotrophic bacteria the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway plays a key role in the assimilation of fixed nitrogen (Merrick and Edwards, 1995). Under non-diazotrophic conditions glutamate dehydrogenase also contributes to the assimilation of ammonium ions. Studies by Alvarez and Martinez-Drets (1995) have shown that indeed the GS-GOGAT pathway is operating in *G. diazotrophicus* and according to their results there was an increase in total GS activity under diazotrophic conditions similar to what has been observed in other diazotrophs, e.g. *Klebsiella pneumoniae* (Merrick and Edwards, 1995) and *Rhodospirillum rubrum* (Johansson and Nordlund, 1996). There was no significant change in GOGAT activity but the activity of glutamate dehydrogenase decreased to about 50%. These authors also found that in extracts from cells grown in N-sufficient medium only 40% of GS activity remained in assay conditions rendering the adenylylated form of GS inactive, i.e. the presence of 60 mM Mg^{2+}. In extracts from cells grown diazotrophically there was no such effect.

In a more recent report GS from *G. diazotrophicus* was purified and partially characterized. As in the study of Alvarez and Martinez-Drets, these authors found that from cells grown in N-sufficient medium, only 40% of the GS activity remained when the assay was run under conditions inhibiting the adenylylated subunits. However, in this study (Ureta and Nordlund, 2001) total GS activity was the same in extracts from cells grown under N-sufficient conditions and in extracts from cells grown diazotrophically. If there was any difference the activity in the diazotrophic extract was lower. The activity measurements were confirmed by Western blot analysis showing that the amount of GS protein was about 70% higher in extracts from cells grown under N-sufficient conditions.

Furthermore Western blot and activity analyses of samples before and after treatment with snake venom phosphodiesterase, which removes AMP from the adenylylated subunits, also confirmed that GS in *G. diazotrophicus* is regulated by adenylylation (Ureta and Nordlund, 2001).

Although no detailed studies on the genetics of glutamine synthetase have been reported, an open reading frame with high similarity to the *glnA* gene in other bacteria has been identified in *G. diazotrophicus* (Perlova et al., 1998; Perlova et al., 2000). The gene is located downstream (less then 200 base pairs) of the *glnB* gene, an arrangement typical for α proteobacteria (Arcondéguy et al., 2001). No obvious promoter sequence can be found between the *glnB* and the *glnA* gene making it likely that the two genes are cotranscribed, again similar to the situation in other proteobacteria of the α-group. The regulation of the transcription of this operon has not been revealed, but judging from the results of the studies at the protein level (Perlova et al., 1998), there is no up-regulation of *glnA* expression during diazotrophy. It thus seems that the regulation of GS activity is mainly controlled by adenylylation, although the final conclusion will have to await detailed studies at the transcriptional level.

4. Regulation in Response to N-Status

Background

In most organisms nitrogen metabolism is tightly controlled in response to the nitrogen source available. The goal is to minimize the utilization of energy requiring pathways and at the same time guarantee a sufficient supply of nitrogenous compounds, e.g. glutamine, for the biosynthesis of the vital nitrogen containing biomolecules. In diazotrophic organisms this means that the highly ATP demanding nitrogen fixing process should only be used in conditions when molecular nitrogen is the only nitrogen source available. It should however be noted that in phototrophic bacteria, e.g. *R. rubrum* and *Rhodobacter capsulatus*, nitrogenase also has the function to relieve the cell of reductants and may thus be present even when nitrogen sources like glutamate are present (Tichi and Tabita, 2000).

The most central enzyme in the nitrogen control system is the bifunctional enzyme uridylyltransferase/uridylyl removing enzyme, encoded by the *glnD* gene. The enzyme catalyzes the modification of the signal protein P_{II} by uridylylation on a specific tyrosine residue (Y51) as well as the demodification, by hydrolysis of the UMP-moiety from the tyrosine residue. The activities of GlnD are regulated by glutamine, so that increasing glutamine concentrations lead to an increase in the uridylyl removing activity, whereas under nitrogen deficient conditions the uridylyltransferase activity

dominates (Merrick and Edwards, 1995; Ninfa and Atkinson, 2000). Consequently, the nitrogen status of the cell as sensed by GlnD is transduced to metabolism through the state of modification of P_{II}.

The first P_{II} protein to be identified was the one encoded by *glnB* in *Escherichia coli* (Shapiro, 1969), but since then it has been shown that a number of bacteria harbour at least two P_{II} paralogs, the second encoded by *glnK* (Arcondéguy et al., 2001). At the protein level these proteins show high similarity and it has been suggested that the P_{II} proteins encoded by genes localized in an operon with *amtB*, should be called GlnK (Arcondéguy et al., 2001) with the *amtB* gene encoding an ammonium transporter. Furthermore a third P_{II} paralog has been demonstrated for two other proteobacterial species. These are encoded by *glnY* in *Azoarcus* (Martin et al., 2000) and *glnJ* in *R. rubrum* (Zhang et al., 2001), and both of them seem to be more related to GlnK proteins than to GlnB proteins.

The targets of the P_{II} paralogs studied in most detail are regulatory proteins controlling either synthesis or activity of enzymes in nitrogen metabolism, such as NtrB, GlnE and NifA/L. NtrB is the sensor of the two-component system NtrBC, which can not bind the uridylylated form of P_{II} and then acts as a kinase catalyzing phosphorylation of NtrC. NtrC-P binds to specific upstream activating sequences of σ^{54}-dependent promoters thereby activating transcription by σ^{54}-RNA-polymerase. However, the unmodified form of P_{II} binds to NtrB stimulating its phosphatase activity and thereby the inactivation of NtrC as transcriptional activator. The *nifLA* operon in *Klebsiella pneumoniae* (Little et al., 2000) and *nifA* in *Rb. capsulatus* (Foster-Hartnett and Kranz, 1992) are examples of genes regulated by NtrBC. In most organisms GlnB is believed to be the P_{II} homolog that interacts with NtrB.

GlnE is the bifunctional enzyme catalyzing the regulation of glutamine synthetase activity by reversible adenylylation. The unmodified form of P_{II} stimulates the adenylylation activity, whereas the uridylylated form is required for deadenylylation (Jaggi et al., 1997). Also in this case GlnB is suggested to be the physiologically important P_{II} protein, with the exception of *Azotobacter vinelandii* which only has one P_{II} protein, GlnK (Meletzus et al., 1998a).

In a number of diazotrophs the expression of the structural *nif* genes is regulated by interaction of a P_{II} protein with either NifA or NifL. This regulatory mechanism is of special importance in organisms where *nifA* expression is constitutive, e.g. *Azospirillum brasilense* (Arsene et al., 1996, 1999). In this organism, activation of NifA is dependent on the presence of uridylyated GlnB. In *K. pneumoniae* and *A. vinelandii* GlnK are required to relieve the inhibitory effect of NifL on NifA (Jack et al., 1999; Little et al., 2000).

Regulation in G. diazotrophicus

The genes for the two-component system NtrBC have been isolated from *G. diazotrophicus* and are located directly downstream of *nifR3*, an organization also found in *A. brasilense* (Meletzus et al., 1998b). Interestingly, just downstream of *ntrBC* open reading frames very similar to *ntrYX* were found. These genes were originally identified in *Azorhizobium caulinodans* and the gene products were suggested to be involved in the control of N-metabolism, possibly acting in concert with NtrBC (Pawlowski et al., 1991). NtrY is homologous to transmembrane sensor proteins, whereas NtrX shows high similarity to NtrC (Pawlowski et al., 1991). The *ntrYX* genes have also been studied in detail in *A. brasilense* (Vitorino et al., 2001). While polar *ntrC-ntrY* mutants of *G. diazotrophicus* were unable to grow with putrescine as the sole nitrogen source, these mutant strains were still Nif^+ when grown under diazotrophic conditions, indicating that both the NtrBC and the NtrYX systems are required for the utilization of certain nitrogen sources, but neither of them is essential for nitrogen fixation (Meletzus et al., 1998b). As it has been shown that transcription of *nifA* is regulated in response to the nitrogen status in *G. diazotrophicus* (Teixeira et al., 1999), the regulatory components sensing the N-status and modulating the level of transcription of this transcriptional activator remain to be identified.

The gene encoding the central sensor in the regulation of nitrogen metabolism, *glnD*, has also been isolated (Meletzus et al., 1998b; Perlova et al., 1998) and recently characterized (Perlova et al., 2002) in *G. diazotrophicus*. From the deduced amino acid sequence it is concluded that GlnD of *G. diazotrophicus* is similar to other GlnD proteins, especially GlnD from *Sinorhizobium meliloti* (Perlova et al., 2002) and *A. brasilense*. Attempts to construct deletion mutants of the entire *glnD* gene were unsuccessful, indicating that such mutations are lethal in *G. diazotrophicus*. However, a mutant created by insertion of a kanamycin resistance cassette in the 3′-end of *glnD* was isolated and characterized (Perlova et al., 2002). This strain showed poor growth even in rich medium, but did show nitrogenase activity under derepressing conditions although the activity was dramatically lower than in wild-type. Interestingly, even under repressing conditions (20 mM NH_4^+) there was a weak expression of nitrogenase. A possible explanation for this is that the mutated GlnD protein has a decreased ability to sense the N-status, i.e. the glutamine concentration. This is reasonable as it has been suggested that the glutamine binding site is in the C-terminal part of GlnD, i.e. the region affected by the mutation (Perlova et al., 2002).

G. diazotrophicus can now be added to the list of those bacteria having three P_{II} paralogs. The *glnB* and *glnK* genes have previously been reported, where *glnB* is located directly upstream of *glnA* and *glnK* is directly followed

by an *amtB* gene (Meletzus et al., 1998b; Perlova et al., 1998), most likely forming two operons like in most proteobacteria studied (Arcondéguy et al., 2001). A third P_{II} gene showing highest similarity to *glnK* genes and located just upstream of a second *amtB* gene has now been isolated (Perlova and Meletzus unpublished). These two genes are suggested to form the $glnK_2amtB_2$ operon. Analysis of the putative promoter regions of these genes indicates that both *glnB* and $glnK_1$ are likely to be expressed from a σ^{70}-type constitutive promoter. Interestingly a highly conserved NtrC-binding site was found to overlap the –10 region of the putative $glnK_1$ promoter. In addition $gusA\text{-}glnK_1$ gene fusions show a 40% reduction of GusA activity when grown under high N conditions. Thus it is likely that NtrC is involved in the N-dependent regulation of the expression of the $glnK_1$ gene in *G. diazotrophicus* (Perlova and Meletzus, unpublished). In the case of $glnK_2$, no conserved promoter sequence could be identified and thus no information on the pattern of expression of this gene is available yet.

To study the role of the three P_{II} paralogs in regulation of nitrogen fixation, the possible single, double and triple mutants were constructed (Perlova and Meletzus, unpublished). Cells were grown in rich medium, washed in nitrogen free defined medium and then resuspended in defined medium with or without nitrogen source. After six hours nitrogenase activity was measured and samples were taken for Western blotting with antibodies against NifH (Ureta, Perlova, Meletzus and Nordlund, unpublished). Interestingly, the triple mutant ($\Delta glnB\ \Delta glnK_1\ \Delta glnK_2$) showed high nitrogenase activity even under repressing conditions (20 mM NH_4^+) and in $\Delta glnB\ \Delta glnK_2$ and $\Delta glnK_1\ \Delta glnK_2$ double mutants, a low activity, about 10% of that in the triple mutant was detected. Under derepressing conditions all strains except the $\Delta glnB\ \Delta glnK_1$ double mutant showed essentially wild-type activity. In this mutant strain no activity or NifH could be detected. It should be noted that a P_{II} protein is present in this mutant as shown by Western blotting. The results indicate that GlnK2 is required for complete control of nitrogenase synthesis under repressing conditions, as the three mutants ($\Delta glnB\ \Delta glnK_2$; $\Delta glnK_1\ \Delta glnK_2$; $\Delta glnB\ \Delta glnK_1\ \Delta glnK_2$) lacking GlnK2 showed induction of nitrogenase. On the other hand it appears that GlnB and/or GlnK1 are required for derepression of nitrogenase synthesis under N-limiting conditions.

In summary, all proteins, with the exception of NifL, that are components of the complex network controlling proteobacterial nitrogen fixation and nitrogen metabolism in general also are present in *G. diazotrophicus*. However, the role(s) of these proteins in *G. diazotrophicus* still needs to be elucidated in detail. One of the important issues to be addressed is how *nifA* expression is regulated, as it clearly is not NtrC(X) dependent. Another major question is the different roles of the three P_{II} proteins in nitrogen fixation and nitrogen metabolism, do they or one of them interact directly with NifA, regulating its

activity, and/or some protein controlling *nifA* expression? In this context it is important to clarify if all three P_{II} paralogs are substrates for GlnD. Finally is there a regulatory interaction between GlnK and AmtB as in *E. coli* and *A. vinelandii* (Coutts et al., 2002)? Understanding the regulatory network and the signal transduction will be a prerequisite to increase the capacity of *G. diazotrophicus* as an efficient supplier of fixed nitrogen to its plant hosts.

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