Posttranslational mechanisms of nitrogenase regulation in *Rhodopseudomonas palustris* CGA009

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ABSTRACT

Post-translational mechanisms of nitrogenase regulation in *Rhodopseudomonas palustris* CGA009

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Nitrogenase catalyzes the conversion of dinitrogen gas (N_2) and protons to ammonia and hydrogen gas (H_2). This is a catalytically difficult reaction that requires large amounts of ATP and reducing power. Thus, nitrogenase is not normally expressed or active in bacteria grown with a readily utilized nitrogen source like ammonium. *nifA** mutants of the purple nonsulfur phototrophic bacterium *Rhodopseudomonas palustris* express nitrogenase genes constitutively and produce H_2 when grown with ammonium as a nitrogen source. This raised the regulatory paradox of why these mutants are apparently resistant to a known posttranslational modification system that should switch off the activity of nitrogenase. In this study I used mutation analysis and gene expression studies to show that posttranslational regulation of nitrogenase activity in *R. palustris* depends on two proteins: DraT2, an ADP-ribosyltransferase, and GlnK2, an NtrC-regulated P(II) protein. GlnK2 was not well expressed in ammonium-grown NifA* cells and thus not available to activate the DraT2 nitrogenase modification enzyme. In addition, the NifA*

strain had elevated nitrogenase activity due to overexpression of the *nif* genes, and this increased amount of expression overwhelmed a basal level of activity of DraT2 in ammonium-grown cells. Thus, insufficient levels of both GlnK2 and DraT2 allow H_2 production by a nifA* mutant strain grown with ammonium. Inactivation of the nitrogenase posttranslational modification system by a *draT2* mutation resulted in increased H_2 production by ammonium-grown NifA* cells. I also showed that the two alternative nitrogenases of *R. palustris* are subject to DraT regulation. The *vnf* cluster has two different phylogenies, the *vnfDGK* genes were horizontally acquired while the *vnfH* gene is the product of a gene duplication event. Transcriptional regulation of V-nitrogenase in *R. palustris* is poorly understood and I have isolated and described a mutant which expresses V-nitrogenase constitutively.

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CHAPTER I:

INTRODUCTION

Regulation of Nitrogenase in Bacteria

Hydrogen

Hydrogen as an energy carrier and current production methods. Hydrogen gas (H_2, called hydrogen for the remainder of this work) could be developed as a sustainable energy carrier and a replacement for fossil fuels in several applications, including as a 'clean' transportation fuel (55, 71, 72). Currently nearly 90% of total hydrogen is produced by steam reformation of methane, a method that is costly and releases CO_2 and other pollutants into the atmosphere (14, 30). In conjunction with the development of H_2 utilization technologies, the development of methods which significantly reduce the cost of hydrogen production could bring this energy carrier economically in line with fossil fuels (55, 71). Because renewable energy sources – including solar, wind, and geothermal – differ by geographical region and all can be used to make hydrogen, hydrogen could also be developed as a universal energy carrier, more easily produced locally than gasoline or natural gas (30). Hydrogen has several non-energy uses as well, including in the refinement of fossil fuels, as an O_2 scrubber to prevent corrosion, and as a coolant in electrical generators (14). The biological conversion of sunlight energy to chemical energy in the form of hydrogen could become a significant portion of future energy production (46, 55).

Biological production of hydrogen. Biological hydrogen production occurs via several mechanisms, including dark fermentation and photobiological production from water or organic electron donors (14, 30, 55). Hydrogen production, powered by

photophosphorylation, can occur via either hydrogenase or nitrogenase (3, 14, 55). Hydrogenase has a low energy requirement for hydrogen production, but is subject to feedback inhibition and is oxygen sensitive (14, 55, 69). Nitrogenase is also oxygen sensitive and requires a large amount of ATP per H_2 formed, but is not inhibited by its product (11, 14, 23, 33). H_2 is an inhibitor of the N_2-fixing activity of nitrogenase, but is not an inhibitor of hydrogen evolution or acetylene reduction; this lack of product inhibition is in contrast to hydrogenases, which are subject to equilibrium chemistry and thus inhibition by H_2 (26, 55, 62). Although the main function of nitrogenase is the production of ammonia, it has long been known that in the absence of N_2 or other substrates, nitrogenase reduces protons exclusively, forming pure H_2, a process that can potentially be exploited for the biological production of hydrogen fuel (34, 46). Photobiological hydrogen production from nitrogenase could be one major method of meeting future global energy demands.

Model Organism

Rhodopseudomonas palustris. I have studied the phototrophic bacterium *Rhodopseudomonas palustris* as a model organism for biological H_2 production via nitrogenase. *R. palustris* is a member of the alpha proteobacterial purple non-sulfur bacteria (PNSB) that is widely distributed in the environment. It is extraordinarily metabolically versatile, capable of chemotrophic as well as phototrophic modes of growth, and has a relatively large genome as a testament to this versatility (39). This

organism can generate the ATP needed for the energy-intensive nitrogen fixation process from the abundant resource of sunlight and can degrade structurally diverse organic compounds, including lignin monomers that are typically found in agricultural waste, as a source of electrons for H_2 production (Figure 1.1) (6, 28, 59). It can also use the inorganic compound thiosulfate, a byproduct of several industrial processes, as an electron source for nitrogen fixation and H_2 production (31). From a bioengineering standpoint, *R. palustris* is a hardy organism that can produce H_2 continuously for months without significant loss of viability (24).

Nitrogenase

Nitrogenase enzyme. Many Bacteria and Archaea can convert N_2 to ammonia, a biologically usable form of nitrogen that sustains life on earth (33). The energetically demanding reaction of reducing the triple bond of N_2 to ammonia is accomplished by the highly conserved enzyme molybdenum nitrogenase with the following stoichimetry:

$$N_2 + 8e^- + 8H^+ + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP \quad (11, 33)$$

Nitrogen fixation is complex. In an initial phase, two protons are reduced to H_2, a catalytic event that is hypothesized to prepare the active site of nitrogenase for subsequent reduction of N_2. H_2 is an obligate product of the nitrogen fixation reaction and its production cannot be excluded, even under 50 atm of N_2 (11, 17, 33, 67). Each of the eight electrons that participate in the overall reaction resulting in H_2 and NH_3 production

is delivered to the active site individually with accompanying hydrolysis of two ATP. Thus the overall rate of catalysis by nitrogenase is very slow (a catalytic turnover of about 5 s^{-1}), necessitating the synthesis of large amounts of enzyme (15, 27). Nitrogenase also contains several complex metal cofactors, whose synthesis and insertion into apoenzyme requires the coordinated activities of multiple accessory proteins (63). As is often the case in other nitrogen fixing organisms, in *R. palustris* genes required for nitrogenase synthesis and function are found in the *nif* cluster on the chromosome (Figure 1.2) (4, 39).

Alternative nitrogenases. It was previously reported that *R. palustris* strain CGA009 encodes three functional nitrogenase isozymes: a *nif*-encoded molybdenum-nitrogenase, a *vnf*-encoded vanadium-nitrogenase, and an *anf*-encoded iron-nitrogenase (Figure 1.2) (51). Mo-nitrogenase is the most commonly found form of the enzyme, while the vanadium and iron isozymes are considered alternative nitrogenases (7, 11). Other isolates of *R. palustris* contain only Mo- and Fe-nitrogenases, but not all three, suggesting that the acquisition of V-nitrogenase by strain CGA009 was a recent event (52). As well as being less common, the alternative nitrogenases are also less efficient for nitrogen fixation, consuming more reducing power and producing more hydrogen per molecule N_2 fixed than Mo-nitrogenase (18). However, this aspect of the alternative nitrogenase enzymes makes them attractive for the biological production of hydrogen gas using *R. palustris* as a biocatalyst.

Regulation of Nitrogenase

Predicted nitrogenase regulation. Free-living, non-symbiotic nitrogen fixers like *R. palustris* face a common set of physiological issues that must be overcome, including substantial demands for ATP, reducing power, and metals; the slow turnover rate of the enzyme, necessitating large quantities of protein synthesis; the O_2 sensitivity of nitrogenase enzyme; and the logic of using fixed nitrogen sources preferentially versus atmospheric N_2 (15). I focus here on nitrogenase regulation in response to ammonium, which has been studied in detail in several different Bacteria and Archaea. Because nitrogen fixation is an energetically demanding and slow process and because nitrogenase is a complicated enzyme to make, its synthesis and activity tend to be strongly repressed by ammonium, a bio-available form of nitrogen. The study of nitrogenase regulation in model organisms allows one to infer the broad outlines of major regulatory control features in a particular organism by inspection of genomic content. Predictions based on its genome sequence show that *R. palustris* regulates nitrogenase synthesis and activity at three levels (Fig. 1.3). Further descriptions of the components governing these three levels of regulation are found below.

P_{II} proteins. Proteomics experiments indicate that *R. palustris* likely senses ammonium availability, as do most Bacteria and Archaea, using P_{II} signal proteins (13, 41). These proteins are uridylylated/deuridylylated by GlnD, a bifunctional uridylyltransferase/uridylyl removing enzyme. When intracellular glutamine (a signal of

nitrogen sufficiency) is low, GlnD adds uridylyl groups to P_{II} proteins to form P_{II}-UMP. This alters the conformation of P_{II}s and changes their ability to interact with target proteins (20, 41, 48). In general, GlnK homologs are found at more abundant quantities than GlnB homologs under low nitrogen conditions, and the same is true for GlnK2 of *R. palustris* (13, 29, 58)

NtrBC. Nitrogenase synthesis is regulated at the highest level by the two-component regulators, NtrBC, which are responsible for activating expression of nitrogen starvation genes in most proteobacteria (Fig. 1.3, level 1). In the presence of fixed nitrogen the histidine kinase NtrB in combination with a bound non-uridylated P_{II} protein stimulates dephosphorylation of the transcription factor NtrC. In ammonium-depleted cells NtrB is not bound by P_{II}, allowing the phosphorylation and activation of NtrC by NtrB (48). NtrC is an enhancer-binding-protein response regulator, which when phosphorylated and thus active allows σ^{54}-RNA polymerase to bind the promoters of NtrC-regulated genes. In the phototrophic bacteria *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* as well as in many non-photosynthetic bacteria, including *Klebsiella* sp., NtrC-P activates expression of *nifA* as well as other nitrogen response genes. (15, 48) The NtrBC regulon of *R. palustris* includes about 270 genes, including *nifA*, which is expressed at about a three-fold lower level in the *ntrBC* mutant (29).

NifA. NifA is the proteobacterial master transcriptional activator protein of the *nif* genes encoding nitrogenase and accessory proteins and the second level of nitrogenase regulation (Fig. 1.3, level 2) (15). NifA is an enhancer DNA binding protein similar to NtrC. It has three major domains – a GAF domain, an AAA ATPase domain, and a helix-turn-helix DNA binding domain – and allows binding of σ^{54}-RNA polymerase to the promoters it activates (15, 68). In *Azotobacter vinelandii*, this GAF domain binds α-ketoglutarate, a small molecule found in the cell when fixed nitrogen is limiting; bound α-ketoglutarate blocks the binding of an anti-activator, NifL (42). In alpha proteobacterial systems, the GAF domain interacts with the ATPase domain, directly inhibiting its activity under non-nitrogen-fixing conditions (5). In *Rhodospirillum rubrum*, which does not encode for NifL, the NifA protein is activated posttranslationally by P_{II}- UMP, which relieves the GAF domain repression (77). The *R. palustris* genome does not encode for a NifL homolog, so presumably its NifA regulation is similar to its close relative, *R. rubrum*, as well as other studied alpha proteobacteria. However, this hypothesis has yet to be proven (39, 51). Previous work showed that NifA of *R. palustris* tightly regulates the *nif* structural genes, activating their expression at 50- to 100-fold higher levels when fixed nitrogen is limiting (29).

Regulation of nitrogenase by posttranslational modification. DraT (Dinitrogenase Reductase ADP-ribosyl Transferase) and DraG (Dinitrogenase Reductase ADP-ribosyl Glycohydrolase) are enzymes that control nitrogenase activity posttranslationally, the

third level of nitrogenase regulation (Fig. 1.3). They accomplish this regulation by adding ADP-ribosyl groups from NAD to a conserved arginine on its dinitrogenase reductase subunit (NifH), and subsequently removing the modification when fixed nitrogen levels decrease (43, 48, 54, 79). This third level of regulation is also modulated by P_{II} proteins. In several purple phototrophic nitrogen fixers, when cells are exposed to a fixed nitrogen source such as NH_4^+, glutamine, or several other nitrogenous compounds, one or more P_{II} isozymes are deuridylylated and are able to interact with DraT to activate its activity (32, 74). In phototrophic organisms, darkness can also cause the posttranslational inactivation of nitrogenase (23, 79). This posttranslational modification mechanism of control over nitrogenase activity saves energy and reducing power by stalling the enzyme when fixed nitrogen levels are high or when energy is limiting, as is the case in darkness. In environmental niches the availability of nutrients, including fixed nitrogen, is often transient. It is more conservative to modify and de-modify nitrogenase to force preferential consumption of the fixed nitrogen source rather than degrade the whole enzyme (up to 20% of total cellular protein) and resynthesize mature metalloenzyme when the fixed nitrogen source is depleted (15). *R. palustris* strain ATCC 17001 has been shown to be subject to loss of nitrogenase activity after ammonium exposure and during darkness (1, 79). The genome sequence of strain CGA009 (the wild-type strain used in this work) revealed *draT* and *draG* genes homologous to those found in *R. rubrum* (39).

Regulation of alternative nitrogenases. While the expression of Mo-nitrogenase is preferred by the organism, it is unclear how the alternative nitrogenases are expressed in response to inactivation of Mo-nitrogenase in *R. palustris*. Several nitrogen fixing bacteria in addition to *R. palustris* contain an alternative nitrogenase (either V or Fe) for use in the event that Mo becomes limiting. It is known that VnfA and AnfA, both NifA homologs, are required for the expression of *vnf* and *anf* genes, respectively. Neither VnfA nor AnfA of *R. palustris* contain the linker region between the GAF and AAA-ATPase domains that when deleted from NifA causes constitutive activity (39, 47). In *Rhodobacter capsulatus*, expression of the Fe alternative nitrogenase is repressed by a regulator that senses the presence of Mo ions (38). However, in *R. palustris*, the availability of Mo does not repress expression of the alternative nitrogenases. Instead, the presence of a functional Mo-nitrogenase enzyme appears to repress expression of the V- and Fe-nitrogenases through an unknown mechanism (51). Unpublished observations of *R. palustris* suggest that long-term nitrogen starvation, which could occur when molybdenum is limiting or the Mo-nitrogenase is otherwise nonfunctional, causes the upregulation of *vnf* and *anf* genes, even in the presence of a functional Mo-nitrogenase. At the post-translational level of regulation, inactivation of Fe-nitrogenase was reported in the PNSB *Rhodobacter capsulatus* by Masepohl et al. in 1993, but was not previously observed in *R. palustris* (45). There are no reported instances of a V-nitrogenase being subject to post-translational modification and inactivation.

Phylogeny of Nitrogenase

Alternative Nitrogenase Phylogenies. It has been hypothesized that all nitrogenases diverged from an early common prokaryotic ancestor, as it is present paraphyletically in a number of bacteria and archaea (57). Recent phylogenetic studies suggest that Mo-nitrogenase evolved from the ancestor of Nfl, an enzyme involved in Ni porphyrin F430 biosynthesis, in an ancestor of methanogenic Archaea (8, 9). It is also related to Bch/Chl chlorophyll biosynthesis genes. All three enzymes contain a chlorophyll or metal-cluster binding pocket which, when modified and improved upon by accessory enzymes, fine-tunes catalytic activity. V-nitrogenase subsequently evolved from Mo-nitrogenase, presumably for use in environments where Mo, but not V, was limiting. This alternative nitrogenase requires the presence of the *nif* accessory proteins as well as its own accessory proteins to insert a FeVco metal cluster into the active site and has never been observed in an organism that does not also have a *nif* gene cluster. Then, Fe-nitrogenase is hypothesized to have evolved from V-nitrogenase in the methanogenic archaeal ancestor (9). Dispersion of nitrogen fixation to bacteria appears to have occurred through lateral gene transfer from a methanogen to a member of the *Firmicutes* (8). *R. palustris* CGA009 is unusual in that it encodes for all three known forms of nitrogenase, a situation seen only in two other organisms, *Azotobacter vinelandii* and *Methanosarcina acetovorans* (22). Of the six *R. palustris* genomes sequenced, only CGA009 encodes for all three nitrogenases; the others encode for either the Mo-nitrogenase alone or the Mo- and Fe-nitrogenases (52). The related bacteria *R. capsulatus* and *R. rubrum* encode for both Mo- and Fe-nitrogenase as well (57, 65). This difference among closely related

strains suggests that V-nitrogenase was a recent horizontal gene transfer into strain CGA009.

In this thesis I sought to address several questions about the posttranslational modification of nitrogenase and about the regulation of the two alternative nitrogenases found in *R. palustris* strain CGA009.

In chapter 3 I explain the apparent paradox suggested by a mutant strain which encodes for ammonium-stimulated posttranslational inactivation of nitrogenase and yet produces high amounts of hydrogen from nitrogenase in the presence of ammonium. I found that the posttranslational regulation of nitrogenase in *R. palustris* depends on a protein that is not well expressed in the presence of ammonium. I also show that the mutation that allows for nitrogenase expression in the presence of ammonium also results in increased expression of nitrogenase, which overwhelms the regulatory apparatus and allows for hydrogen production.

In chapter 4 I investigated the posttranslational modification and inactivation of the two alternative nitrogenases encoded for by *R. palustris* CGA009. I found that both nitrogenases are modified and inactivated in the presence of ammonium. I also conclude that the modified subunit of V-nitrogenase is the product of a gene duplication event

which I hypothesize occurred in order to bring the V-nitrogenase under better posttranslational control.

In chapter 5 I describe how I selected for and observed a mutant of *R. palustris* which expresses V-nitrogenase constitutively and which may be used to understand how the alternative nitrogenases are transcriptionally regulated.

In Appendix A I describe the work I've done for papers published by collaborators both inside and outside the Harwood lab.

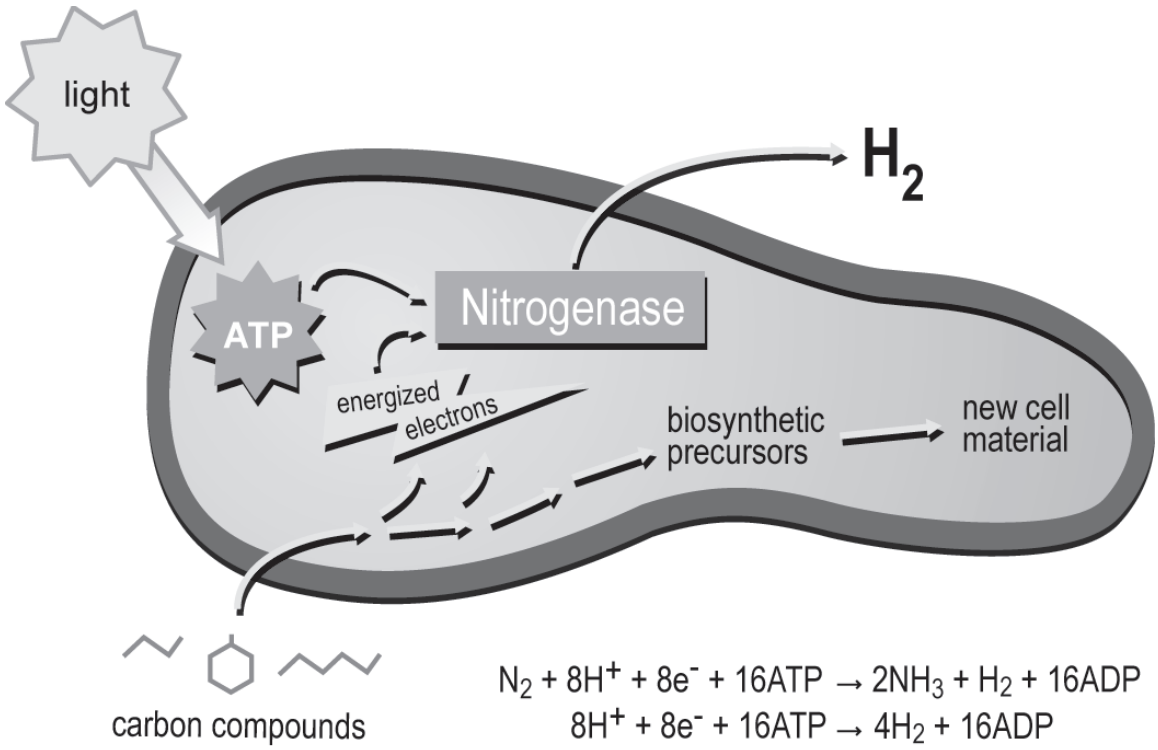

Figure 1.1. *R. palustris* produces hydrogen from nitrogenase by harvesting light for ATP and carbon compounds for electrons. N_2 is fixed according to the first equation. If N_2 is excluded, nitrogenase produces pure hydrogen.

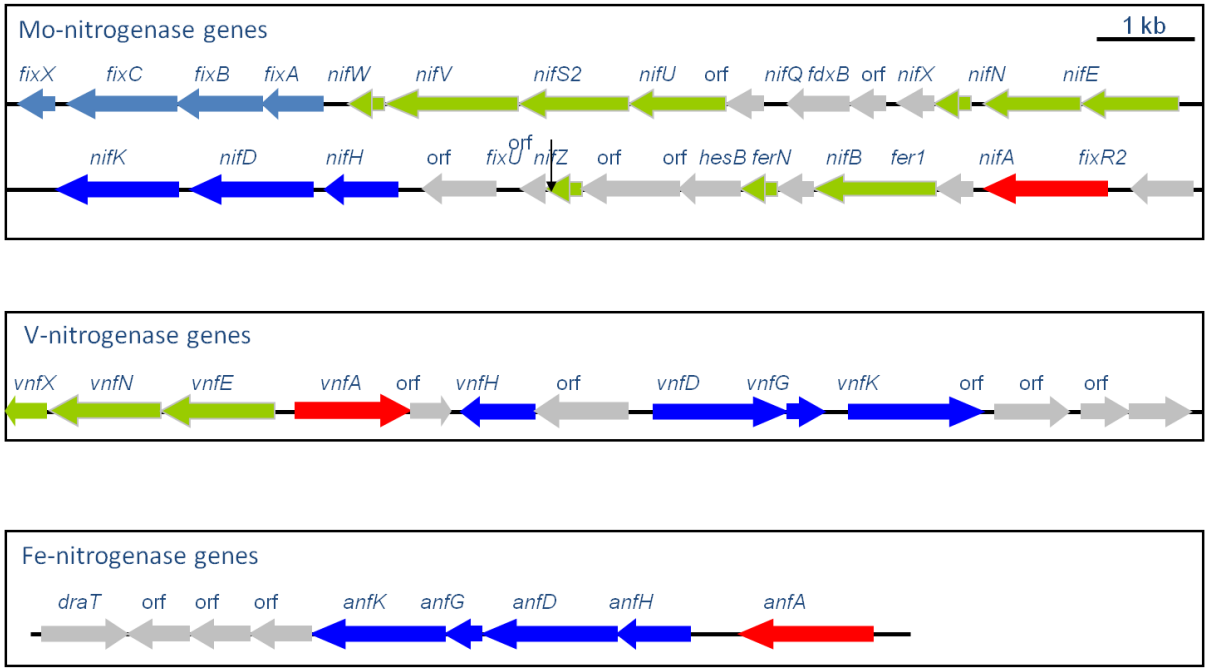

Figure 1.2. Genes for nitrogenase structural and accessory genes are found in three locations on the *R. palustris* chromosome. The Mo-nitrogenase genes are encoded by the *nif* gene cluster (top); the V-nitrogenase genes are encoded by the *vnf* gene cluster (middle); and the Fe-nitrogenase genes are encoded by the *anf* gene cluster (bottom). Structural genes are shown in blue, regulatory genes in red, cofactor assembly and insertion genes in green, and electron transfer genes in light blue. Other genes and genes of unknown function are shown in gray.

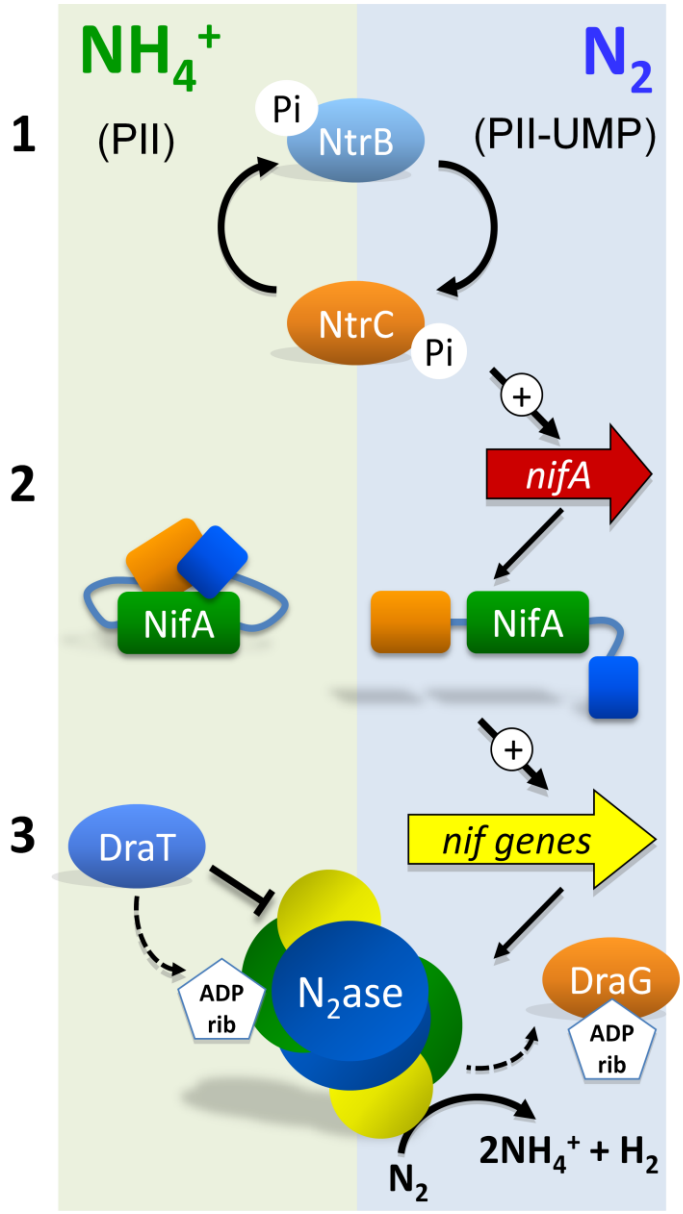

Figure 1.3. *R. palustris* nitrogenase synthesis and activity is predicted to be regulated at three levels in response to fixed nitrogen availability. The predicted activities of regulatory proteins NtrBC, NifA and DraT in ammonia-grown cells are shown on the left side, whereas the predicted activities of NtrBC, NifA and DraG in cells that are starved for fixed nitrogen (nitrogen-fixing cells) are shown on the right side. As described in the text, the activities of these proteins are influenced by interactions with P_{II} proteins which are either unuridylylated or uridylyated depending on cellular nitrogen status.

CHAPTER II:

MATERIALS AND METHODS

Bacterial Growth. *R. palustris* was grown anaerobically in sealed tubes in nitrogen-fixing (NF) medium, a nitrogen-free mineral-based minimal medium with N_2 in the headspace, for nitrogen-fixing conditions (51). When a fixed source of nitrogen was used, ammonium sulfate was added to 0.1% (PM medium) (36). Cells were grown with 20 mM sodium acetate, 10 mM sodium succinate, or 3 mM cyclohexanecarboxylic acid, pH 7.0, as indicated. In some experiments strains were grown in NF or PM medium supplemented with 0.3% yeast extract and succinate in an argon (Ar) atmosphere, as indicated. *R. palustris* strains were grown anaerobically in light at 30 ± 2°C. For mutant selections, *R. palustris* was grown aerobically on CA plates (NF medium, 4 g/L yeast extract, 5 g/L casamino acids, 1.5% Bacto agar). Experiments in Chapters 4 and 5 were carried out by growing *R. palustris* anaerobically in NF medium with N_2 in the headspace, as well as with 1 mM VCl_3 and 1X Wolfe's vitamins (35, 36). *E. coli* was grown in Luria-Bertani (LB) medium at 37°C with shaking at 250 rpm. Where indicated, *R. palustris* was grown in the presence of 70 or 100 µg gentamicin (Gm) sulfate per ml or 100 µg kanamycin sulfate (Km) per ml. *E. coli* was grown with 100 µg per ml ampicillin (Ap) or 20 µg per ml Gm.

Strain constructions. All *R. palustris* strains described in this work are either wild type CGA009 or derivatives thereof (Table 2.1). The *ntrBC* and *nifA* mutant strains used for microarray analysis were generated in CGA010, a derivative of CGA009 in which a *hupV* frameshift mutation was repaired (60). Gene deletions or replacements were done via

homologous recombination. For *glnB, glnK1* and *draT2* mutations, DNA sequences of approximately 1 kb in size that flanked either side of the desired deletion were cloned into pUC19. A Km resistance cassette from plasmid pBSL15 was cloned between the two flanking regions. This construct was then cloned into the suicide vector pJQ200SK (56). For *ntrBC, nifA, glnK2*, and *draT1* mutations, flanking regions were cloned into pJQ200SK, but no Km resistance gene was added. The *ntrBC, nifA, glnB, glnK2, draT1*, and *draT2* deletion suicide vectors were introduced into *R. palustris* from *E. coli* S17-1 by conjugation. The *glnK1* deletion suicide vector was electroporated into *R. palustris* as described (53). Single recombinants were selected for on CA plates containing Gm. Double recombinants were selected on PM - succinate plates containing 10% sucrose for *ntrBC, nifA, glnK2,* and *draT1,* or 10% sucrose and Km for *draT2, glnB* and *glnK1*. Complementation vectors were constructed using pBBR1-MCS5 (37), and constructs were amplified in and purified from *E. coli* strain DH5α before either being transferred into *E. coli* S17-1 and used for conjugation or directly electroporated into *R. palustris*.

Nitrogenase assays. Nitrogenase activity was measured by monitoring the production of ethylene from acetylene. Cultures of *R. palustris* were grown to an OD_{660} of 0.35 to 0.45. For the acetylene reduction assay, 10 ml of cells were harvested anaerobically and resuspended in 10 ml of 25 mM sodium phosphate and potassium phosphate buffer (phosphate buffer), pH 7.0. Resuspended cells were transferred to 16 mL rubber septum sealed tubes filled with Ar. Sodium acetate was added to 10 mM and cells were allowed

to recover at 30°C in light for 60 min. Then, 250 µl of 100% acetylene gas was added to the headspace of tubes to initiate the assay. Ethylene produced by nitrogenase activity was measured over time by gas chromatography as previously described (51). After linear ethylene production had been established (about 20 min after acetylene injection), either sodium chloride or ammonium chloride was injected into the assay tube to a final concentration of 100 µM. Stocks of 10 mM sodium chloride and ammonium chloride were prepared and stored anaerobically. Ethylene production was measured for at least 40 min after addition of sodium chloride or ammonium chloride and normalized to total protein. Nitrogenase specific activity was also measured by acetylene reduction. In this assay, cells were grown in 16 mL rubber septum sealed tubes filled with Ar to an OD_{660} of 0.35 to 0.45. Ethylene and ethane production was measured for at least 2 hours and results normalized to total cellular protein. The total protein content of cell suspensions was estimated from the OD_{660} of the suspension using a standard curve prepared with whole *R. palustris* CGA009 cells grown under nitrogen-fixing conditions. To generate this curve, the Bio-Rad Protein Assay was used to measure total protein from NaOH lysed cells (10).

Hydrogen assays. Measurement of nitrogenase activity in Chapter 4 was done by monitoring the accumulation of hydrogen gas in the headspace above the cultures. Cultures of *R. palustris* were allowed to grow to an OD660 of 0.35 to 0.45. Cells (60 mL) were harvested anaerobically and resuspended in 25 mM sodium phosphate / potassium

phosphate buffer, pH 7.0. Cells were transferred to sealed 27 mL tubes containing an argon atmosphere and allowed to recover for 60 min before hydrogen production was measured. Hydrogen evolution was measured by gas chromatography as previously described (51)**.** Hydrogen production was established for approximately 20 min before either sodium chloride or ammonium chloride was added to the assay tube to a final concentration of 100 μM. Stocks of 10 mM sodium chloride and ammonium chloride were prepared and stored anaerobically. Hydrogen production was monitored for at least 40 min after salt addition. Non-switch off hydrogen production assays were also carried out by measuring the evolution of H_2 during exponential growth (OD660 0.2 to 0.7). H_2 produced was normalized to total cellular protein, the measurement of which is described above.

Protein modification assay. Protein for visualization of NifH post-translational modification was prepared from cells that had been grown under nitrogen-fixing conditions and exposed to either 100 μM sodium chloride or 100 μM ammonium chloride 30 min prior to harvest. The NifA* strain was grown in PM-acetate medium. To arrest cell metabolism, 5 ml of treated cell suspension was placed an ice water bath for 5 min and then centrifuged for 7 min at 4°C. The supernatant was decanted and the cell pellet was frozen quickly in liquid nitrogen and stored at -20°C. Frozen cell pellets were thawed on ice and resuspended in 300 μl lysis buffer [25 mM Tris-HCl pH 7.8, 150 mM NaCl, 1 mM EDTA, 1 mM fresh DTT, 0.5% Tween, 10% glycerol, 1X HALT protease

inhibitor cocktail (Thermofisher)]. Cells were lysed by sonication and cell debris was removed by centrifugation at 4°C. Cell extracts were measured for total protein content using the Bio-Rad Protein Assay. Approximately 1 or 2 µg of protein was loaded per SDS-PAGE well. Low crosslinker SDS-PAGE gels were used to resolve the ADP-ribose modified protein from the unmodified protein (acrylamide: bisacrylamide ratio was 171:1). Proteins were transferred to a PVDF membrane and incubated with rabbit antiserum prepared against NifH purified from *Azotobacter vinelandii*. Antibodies to NifH cross-reacted with VnfH and AnfH, though a 10-fold higher concentration of antibody was required to visualize AnfH than either VnfH or NifH. Anti-rabbit horse radish peroxidase secondary antibody was hybridized to the primary antibody and Pierce/ThermoFisher ECL femto-substrate was used for visualization.

Quantitative reverse transcription real-time PCR (qRT-PCR). cDNA was synthesized as in the Affymetrix GeneChip experiment (without adding eukaryotic poly-A RNA control) and purified with the QIAquick PCR Purification kit (QIAGEN) (59). Primers were designed using the Primer Express version 2.0.0 software (Applied Biosystems). PCR reactions included 1 ng of cDNA and primers at a concentration of 500 nM in 20 µl of SsoFast Evagreen PCR amplification Supermix (Bio-Rad). Genomic DNA was used as a standard. The temperature profile was as follows: 98°C for 2 min, and then 45 cycles at 98°C for 5 sec (denaturation) and 60°C for 10 sec (annealing and extension). Amplification and the amount of PCR products were monitored by using the

Bio-Rad CFX96 Real-Time PCR System. Each reaction was performed in triplicate, and average data from at least two experimental replicates are reported.

Protein alignment and phylogenetic tree generation. Protein sequences were accessed from the GenBank database and input in the ClustalW alignment tool (40). The resulting alignment was trimmed to show only the region of interest, including the modified subunit, Arginine 101 on the *R. palustris* NifH protein. Phylogenetic trees were built using Mega5 software (70). Protein sequences were aligned using the ClustalW algorithm and phylogenies inferred using the neighbor-joining method with all gaps completely deleted (64). Bootstrap values were calculated as described in the figure legends.

Table 2.1. Strains, plasmids and primers used in this study

Strain, plasmid or primers	Genotype or phenotype	Reference origin or description
R. palustris strains		
CGA009	Wild-type strain; spontaneous Cm^r derivative of CGA001	(36)
CGA010	CGA009 *hupS*$^+$ derivative	(60)
CGA552	Δ*nifA*	This study
CGA676	NifA*, $nifA_{\Delta 48bp}$, constitutive mutation	(47)
CGA653	*glnB*::Km^r in a CGA009 background	This study
CGA720	*glnK1*::Km^r in a CGA009 background	This study
CGA721	Δ*glnK2* in a CGA009 background	This study
CGA722	Δ*draT1* (RPA1431) in a CGA009 background	This study
CGA723	Δ*draT2* (RPA2405) in a CGA009 background	This study
CGA724	Δ*draT2* in CGA676 (NifA*) background	This study
CGA725	Δ*glnK2* in CGA676 (NifA*) background	This study
CGA726	Δ*ntrBC* in CGA676 (NifA*) background	This study
CGA730	Δ*nifH, nifD::*Tn5, $nifA_{L208P}$, evolved strain	This study
CGA753	Δ*vnfH*, Δ*anfH*, Mo nitrogenase strain	(51)
CGA755	Δ*nifH* Δ*vnfH*; Fe nitrogenase strain	(51)
CGA762	Δ*nifH, nifD::*Tn5	(51)
CGA763	Δ*nifH, nifD::*Tn5, $nifA_{\Delta 48bp}$, constitutive mutation	
CGA766	Δ*nifH, nifD::*Tn5, Δ*anfA*, V nitrogenase strain	(51)
CGA805	CGA010 *hupS*$^+$ Δ*ntrBC*	This study
CGA806	CGA009 Δ*ntrBC*	This study
E. coli strains		
DH5α	*F- λ- recA1 Δ(lacZYA-argF)U169 hsdR17 thi-1 gyrA96 supE44 endA relA1 φ80dlacZΔM15*	GIBCO-BRL
S17-1	*thi pro hdsR hdsM+ recA*; chromosomal insertion of RP4-2 (Tc::Mu Km::Tn7)	(66)
Plasmid		
pJQ200SK	Gm^r, *sacB* suicide vector	(56)
pUC19	Ap^r high-copy-number cloning vector	(73)
pBSL15	Km^r cassette containing plasmid	(2)
pJQntrBC	pJQ200SK containing a *ntrBC* in-frame deletion construct	This study
pJQnifA	pJQ200SK containing a *nifA* in-frame deletion construct	This study
pJQglnB	pJQ200SK containing a *glnB*::Km^r deletion construct	This study
pJQglnK1	pJQ200SK containing a *glnK1*::Km^r deletion construct	This study
pJQglnK2	pJQ200SK containing a *glnK2* in-frame deletion construct	This study
pEH012	pJQ200SK containing a *draT1* in-frame deletion construct	This study
pEH016	pJQ200SK containing a *draT2*::Km^r deletion construct	This study

pBBR1MCS-5	Broad host range expression vector, Gm^r	(37)
pEH023	pBBR containing *glnK2* as well as its predicted promoter region	This study
pEH024	pBBR containing the coding region of *draT2*	This study
pEH025	pBBR containing the SD consensus and the coding region of *glnK2*	This study
pEH026	pBBR containing the SD consensus and coding regions of *glnK2* and *draT2*	This study
Primer for qRT-PCR		
EH219	TGACGGAAGTGAAGGGATACG	*glnK2* forward
EH220	CAGCGCCGCGGTAGATT	*glnK2* reverse

CHAPTER III:

How posttranslational modification of nitrogenase is circumvented in *Rhodopseudomonas palustris* strains that produce hydrogen gas in the presence of fixed nitrogen.

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INTRODUCTION

Nitrogenase is an expensive enzyme to synthesize and supply with ATP and reducing power. Therefore, this enzyme is tightly regulated at several levels (Figure 1.3). Transcriptional regulation of nitrogenase occurs under low-ammonium conditions as NtrC activates *nifA* expression and NifA in turn activates *nif* gene expression. Basal expression of *nifA* allows low levels of expression of the *nif* genes in the *ntrBC* mutant strain, but at much lower levels in than in fully induced wild-type cells (29). At the third level of regulation, nitrogenase is inactivated by DraT-mediated posttranslational modification of the NifH subunit. This regulatory system has been observed from several species of α-proteobacterial nitrogen fixers. DraT's enzymatic activity in these observed systems is regulated by the nitrogen-responsive P_{II} proteins, which often have redundant and overlapping targets (75). There are predicted to be two DraT enzymes (DraT1 and DraT2) and three P_{II} proteins (GlnB, GlnK1 and GlnK2) encoded for in the *R. palustris* genome (39). It had previously been reported from *R. palustris* strain ATC17001 that nitrogenase activity was rapidly lost after exposure to ammonium, but posttranslational inactivation of nitrogenase in strain CGA009 had not been reported (1).

In initial work, my collaborators and I sought to identify *R. palustris* mutants that synthesized nitrogenase constitutively, even when ammonium was present. Such mutants would be required in an applied situation because ammonium-containing agricultural or industrial wastes would most likely be used as organic feed stocks for a H_2 production

process (34). Cells were cultured anaerobically in the presence of ammonium and a carbon source that is more reduced than cell material. In order to maintain cellular redox balance and a functional $NAD(P)^+$/NAD(P)H ratio, the cell must release electrons in order to grow. *R. palustris* is able to do this anaerobically either by fixing CO_2 or producing H_2 via nitrogenase activity (47). In the absence of available CO_2, nitrogenase must be activated, despite the presence of ammonium, for growth to occur (59).

We obtained four mutants and found that the responsible mutations caused four different single amino acid changes that probably locked the NifA transcriptional regulator for nitrogenase gene expression into a constitutively active state. Activation of NifA can be mimicked by mutations either in the GAF domain or in the linker between the GAF and AAA domain, mutations which presumably interfere with how these two domains interact, causing constitutive NifA activity (Figure 3.1) (59, 78). We named these NifA* mutants. Such mutants synthesize large amounts of H_2 when grown with ammonium as a nitrogen source, whereas the wild-type parent grown under the same conditions does not produce H_2 (59).

After identifying and recreating the NifA* mutation we were left with a question – how does a mutation in one transcriptional regulator allow the mutant strain to circumvent all three levels of nitrogenase regulation (Figure 1.3)? Transcriptional profiles of *nifA**,

Δ*ntrBC* and Δ*nifA* mutant strains showed that *nifA** mutants express nitrogenase and several other hydrogen production genes in the presence of ammonium. However they do not express non-NifA regulated members of the NtrBC nitrogen-starvation response regulon (29, 59). Basal expression of *nifA** in the absence of activated NtrC is sufficient to activate expression of *nifHDK* and other *nif* genes in NifA* strains (59). However, if *R. palustris* produces and uses posttranslational modification and inactivation of nitrogenase as indicated by the presence of these genes on the genome, and this regulatory mechanism is stimulated by the presence of ammonium, how do *nifA** strains produce hydrogen from active nitrogenase? My work presented here answers this question.

RESULTS

***R. palustris* nitrogenase activity is inhibited by ammonium and NifH is posttranslationally modified.** A number of nitrogen-fixing bacteria posttranslationally regulate nitrogenase activity by ADP-ribosylation. This regulation has been characterized primarily in the purple non-sulfur bacteria *Azospirillum brasilense*, *R. rubrum* and *R. capsulatus* (32, 48). Transfer of an ADP-ribose moiety by dinitrogenase reductase ADP-ribosyltransferase (DraT) from NAD to a conserved arginine on dinitrogenase reductase (NifH) inactivates nitrogenase. This modification occurs when cells that express active nitrogenase are exposed to ammonium and is reversed by dinitrogenase reductase-activating glycohydrolase (DraG) in response to removal of ammonium. *R. palustris* encodes two predicted DraTs and one predicted DraG (39). Inhibition of nitrogenase

activity in response to ammonium has been reported in *R. palustris* (1, 79). To verify that this regulation occurs in strain CGA009, I grew cells under nitrogen-fixing conditions, washed them and assayed for inhibition of nitrogenase activity in response to addition of ammonium. I found that nitrogenase activity was inhibited by about 70% after addition of 100 μM ammonium chloride compared to addition of 100 μM sodium chloride (Fig 3.2A). Addition of 100 μM sodium chloride resulted in slightly reduced nitrogenase activity, possibly due to a non-specific response to salt or to introduction of trace amounts of oxygen. Immunoblot analysis showed that the NifH protein was modified when cells were exposed to ammonium as evidenced by the appearance of a slower migrating form of NifH on SDS-PAGE gels (Fig 3.2B). The pattern of modification looks very similar to that previously reported for NifH modification by ADP-ribosylation in other bacteria (45). Cells treated with sodium chloride did not show such a NifH protein modification.

The NifH protein from an ammonium-grown NifA* strain is slightly modified. The original *nifA** mutants were missense mutants that reverted to wild type with detectable frequency. I used a subsequently constructed a stable *nifA** mutant that had a 16 amino acid deletion in the Q-linker region of the gene (47). This NifA* strain (CGA676) produces H_2 and behaves as the previously described *nifA** mutants and I will refer to it as the NifA* strain here. When I harvested CGA676 cells grown in ammonium-containing medium and examined the NifH protein in immunoblots, I found that it was

modified, but to a much lesser extent than NifH from nitrogen fixing-grown wild-type cells that have been switched-off by ammonium (Fig. 3.2).

DraT2 and GlnK2 are each required for nitrogenase switch-off by ammonium. To understand why nitrogenase activity from the NifA* strain was relatively resistant to ammonium, I needed to investigate the specific mechanism governing posttranslational modification of nitrogenase in wild-type *R. palustris*. Towards this end I constructed *draT1* (RPA2405) and *draT2* (RPA2406) mutants and tested their sensitivity to ammonium. I found that as with the wild type, nitrogenase activity in the *draT1* (RPA1431) mutant was inhibited by ammonium addition. By contrast, *draT2* mutant nitrogenase activity was relatively insensitive to ammonium addition (Fig 3.3A and C). This mutant phenotype was complemented when the *draT2* gene was expressed *in trans* from a broad-host range plasmid (Fig. 3.3B and C). Previous work indicates that *draT2* is constitutively expressed in wild-type and NifA* strains. Levels of expression are the same in ammonium-grown wild type and NifA* strains as well as in the wild type grown under nitrogen-fixing conditions (51, 59).

Bacteria studied to date that have the DraG/DraT system also have two or three P_{II} isozymes. P_{II} proteins have been shown to interact with DraT to activate its ADP-ribosylation activity, but the P_{II} isozyme specificity for this varies depending on bacterial

species. I investigated the role of each of the three P_{II}-protein isozymes encoded by *R. palustris* by constructing several of the mutants and assaying all of them for nitrogenase inhibition in response to ammonium. Nitrogenase activities in *glnB* and *glnK1* mutants were each sensitive to ammonium addition (Fig. 3.4A). The NifH proteins from each strain were also modified in response to ammonium (Fig. 3.4D). However, the nitrogenase activity of the *glnK2* mutant was insensitive to ammonium addition and its NifH protein did not become modified (Fig. 3.4B and D). This mutant phenotype was complemented by provision of the *glnK2* gene *in trans* (Figs. 3.4 C and D). These results suggest that GlnK2 is required to activate DraT2.

The *glnK2* gene is expressed at low levels in ammonium-grown NifA* cells. The *glnK2* gene is expressed at 30-fold higher levels while fixing nitrogen, compared to ammonium-grown, wild-type cells, an expression profile which is common among proteobacteria with GlnK homologs (15, 51). Previous transcriptome data showed that GlnK2 expression levels in the NifA* strains are not elevated in cells grown with ammonium, even though these strains have high levels of nitrogenase gene expression (59). I verified these results in CGA676, the NifA* strain used in this study, by qRT-PCR (Table 3.1). The observed low levels of GlnK2 protein in the NifA* strain could explain why its nitrogenase is resistant to posttranslational modification and inhibition when cells are grown with ammonium because I have shown above that GlnK2 is required for DraT2 activation. Inspection of the *ntrBC* mutant microarray data provides an explanation for

this regulatory pattern. Transcription of the NtrBC regulon is not activated in wild-type or NifA* cells grown with ammonium, and *glnK2* is part of the NtrBC regulon (29, 59).

Lack of *glnK2* expression in NifA* strain does not fully explain its resistance to switch-off. To further probe my hypothesis that the incomplete switch-off of nitrogenase in ammonium-grown NifA* was due to a lack of adequate *glnK2* expression, I expressed *glnK2* from a constitutive promoter *in trans* and measured production of H_2 by ammonium-grown cells. I verified by qRT-PCR that *glnK2* was well expressed (Table 3.1). Surprisingly, the amount of H_2 produced by the strain dropped only slightly compared to the vector control strain (Table 3.2). In complementary experiments I found that the NifA* strain grown with N_2 as its sole nitrogen source (a condition which should up-regulate GlnK2 expression) and subsequently exposed to exogenous ammonium chloride retained most of its nitrogenase activity. Thus it appears that the resistance of the NifA* strain to post-translational modification by ADP-ribosylation cannot be solely due to low expression levels of *glnK2*.

It has been previously reported that NifA* strains express nitrogenase genes at higher levels than wild-type cells grown under nitrogen-fixing conditions (59). In agreement with this, I found that the nitrogenase activity of the NifA* strain was three times higher than that of the wild type (not shown). Thus a possible explanation for the inability of the

NifA* strain expressing *glnK2* to significantly switch-off nitrogenase activity is that the amount of DraT2 that it produces is insufficient to completely inactivate the increased amount of nitrogenase that it synthesizes. To test this I expressed *draT2* from a constitutive promoter *in trans* in the NifA* strain. The nitrogenase activity and amount of H_2 produced in this strain were reduced relative to the vector control (Table 3.2). When I expressed both *glnK2* and *draT2* from the same plasmid in the NifA* strain, I observed a substantial reduction in the amount of H_2 produced relative to the vector control strain. Thus inadequate expression levels of both *glnK2* and *draT2* in the NifA* strain each contribute to the resistance of this strain to switch-off of nitrogenase by ADP-ribosylation.

Introduction of a *draT2* mutation into the NifA* strain results in increased production of hydrogen. The initial observation that nitrogenase from the NifA* strain grown with ammonium was partially modified by ADP-ribosylation (Fig. 3.2) suggested there was some active DraT2 present. To test this I constructed a *nifA*draT2* double mutant. Protein immunoblots showed that this strain did not modify its nitrogenase protein (data not shown). I also observed that the *nifA*draT2* double mutant produced more H_2 than the *nifA** strain (Table 3.3).

DISCUSSION

In this work I found that nitrogenase is posttranslationally modified by DraT2 in *R. palustris* as was predicted from its genome sequence. I observed that modification of nitrogenase in wild-type cells resulted in a decrease in nitrogenase activity and the appearance of higher molecular weight bands in anti-NifH immunoblots. This is consistent with posttranslational modification by ADP-ribosylation. The substantial amount of unmodified NifH that is present in inactivated samples is in keeping with past studies in other bacteria showing that only one subunit of the NifH dimer is modified at any one time, presumably because modification at one dimer sterically blocks modification on the opposing dimer (25, 54).

A revised model for three-tiered regulation of nitrogenase expression and activity in *R. palustris* derived from my data is shown in Fig. 3.5. At the top level, in wild type cells, NtrB responds to fixed nitrogen limitation when P_{II} protein fails to interact with it and allows NtrB to phosphorylate NtrC. NtrC-P is the active form, allowing the transcription of genes associated with nitrogen starvation, including *nifA*. In ammonium-grown cells, *nifA*, which is normally up-regulated 2- to 3-fold by NtrBC, is not induced. On level two, NifA is activated under low nitrogen growth conditions, presumably by P_{II}-UMP to become proficient to turn on *nif* gene expression. The NifA* variant does not need to be activated by P_{II}-UMP. Moreover non-NtrC activated basal levels of NifA* protein are sufficient for activation of nitrogenase genes during growth with ammonium. Thus,

nitrogenase genes, but not NtrBC regulated genes including *glnK2*, are expressed in the NifA* strain grown with ammonium. At the third level, the constitutively expressed DraT2 posttranslationally modifies and inactivates nitrogenase in response to exogenous addition of ammonium, when it interacts with unmodified GlnK2. GlnK2 is not well expressed in ammonia-grown NifA* cells because the NtrC regulon is not expressed. Furthermore nitrogenase is expressed at elevated levels in the NifA* strain, overwhelming the switch-off apparatus. These two factors allow continued activity of nitrogenase and H_2 production in the presence of ammonium. Therefore, a single mutation in *nifA* is sufficient to bypass three levels of regulatory control and allow H_2 production by *R. palustris* in the presence of ammonium.

I have shown that *R. palustris* posttranslational regulation of nitrogenase depends on GlnK2, one of three P_{II}-proteins found in this species. It is not possible to predict based on homology to other systems which functions a given P_{II} protein will perform. Also P_{II} isozymes in a single bacterium can have redundant functions. The use of only one P_{II} protein to activate DraT is dissimilar to what has been observed previously among purple nonsulfur bacteria. *R. palustris* is so far unique in that only GlnK2, which is part of the NtrBC regulon and is not expressed when the cells are grown in the presence of ammonium, can activate the nitrogenase switch-off system. When NifA* mutations were introduced in *R. rubrum*, it was necessary to also inactivate the *draT* gene in order to get H_2 production in the presence of ammonium because its activity is controlled by a

constitutively expressed P_{II} protein (78). I also found that inactivation of the switch-off system by mutation of the ADP-ribosyltransferase gene *draT2* in a NifA* *R. palustris* strain resulted in increased H_2 production in ammonia-grown cells. This is in keeping with my observation that nitrogenase expressed in NifA* cells was post-translationally modified to some degree (Fig. 3.2).

This information presented here has helped to elucidate the regulatory hierarchy controlling nitrogenase gene expression in *R. palustris* (Fig. 3.5). I have shown that even when the components of a regulatory network have been well studied in other bacteria, understanding exactly how these components work together in a bacterium of interest can be important for determining how to engineer such a bacterium to meet bioengineering goals. I have also solved a paradox posed by our previous work on the NifA* mutant and gained a better understanding of nitrogen regulation in general in *R. palustris*.

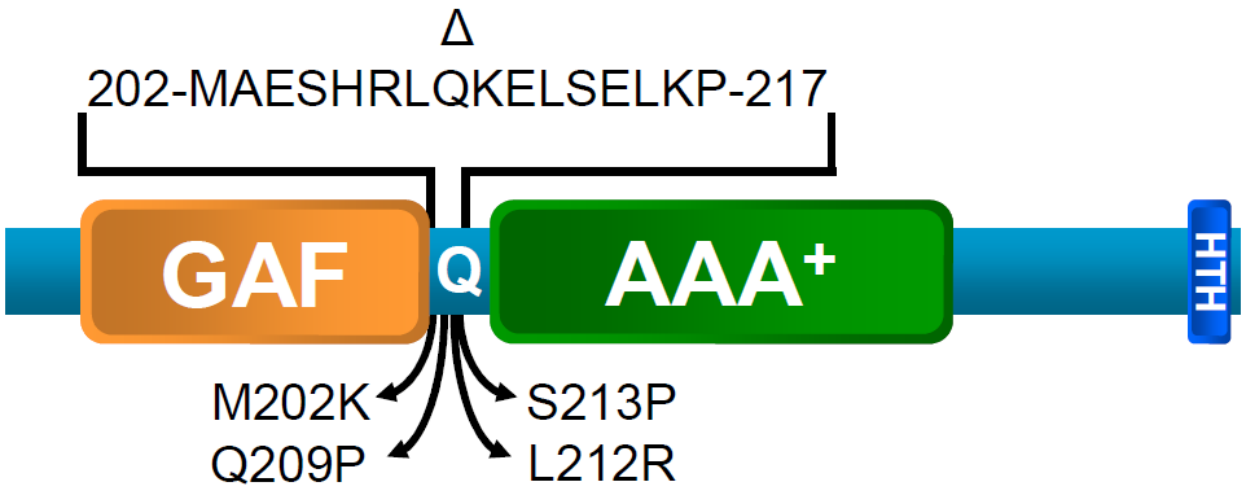

Figure 3.1. Variants of *R. palustris* NifA that have a constitutive phenotype have amino acid changes in the Q-linker domain between the GAF and AAA^+ domains. Mutants with single nucleotide substitutions resulting in single amino acid changes were selected for as described in (59). The 16 amino acid deletion variant was constructed to reduce the frequency of revertants (47).

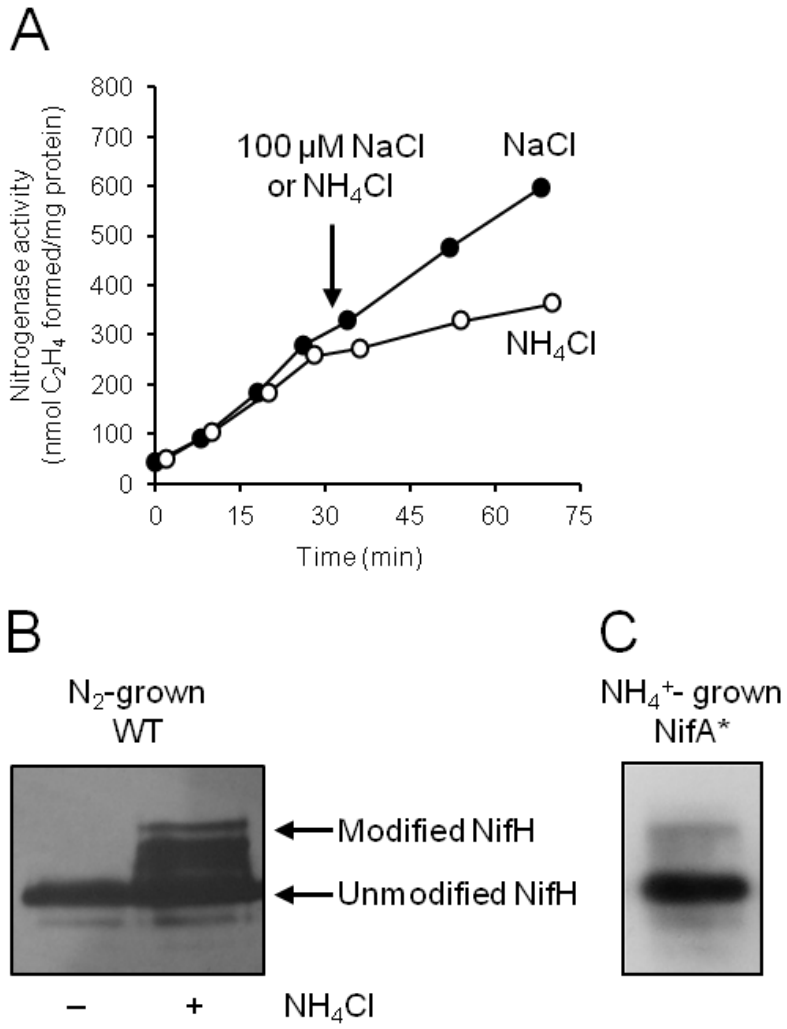

Figure 3.2. Nitrogenase inactivation and modification in wild-type and *nifA* R. palustris*. (A) Nitrogenase activities of wild-type cells grown under nitrogen-fixing conditions were measured before and after exposure to 100 µM ammonium chloride (open symbols) or sodium chloride (closed symbols). Only the ammonium-exposed cells lost the majority of their nitrogenase activity. Data are representative of at least four different experiments. (B) Post-translational modification of NifH protein by addition of ammonium chloride. Cell proteins were separated on low-crosslinker polyacrylimide gel in order to resolve the ADP-ribosylated subunit from the unmodified subunit of NifH. NifH was visualized by immunoblotting. (C) NifH protein from the NifA* strain grown with ammonium is modified slightly. NifH protein from wild type cells grown under nitrogen-fixing conditions.

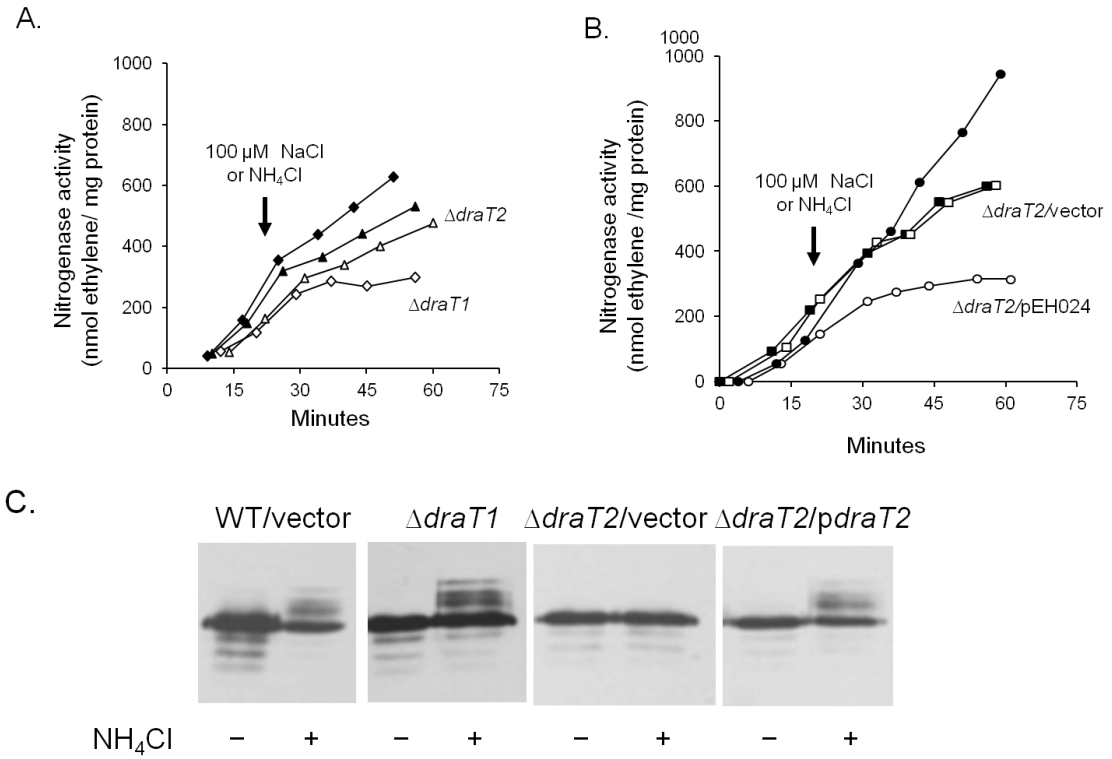

Figure 3.3. DraT2 ADP-ribosyltransferase is responsible for nitrogenase inactivation in *R. palustris*. (A) Switch-off of nitrogenase activities of *draT1* (diamonds) and *draT2* (triangles) mutants. Open symbols indicate ammonium chloride addition and closed symbols are sodium chloride addition. *draT1* cells exposed to ammonium chloride lost significant nitrogenase activity whereas cells exposed to sodium chloride lost little activity. The *draT2* mutant nitrogenase by contrast was not inactivated by addition of ammonium chloride. Data are representative of at least four independent experiments. (B) The inability of the *draT2* mutant (squares) to inactivate nitrogenase activity in response to ammonium chloride was restored by provision of the *draT2* gene *in trans* (circles). (C) Anti-NifH immunoblots indicate that in the absence of DraT2, NifH is not ADP-ribosylated and that this phenotype can be complemented.

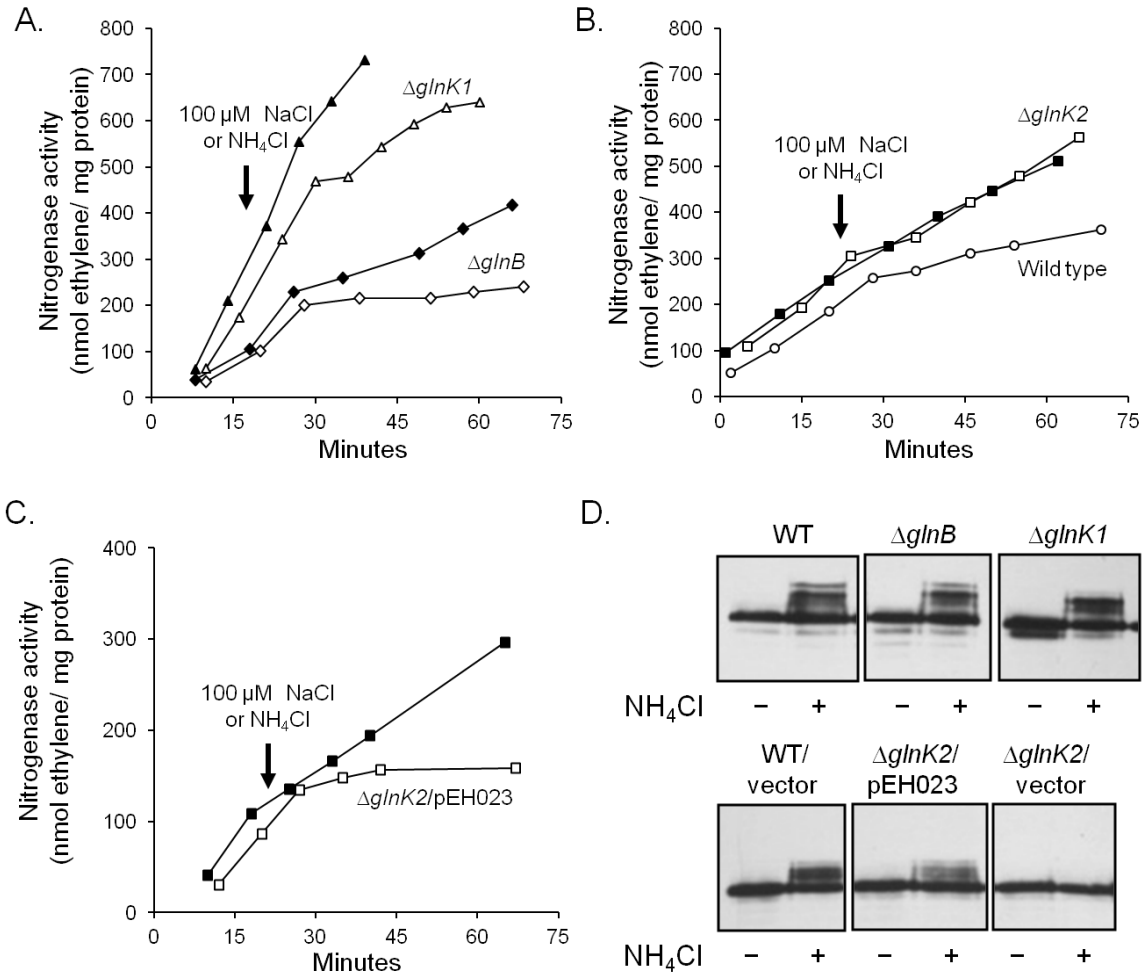

Figure 3.4. The P_{II} protein GlnK2 is essential for nitrogenase inactivation in *R. palustris*. (A) Switch-off of nitrogenase activities of *glnB* (triangles) and *glnK1* (diamonds) mutants. Sodium chloride (closed symbols) exposed cells lost little activity whereas ammonium chloride (open symbols) exposed cells lost significant nitrogenase activity. Data are representative of at least four independent experiments. (B) The *glnK2* mutant (triangles) did not lose nitrogenase activity after ammonium chloride addition, as did the wild-type (diamonds). (C) The *glnK2* phenotype was complemented by provision of the pEH023 *in trans*. (D) Anti-NifH immunoblots corroborate the results seen in the nitrogenase activity assays, showing that GlnK2 is required for NifH protein modification.

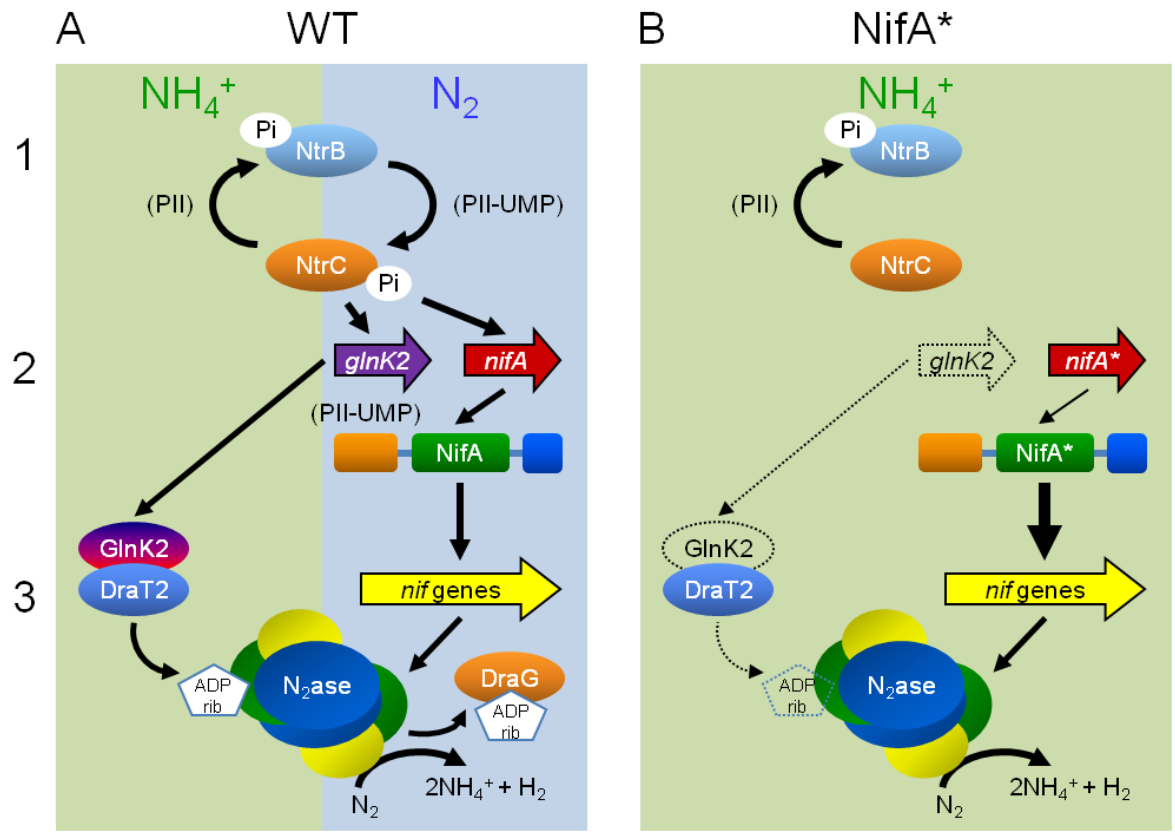

Figure 3.5. A model for the regulation of nitrogenase in wild-type and NifA* *R. palustris* strains. (A) In N_2-grown wild type cells, NtrB trans-phosphorylates NtrC, which then up-regulates genes of the NtrBC regulon, including *glnK2* (encoding a PII isozyme) and *nifA*. NifA undergoes a conformational change which may be promoted by a PII-UMP isozyme. This allows it to upregulate *nif* gene expression and the synthesis of nitrogenase. DraG, which is constitutively synthesized in *R. palustris* ensures the continued activity of nitrogenase by removing any ADP-ribose moieties that may be attached. When wild type cells are exposed to ammonia, GlnK2 binds to DraT2 (also constitutively synthesized) to activate its ability to ADP-ribosylate nitrogenase. (B) When the NifA* strain is grown with ammonium as a nitrogen source, NtrB protein is inactivated by interaction with P_{II} protein (an unknown combination of GlnB, GlnK1, or GlnK2) and it cannot promote NtrC phosphorylation. As a consequence neither the P_{II} gene, *glnK2*, nor *nifA* is up-regulated. However, sufficient basal expression levels in the NifA* strain allow the over-expression of the *nif* genes. Without adequate GlnK2 levels and over-expression of nitrogenase, the majority of nitrogenase escapes ADP-ribosylation by DraT2 and as a result H_2 is produced.

Table 3.1. Expression of *glnK2* genes determined by qRT-PCR

Strain	Genotype	Plasmid	Growth condition	pg *glnK2* cDNA[a]
CGA009	wild type		NFM	589 (61)
CGA009	wild type	pBBR1MCS-5 (control)	NFM	409 (29)
CGA009	wild type		PM	9 (2)
CGA009	wild type	pBBR1MCS-5 (control)	PM	10 (1)
CGA676	NifA*		NFM	289 (21)
CGA676	NifA*		PM	32 (6)
CGA676	NifA*	pBBR1MCS-5 (control)	PM	10 (2)
CGA676	NifA*	pEH025 (*glnK2*)	PM	650 (33)
CGA725	NifA* Δ*glnK2*		NFM	< 1
CGA725	NifA* Δ*glnK2*		PM	< 1
CGA726	NifA* Δ*ntrBC*		NFM	11 (2)
CGA726	NifA* Δ*ntrBC*		PM	7 (1)

[a] The data shown are the average pg of *glnK2* cDNA measured from at least two experiments as compared to a genomic DNA standard curve. Standard errors of the mean are shown in parentheses.

Table 3.2. Expression of GlnK2 or DraT2 *in trans* reduces H_2 production by ammonium-grown NifA* strain

Strain	Plasmid	Hydrogen production (µmol/mg total protein)[a]	% relative to NifA*(pBBR1MCS-5)
CGA009	pBBR1MCS-5 (control)	<1	0
CGA676	pBBR1MCS-5 (control)	81 (3.4)	100
CGA676	pEH025 (*glnK2*)	66 (2.6)	81
CGA676	pEH024 (*draT2*)	50 (3.4)	62
CGA676	pEH026 (*glnK2* and *draT2*)	18 (2.1)	22

[a]The data shown are the average of at least three experiments. Standard errors of the mean are shown in parentheses.

Table 3.3. H_2 production by ammonium-grown NifA* cells is increased by elimination of DraT2

Strain	Genotype	Hydrogen production (μmol/mg total protein)[a]	% relative to NifA*(pBBR1MCS-5)
CGA009	wild type	<1	0
CGA676	NifA*	80 (3.6)	100
CGA724	NifA* Δ*draT2*	107 (10.0)	134

[a]The data shown are the average of at least three experiments. Standard errors of the mean are shown in parentheses.

CHAPTER IV:

Posttranslational inactivation and modification of alternative V- and Fe-nitrogenases of *R. palustris*

INTRODUCTION

Previous work showed that *R. palustris* strain CGA009 encodes for three functional nitrogenase isozymes: a *nif*-encoded molybdenum-nitrogenase, a *vnf*-encoded vanadium-nitrogenase, and an *anf*-encoded iron-nitrogenase (51). While the expression of Mo-nitrogenase is preferred by the organism, it is unclear how the alternative nitrogenases are expressed in response to inactivation of Mo-nitrogenase in *R. palustris*. At the post-translational level of regulation, inactivation of Fe-nitrogenase was reported in the PNSB *Rhodobacter capsulatus* by Masepohl et al. in 1993, but was not previously observed from *R. palustris* (45). There are no reported instances of a V-nitrogenase subject to post-translational modification and inactivation.

R. palustris encodes for two ADP-ribosyltransferase enzymes, DraT1 and DraT2 (39). In Chapter 3, I showed that DraT2 is responsible for inactivating Mo-nitrogenase in the wild-type strain. Whereas DraT2 is constitutively expressed and found in an operon with the only DraG homolog on the chromosome, DraT1 is encoded on the chromosome near the *anf* Fe-nitrogenase genes. DraT1 is upregulated in CGA755, the iron-only strain of *R. palustris* and during long-term N-starvation (51).

This work was done to test the hypothesis that the DraT-mediated system is capable of inactivating the alternative nitrogenases of *R. palustris* in response to the addition of the fixed nitrogen source ammonium to cells.

RESULTS

Previously we reported the posttranslational modification of molybdenum nitrogenase activity in response to the addition of ammonium, but not in response to a sodium control (29). However, acetylene, which used in that study, is a poor substrate for V- and Fe-nitrogenases, therefore the posttranslational modification phenotype was tested in these strains by measuring hydrogen production as a proxy for nitrogenase activity. As a control, we also assayed posttranslational inactivation in strain CGA753, which is deleted for *vnfH* and *anfH* and thus only produces molybdenum nitrogenase (51). We found that after the addition of ammonium chloride, CGA753 lost significantly more hydrogen production activity than the sodium control (Fig. 4.1A, Table 4.1). We also found that NifH protein harvested from cells exposed to ammonium migrated more slowly during PAGE analysis, which is consistent with the protein becoming modified (Fig. 4.1D).

The previously constructed strain CGA766 is mutated in *nifH, nifD, and anfA* and consequently produces only the vanadium nitrogenase (51). This strain also responded to ammonium by the loss of hydrogen production and modification of the VnfH subunit of the enzyme (Fig. 4.1B and D, Table 4.1). Furthermore, the previously constructed strain CGA755, which is inactivated in *nifH* and *vnfH* and consequently produces only the iron nitrogenase, is also subject to nitrogenase inactivation after ammonium addition (51) (Fig. 4.1C, Table 4.1). AnfH is slightly smaller than NifH and VnfH, but a slower-

migrating protein band corresponding to modified AnfH was detected from cells exposed to ammonium (Fig. 4.1D).

I aligned the protein sequences of five dinitrogenase reductase proteins and found that the region containing the modified arginine is highly conserved (Fig. 4.2). The V-nitrogenase enzyme is composed of four gene products, VnfD, VnfG, VnfK, and VnfH. Phylogenetic trees were built using neighbor-joining to infer the relationship of *R. palustris* alternative nitrogenase genes to other sequenced nitrogenases. The tree shown in Figure 4.3 suggests that the VnfD protein from *R. palustris* shares a common ancestor with vanadium nitrogenases from *Azotobacter* species. However, VnfH appears to share a common ancestor with the *R. palustris* NifH protein, suggesting that it is the product of a gene duplication event (Fig. 4.4).

DISCUSSION

I have shown that both alternative nitrogenase enzymes of *R. palustris* are subject to posttranslational modification in the presence of ammonium. It is likely that the vanadium alternative nitrogenase of *R. palustris* was acquired by horizontal gene transfer as all sequenced strains of *R. palustris* contain the *nif*-encoded Mo-nitrogenase and the Fe-nitrogenase, but only CGA009 contains the V-nitrogenase as well (52). Despite this probable horizontal acquisition, all three nitrogenases in *R. palustris* CGA009 rely on

several common components for production of metal cofactors and their insertion into the holoenzyme and all nitrogenase genes rely on NifA for transcriptional activation (51, 65). The vanadium nitrogenase VnfH is 94% identical and 98% similar to NifH while AnfH is only 58% identical and 75% similar to NifH, and yet both were modified after ammonium addition. I have found the degree of modification of AnfH to be qualitatively less than the degree to which NifH and VnfH were modified (Fig. 4.1D). Full inactivation of NifH (and presumably VnfH and AnfH) occurs when 50% of the subunits are modified, as NifH is found as a dimer and modification of one subunit sterically blocks modification of the second subunit (43). I also found that the Fe-nitrogenase only strain did not lose as much hydrogen production activity as the Mo- and V-nitrogenase expressing strains (Table 4.1). Previously it was found by that modification of the *Rhodospirillum rubrum* NifH protein occurs on Arginine102 (44). An alignment of *R. rubrum* NifH with all three dinitrogenase reductase proteins from *R. palustris* shows that the modified arginine is found in a conserved portion of the protein (Figure 4.2). In fact, when DraT from *R. rubrum* is expressed in *K. pneumoniae*, a nitrogen-fixer which does not encode for the switch-off system, heterologous nitrogenase becomes switched off upon the addition of ammonium (21). These results suggest that though the site of modification is well conserved, more highly divergent NifH homologs are less efficiently modified by DraT.

While VnfD, AnfD, and NifD from a wide variety of organisms fall into clades separated by the metal they use at the active site, as seen in Figure 4.3, the dinitrogenase reductase proteins (VnfH, AnfH, and NifH) do not separate similarly in our analysis (Figure 4.4). These results confirm previous analyses which noted but did not address this discrepancy (57). The *R. palustris* VnfH and NifH proteins are so similar that they appear more related to each other than to any other dinitrogenase reductase protein analyzed. However, the *R. palustris* VnfD protein groups with other VnfD proteins and not the *R. palustris* NifD. I believe this result suggests that VnfH in *R. palustris* is the product of a gene duplication event. A possible scenario for this gene duplication is as follows. *R. palustris* CGA009 expanded its nitrogen fixation abilities by horizontally acquiring the *vnf* gene cluster, which includes structural as well as accessory genes, perhaps from a species similar to *A. vinelandii* as Figure 4.3 suggests. The acquired VnfH would be approximately 71% identical and 86% similar to *R. palustris* NifH and possibly be less efficiently modified by DraT, even though it has an identical site of modification (Figure 4.2). To bring V-nitrogenase under tight regulatory control, increasing its efficiency in a competitive environment, recombination between the *vnf* and *nif* gene clusters could result in duplication of *nifH*. Inspection of the *vnf* gene cluster shows that VnfH does not conform to the expected gene organization (VnfH, VnfD, VnfG, VnfH) as seen in the *nif* and *anf* gene clusters (Figure 1.2), another point of evidence which suggests a recombination event occurred. Not only are the *vnfH* and *nifH* genes of *R. palustris* very similar, but the currently-encoded-for VnfH can substitute for NifH and allow NifDK activity *in vivo* (data not shown). The ability of VnfH to substitute for NifH was

demonstrated in *A. vinelandii* (12). Presumably, NifH can substitute for VnfH when Mo is scarce, though this has not been tested. However, the *nif* structural genes are expressed at different times and stoichiometric amounts than the *vnf* genes (51). Expression of a copy of *nifH* from the *vnf* cluster, under conditions where the rest of the vanadium nitrogenase structural genes are also expressed could allow for more efficient assembly of the V-nitrogenase enzyme and better function *in vivo*.

The posttranslational modification of V-nitrogenase by DraT has been first described here because the vanadium isozyme is found primarily in *Azotobacter* species, cyanobacteria, and methanogens – phyla that do not carry the DraT/G posttranslational mechanism of control. I have shown that all three nitrogenase isozymes are subject to this level of regulation. The ability to quickly inactivate nitrogenases that are less efficient at fixing nitrogen, and thus waste more energy than the Mo-nitrogenase, would be a great advantage during competitive growth in the environment. These results also show that if a strain of *R. palustris* were engineered to express all three nitrogenases concurrently for maximum hydrogen production, the posttranslational regulation mechanism must be removed.

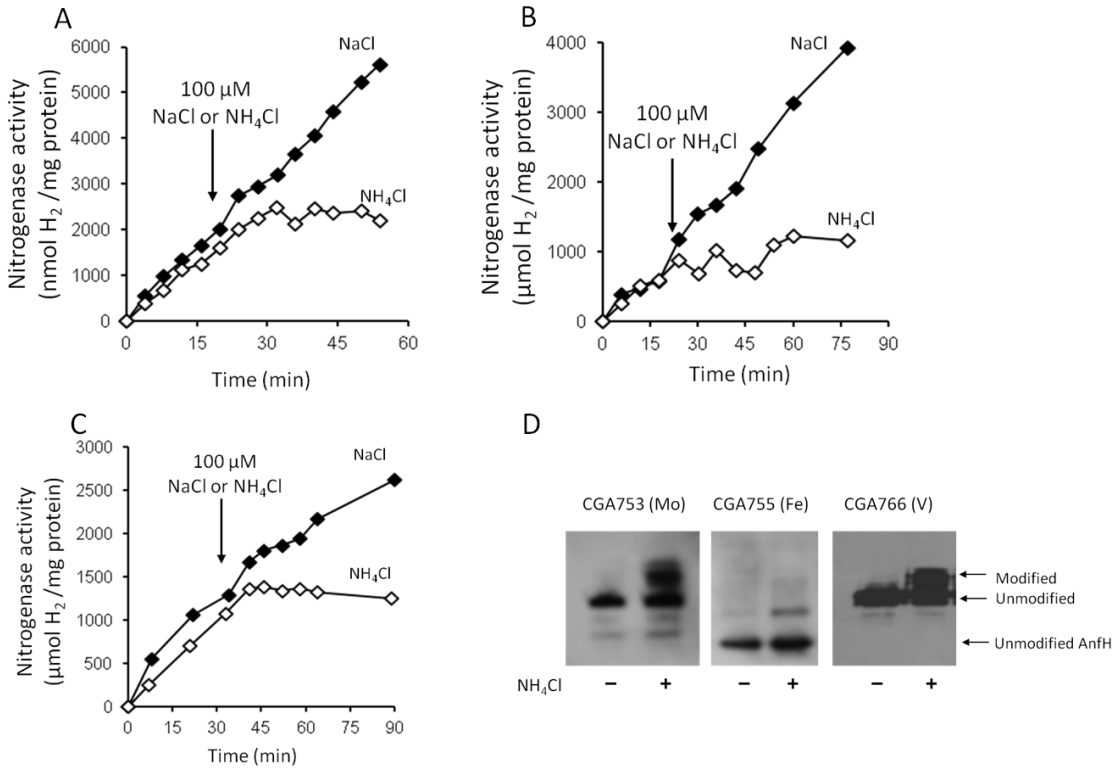

Figure 4.1. All three nitrogenase isozymes are subject to posttranslational inactivation. A) Hydrogen produced over time by the Mo-nitrogenase expressing strain CGA753. At the arrow, either NaCl (closed symbols) or NH_4Cl (open symbols) was added to the cell suspension at a final concentration of 100 µM. The data shown are one representative experiment of at least four experiments, all of which showed similar results. B) Hydrogen-production switch-off of the Fe-nitrogenase-only expressing strain CGA755. C) Hydrogen-production switch-off of the V-nitrogenase-only expressing strain CGA766. D) Anti-NifH immunoblot of protein harvested from cells exposed for 30 min to either NaCl (-) or NH_4Cl (+). The unmodified forms of NifH and VnfH run with the 32 kDa size marker, while umodified AnfH runs at 30 kDa.

Figure 4.2. Clustal W alignment of five nitrogenase reductase proteins shows ADP-ribosylation target R101 is found in a conserved region of the protein. Included are NifH, VnfH, and AnfH encoded by the *R. palustris* genome as well as the NifH protein of *Rhodospirillum rubrum* and the VnfH protein of *Azotobacter vinelandii* DJ. The residue shown to be modified in the *R. rubrum* protein, Arginine 102, is in bold (44).

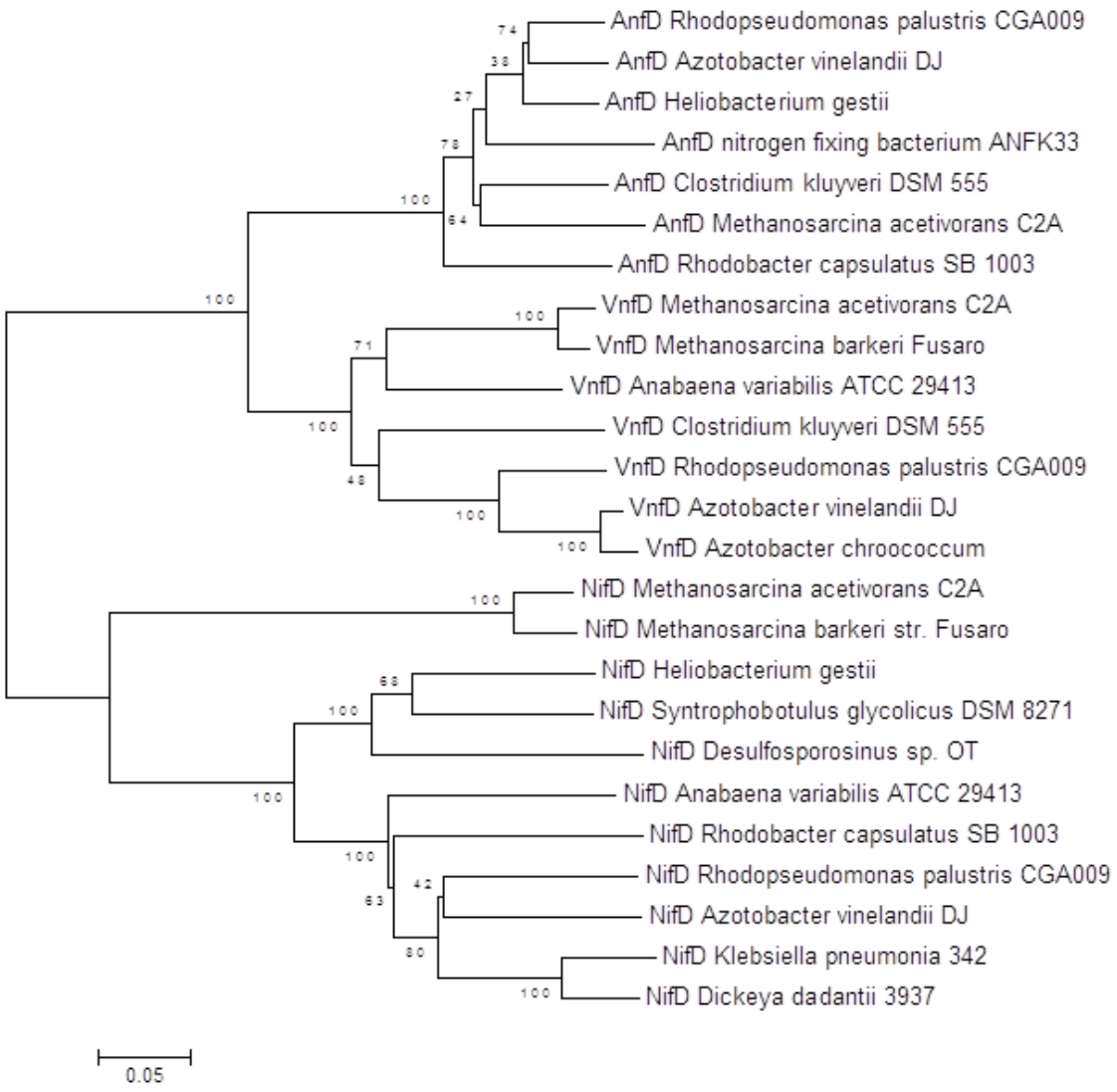

Figure 4.3. Evolutionary relationships of all three nitrogenase isozymes show separate phylogenies for each type of nitrogenase. The evolutionary histories of NifD, VnfD and AnfD were inferred using the Neighbor-Joining method (64). The bootstrap consensus trees inferred from 500 replicates are taken to represent the evolutionary history of the taxa analyzed (19). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (19). The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site (50). The analysis involved 25 (B) or 24 (C) amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 275 (B) and 224 (C) positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (70).

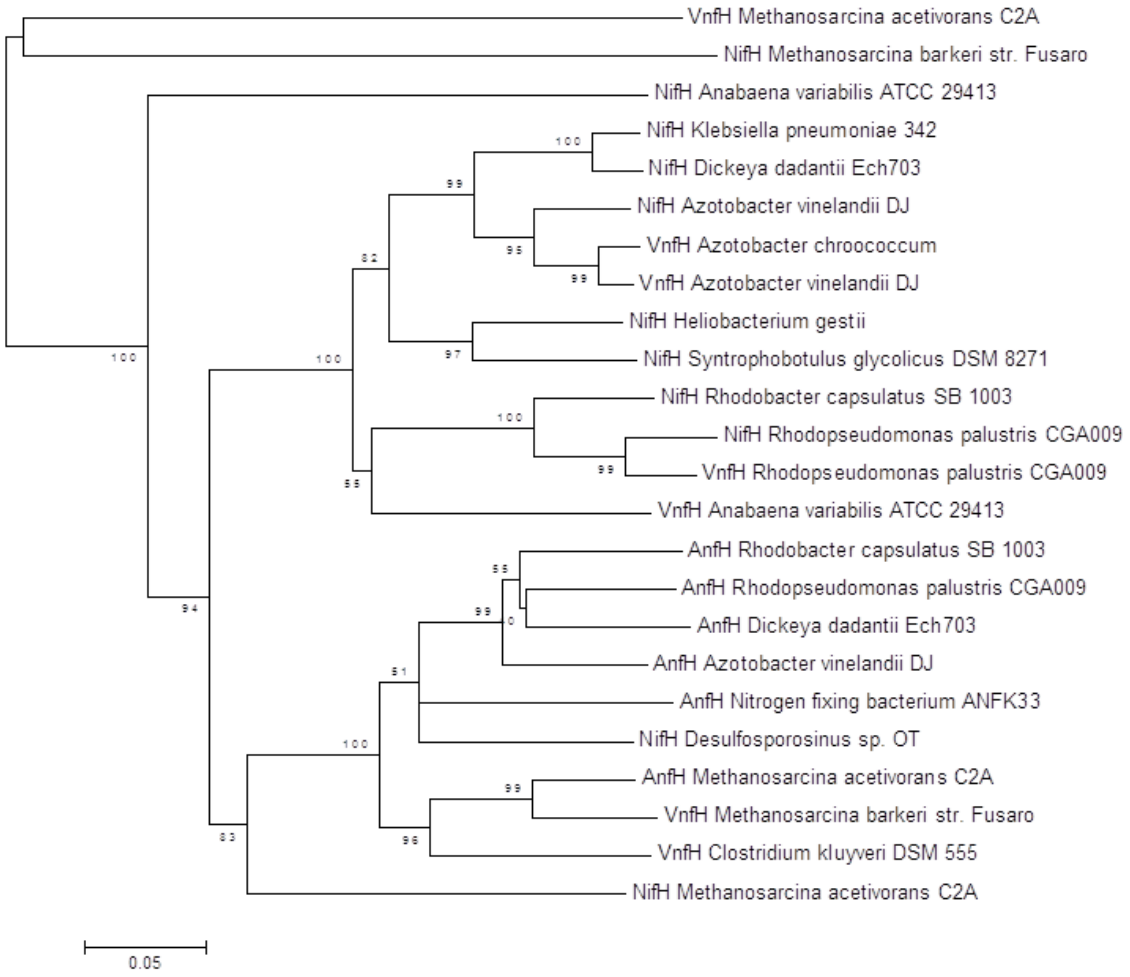

Figure 4.4. Evolutionary relationships of all three nitrogenase isozymes show disparate origins of the VnfH proteins compared to VnfD (Figure 4.3). The evolutionary histories of NifH, VnfH, and AnfH were inferred as described for Figure 4.3. VnfH in *R. palustris* is more related to the NifH protein of *R. palustris* than any other NifH homolog.

Table 4.1. Fe-nitrogenase is incompletely inactivated after ammonium addition

Strain	Genotype	Percent activity remaining (SEM)[a]	p-value
CGA753 (Mo)	Δ*anfH* Δ*vnfH*	4 (3)	0.05
CGA755 (Fe)	Δ*nifH* Δ*vnfH*	35 (12)	0.002
CGA766 (V)	*nifD*::Tn5 Δ*nifH* Δ*anfA*	1 (1)	0.02

[a] Value is the percent activity remaining after NH_4Cl addition, averaged over four experiments. The standard error of the mean is shown in parentheses.

CHAPTER V:

Selection for and phenotypic observation of a V-nitrogenase-utilizing constitutive hydrogen producing mutant of *R. palustris*

INTRODUCTION

The *Rhodopseudomonas palustris* CGA009 genome encodes for three functional nitrogenases – one commonly-found Mo-nitrogenase and two alternative nitrogenases which rely on either V or Fe for their enzymatic function (51). Several nitrogen fixing bacteria contain an alternative nitrogenase (either V or Fe) for use in the event that Mo becomes limiting. In *Rhodobacter capsulatus*, expression of the Fe alternative nitrogenase is repressed by a regulator that senses the presence of molybdate ions (38). However, in *R. palustris*, the availability of Mo does not repress expression of the alternative nitrogenases. Instead, the presence of a functional Mo-nitrogenase enzyme appears to repress expression of the V- and Fe-nitrogenases through an unknown mechanism (51).

Nitrogenase expression and synthesis is an energy and resource intensive process, and thus is tightly controlled by the cell. Transcription of nitrogenase is controlled hierarchically by the two component regulator NtrBC and by the transcriptional activator NifA (Fig. 1.3). NtrBC responds to the nitrogen status of the cell by its interaction with at least two of the three PII-protein homologs encoded on the *R. palustris* genome to activate a wide variety of genes useful for survival during nitrogen starvation conditions (29). NifA responds to the nitrogen status of the cell in an unknown manner to activate expression of the genes required for Mo-nitrogenase function as well as a few genes outside the *nif* gene cluster (59).

Previously we selected for mutants that constitutively express Mo-nitrogenase. During this selection cells are cultured anaerobically in the presence of ammonium and a carbon source that is more reduced than cell material. In order to maintain redox balance and a functional $NAD(P)^+$/NAD(P)H ratio, the cell must release electrons in order to grow (59). *R. palustris* is able to do this anaerobically either by fixing CO_2 or producing H_2 via nitrogenase function (47). In the absence of available CO_2, nitrogenase must be activated, despite the presence of ammonium, for growth to occur. I used this method to select for and isolate mutants of an *R. palustris* strain that is deleted for the Mo-nitrogenase, but which expresses the vanadium nitrogenase constitutively.

RESULTS

Several tubes of medium containing cyclohexanecarboxylate (chc) and ammonium, with or without 1 mM vanadium, were inoculated with strain CGA762, which is deleted for *nifH* and carries an inactivating transposon insertion in *nifD*. These two mutations make the cell incapable of producing a functional Mo-nitrogenase. However, the *vnf* and *anf* gene clusters were intact. Strain CGA762 cannot make hydrogen from acetate in the presence of ammonium (Table 5.1). After seven months of incubation, one of the cultures grew to a high OD. Seven of the twenty-four strains isolated from this culture produced hydrogen in the presence of ammonuim, using either acetate or chc as the carbon and electron source, with varying efficiencies. One of the best hydrogen-producing mutants,

strain CGA730, was selected for further study. This mutant is able to grow on chc in the presence of ammonium with a doubling time of 41 ± 1.3 hours. It is also able to produce hydrogen in the presence of ammonium and hydrogen production is stimulated when vanadium was also present (Table 5.1) These results suggest that the vanadium nitrogenase is constitutively expressed in this mutant strain.

Sequencing of selected genes and promoter regions expected to have a role in constitutive expression of vanadium nitrogenase (Table 5.2) revealed only one mutation – a leucine to phenylalanine mutation in the Q-linker region of NifA which resembles those NifA mutations that result in constitutive activation of Mo-nitrogenase expression (59). When the Mo-nitrogenase genes *nifHD* were deleted from the $NifA_{\Delta Q\text{-linker}}$ mutant (Figure 3.1), this strain (CGA763) was unable to produce hydrogen in the presence of ammonium and vanadium (Table 5.1). In contrast, CGA730 was able to produce hydrogen from the alternative nitrogenases in the presence of ammonium and vanadium.

Acetylene is reduced by Mo-nitrogenase to ethylene and by V- and Fe-nitrogenases to ethylene and ethane at specific ratios (51). Acetylene reduction assays performed with CGA730 gave an ethylene: ethane ratio consistent with V-nitrogenase activity (data not shown).

DISCUSSION

In order to learn more about how the alternative nitrogenases of *R. palustris* are regulated, I disrupted this regulation by applying selective pressure that required the bacterium to constitutively fix nitrogen from either of the alternative nitrogenases. The resulting culture yielded a mutant strain that requires vanadium for hydrogen production and growth on chc in the presence of ammonium. I found that only 29% of the isolates from this culture were hydrogen producing mutants, while the rest were the parent strain. These parent strain cells were presumably able to grow once the mutant began producing hydrogen because they "cheated" and were able to maintain redox balance by fixing CO_2 released by the mutant cells during growth. The master nitrogenase regulator, NifA was found to be mutated in this strain. However, a similar mutation, $nifA_{\Delta Q\text{-linker}}$, when introduced into the Mo-nitrogenase deleted parent strain (strain CGA763), was unable to cause constitutive hydrogen production from either alternative nitrogenase.

I have two hypotheses which may explain these results. (I) The L208P mutation of *nifA* has allowed NifA to expand its target range to include VnfA responsive promoters and is able to activate the constitutive transcription of the *vnf* gene cluster. And (II) a constitutive version of NifA was required for expression of the *vnf* genes in the presence of ammonium, as well as a second-site regulatory mutation.

To determine which, if either, of these hypotheses is correct, I would propose several experimental lines of inquiry. First would be to introduce the $NifA_{L208P}$ mutation onto the parental Δ*nifHD* strain. I was unable to obtain this mutant after several attempts and sequencing many double recombinants. Constitutive *nifA* mutations cause a decrease in growth rate, likely due to overexpression of nitrogenase and its accessory genes (59). I hypothesize that my efforts to isolate a mutant were hampered by the presence of single recombinants that contained wild-type *nifA*, which presumably has a growth advantage over the mutant, as it grows more quickly in pure culture. If the same is true during selection of double recombinants, *nifA* wild-type cells would dominate the population. In the future, I would suggest constructing this strain by starting with $NifA_{\Delta Q\text{-linker}}$ Δ*nifHD* and introducing the $NifA_{L208P}$ subsequently. This starting strain should have less of a growth advantage over the desired mutant than Δ*nifHD* with no NifA mutation. If a $NifA_{L208P}$ Δ*nifHD* strain is able to activate transcription of the *vnf* genes in the presence of ammonium, this result would support hypothesis (I).

If the $NifA_{L208P}$ Δ*nifHD* strain does not produce hydrogen in the presence of ammonium and vanadium, this result would support hypothesis (II) and I would propose sequencing the entire genome of the mutant strain in search of the second-site mutation responsible for constitutive hydrogen production. To reduce the number of false leads that may arise from this method, I would recommend sequencing the genomes of at least two other constitutive *vnf* hydrogen-producing sibling isolates, to find the causal mutation they

should all have in common and rule out any incidental mutations that they may or may not share, depending on the ancestral history of each isolate.

Regardless of which hypothesis is correct, it would also be prudent to measure the transcriptional upregulation of the *vnf* genes in this mutant in the presence and absence of both ammonium and vanadium as compared to the parent strain. This would be done using quantitative RT-PCR, with primers that have been designed to anneal specifically to the *vnf* genes and not to the very similar *nif* and *anf* genes. I would also use immunoblotting to check for the accumulation of Vnf proteins. I have already found that the antibody raised against *Azotobacter vinelandii* NifH cross-reacts with *R. palustris* VnfH to allow for detection of this protein (discussed in Chapter 4).

Table 5.1. Hydrogen production from the evolved strain is stimulated by the presence of vanadium.

	Hydrogen production (umol/mg protein)	
	(no VCl3)	(10 uM VCl3)
CGA009	<1	<1
CGA676 (NifA*$_{\Delta Q\text{-linker}}$)	75.5 (9.9)	67.5 (3.8)
CGA762 (ΔnifDH)	<1	<1
CGA763 (ΔnifDH NifA*$_{\Delta Q\text{-linker}}$)	<1	<1
CGA730 (ΔnifDH NifA*$_{L208P}$ Vnf*)	0.4 (0.2)	10.5 (2.1)

Cells were grown on PM-20mM acetate with Wolfe's vitamins.

Table 5.2. List of genes and promoter regions sequenced in strain CGA730.

Gene name	Description
VnfA ORF and promoter region	σ^{54}- dependant transcriptional activator of *vnf*
NifA ORF and promoter region	σ^{54}- dependant transcriptional activator of *nif*, required for *vnf* and *anf* expression
NtrBC	Two-component nitrogen-starvation response regulator
NifR3	A predicted regulator often found in the NtrBC operon
GlnD	A bifunctional uridylyl transferase/removase that senses cellular nitrogen status and modifies P_{II} accordingly
GlnB	P_{II} homolog
AmtB1GlnK1	Ammonium transporter/P_{II} homolog operon
AmtB2GlnK2	Ammonium transporter/P_{II} homolog operon
RPA1383	A ModE-family transcriptional regulator of unknown function in *R. palustris*

CHAPTER VI:

Conclusions and future perspectives

CONCLUSIONS

The expression and activity of the nitrogen-fixing and energetically costly enzyme nitrogenase is tightly controlled. In the fluctuating environment, direct enzymatic control, such as posttranslational modifications, allow for rapid adaption and growth in a competitive environment. Tight control of alternative nitrogenases – which due to their inefficiency should only be used when Mo becomes limiting – also presumably increases competitive advantage.

In this work I have shown that *R. palustris* tightly controls activity of all three nitrogenase isozymes at several levels. Given the energetic costs that accompany synthesizing and running nitrogenase, these results were expected. However, I have shown that though *R. palustris* encodes for a similar set of regulators as the related bacterium *R. rubrum*, there are important differences between the two species (61). Therefore, one cannot assume that the specific gene products involved in one well-studied system applies unilaterally to all similar systems. For example, in *R. rubrum*, both GlnB and GlnJ are capable of regulating the DraT/G system (61). In *R. palustris* only GlnK2 is required. In *R. capsulatus*, but not *R. rubrum*, NtrC activity is absolutely required for nitrogenase activation (16, 76). In *R. palustris*, nitrogen fixation is greatly reduced in an *ntrBC* mutant, but the increased activity of the NifA* variant as well as adequate basal expression of *nifA* allows hydrogen production from *R. palustris* under conditions where NtrC is not activated (29). A deep understanding of these differences

between the model organism and related well-studied organisms is important for effectively engineering a strain of *R. palustris* to produce the greatest amount of hydrogen possible.

Additionally, I have shown that *R. palustris* CGA009 was able to horizontally acquire a third nitrogenase isozyme, V-nitrogenase, and bring it under tight regulatory control. Encoding for V-nitrogenase allows *R. palustris* CGA009 to fix nitrogenase when vanadium, but not molybdenum, is available in the environment. The V-nitrogenase is a more efficient enzyme for nitrogen fixed per reducing power used than the heterometal free Fe-nitrogenase and is also more effective at lower temperatures than the Mo-nitrogenase (18, 49). This expanded nitrogen-fixation capability would only be an advantage in competitive environments if it were tightly controlled and used secondarily compared to the Mo-nitrogenase, which is the most efficient enzyme for nitrogen fixation.

Not only have I shown that *R. palustris* controls V-nitrogenase posttranslationally in response to ammonium, but I have also isolated a mutant which expresses V-nitrogenase constitutively. This mutant should be a good tool for further investigations into how the V-nitrogenase is regulated. Its expression appears to be tied to degree of nitrogen starvation, but the mechanism of this regulation has yet to be determined. Finding the mutation that allows the evolved strain to produce hydrogen from V-nitrogenase

constitutively could allow for the construction of a strain that produces both Mo- and V-nitrogenase, and makes high amounts of pure H_2, even when ammonium is present in the feedstock.

FUTURE QUESTIONS

What role does DraT1 play in R. palustris *nitrogenase regulation?*

I found that DraT1 is not required for posttranslational modification and inactivation of Mo-nitrogenase. This was unsurprising as only DraT2 is constitutively expressed, while DraT1 is expressed concurrently with the *anf* gene cluster (51). AnfH is considerably less similar to NifH than VnfH and DraT2 may have less activity against it. Perhaps DraT1 is more specific for AnfH and less specific for NifH. The experiments I performed did not address this hypothesis. A *draT1* mutant of Fe-nitrogense strain CGA755 would allow one to address this question. I was unable to construct this mutant during the course of my work.

Is a greater degree of N-starvation the signal for alternative nitrogenase expression?

Transcriptional studies suggest that *vnf* and *anf* genes are upregulated after long-term nitrogen starvation, even in the presence of functional Mo-nitrogenase and Mo-ion. It is known that P_{II} protein modification increases with N-starvation (13, 58). Unpublished transcriptional profiles of N-starved *R. palustris* show that *vnf* expression also increases

with N-starvation. I hypothesize that the V-nitrogenase expressing strain has a P_{II} modification profile that resembles the N-starvation state, that is to say more fully modified. As NtrB kinase activity is repressed by unmodified-P_{II}, cells containing completely modified P_{II} could have greater NtrC phosphorylation and activity. More NtrC activity could lead to more NifA expression and if PII proteins are also responsible for activating NifA, more VnfA expression. VnfA is expressed at 78-fold higher levels in the V-nitrogenase strain than in wild-type *R. palustris* when grown under nitrogenase-stimulating conditions. Whether this upregulation is due to increased NtrC and NifA activity, or the action of another regulator has yet to be determined.

Can all three nitrogenases of R. palustris *CGA009 be expressed and active concurrently for maximum hydrogen production?*

The mutation that allows for constitutive expression of V-nitrogenase in a Δ*nifHD* mutant of *R. palustris* CGA009 did not occur in any of the expected regulatory genes, unless the specific $NifA_{L208P}$ mutation is responsible. If there is a second-site mutation responsible, it will be interesting to see if this mutation can be applied to constitutive expression of the Fe-nitrogenase as well. Other obstacles to overcome are the posttranslational modification and inactivation in response to ammonium as well as the energetic burden placed on a cell synthesizing and running three nitrogenases at once. Posttranslational modification is easy to remove by deleting both *draT* genes from the chromosome. If the cells are provided ample sunlight for energy and are used as non-growing biocatalysts,

energetic burden shouldn't be a grave concern. For initial increase in biomass before input into a bioreactor, these engineered cells may be grown aerobically, a condition which represses nitrogenase expression, even in the *nifA** mutant, and which would reduce the burden of nitrogenase expression on growth rate.

CHAPTER VII:

References

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APPENDIX

Contributions to other works performed during the course of doctoral training.

Rey et al. 2007. I performed sequencing and identified three of the *nifA* mutations described in the paper. I assisted in gathering growth data and preparing samples for transcriptome profiling.

Crosby et al. 2010. I constructed three key mutants used in this work as well as performed a subset of the growth experiments.

Huang et al. 2010. I constructed several *fix* mutants, and designed as well as executed many of the preliminary *fix* mutant growth experiments.