Université de Montréal

The Role of Specific Amino Acids in the Formation of Ternary Complexes in Nitrogenase Regulation in the Photosynthetic Bacterium *Rhodobacter capsulatus*

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Résumé

L'azote est l'un des éléments les plus essentiels dans le monde pour les êtres vivants, car il est essentiel pour la production des éléments de base de la cellule, les acides aminés, les acides nucléiques et les autres constituants cellulaires. L'atmosphère est composé de 78% d'azote gazeux, une source d'azote inutilisable par la plupart des organismes à l'exception de ceux qui possèdent l'enzyme nitrogénase, tels que les bactéries diazotrophique. Ces micro-organismes sont capables de convertir l'azote atmosphérique en ammoniac (NH_3), qui est l'une des sources d'azote les plus préférables. Cette réaction exigeant l'ATP, appelée fixation de l'azote, est catalysée par une enzyme, nitrogénase, qui est l'enzyme la plus importante dans le cycle de l'azote. Certaines protéines sont des régulateurs potentiels de la synthèse de la nitrogénase et de son activité; AmtB, DraT, DraG, les protéines PII, etc.. Dans cette thèse, j'ai effectué diverses expériences afin de mieux comprendre leurs rôles détailés dans *Rhodobacter capsulatus*.

La protéine membranaire AmtB, très répandue chez les *archaea*, les bactéries et les eucaryotes, est un membre de la famille MEP / Amt / Rh. Les protéines AmtB sont des transporteurs d'ammonium, importateurs d'ammonium externe, et ont également été suggéré d'agir comme des senseurs d'ammonium. Il a été montré que l'AmtB de *Rhodobacter capsulatus* fonctionne comme un capteur pour détecter la présence d'ammonium externe pour réguler la nitrogénase. La nitrogénase est constituée de deux métalloprotéines nommées MoFe-protéine et Fe-protéine. L'addition d'ammoniaque à une culture *R. capsulatus* conduit à une série de réactions qui mènent à la désactivation de la nitrogénase, appelé "nitrogénase switch-off". Une réaction critique dans ce processus est l'ajout d'un groupe ADP-ribose à la Fe-protéine par DraT. L'entrée de l'ammoniac dans la cellule à travers le pore AmtB est contrôlée par la séquestration de GlnK. GlnK est une protéine PII et les protéines PII sont des protéines centrales dans la régulation du métabolisme de l'azote. Non seulement la séquestration de GlnK par AmtB est importante dans la régulation nitrogénase, mais la liaison de l'ammonium par AmtB ou de son transport partiel est également nécessaire. Les complexes AmtB-GlnK sont supposés de lier DraG, l'enzyme responsable pour enlever l'ADP-ribose ajouté à la nitrogénase par DraT, ainsi formant un complexe ternaire.

Dans cette thèse certains détails du mécanisme de transduction du signal et de

transport d'ammonium ont été examinés par la génération et la caractérisation d'un mutant dirigé, RCZC, (D335A). La capacité de ce mutant, ainsi que des mutants construits précédemment, RCIA1 (D338A), RCIA2 (G344C), RCIA3 (H193E) et RCIA4 (W237A), d'effectuer le « switch-off » de la nitrogénase a été mesurée par chromatographie en phase gazeuse. Les résultats ont révélé que tous les résidus d'acides aminés ci-dessus ont un rôle essentiel dans la régulation de la nitrogénase. L'immunobuvardage a également été effectués afin de vérifier la présence de la Fe-protéine l'ADP-ribosylée. D335, D388 et W237 semblent être cruciales pour l'ADP-ribosylation, puisque les mutants RCZC, RCIA1 et RCIA4 n'a pas montré de l'ADP-ribosylation de la Fe-protéine. En outre, même si une légère ADP-ribosylation a été observée pour RCIA2 (G344C), nous le considérons comme un résidu d'acide aminé important dans la régulation de la nitrogénase. D'un autre coté, le mutant RCIA3 (H193E) a montré une ADP-ribosylation de la Fe-protéine après un choc d'ammonium, par conséquent, il ne semble pas jouer un rôle important dans l'ADP-ribosylation.

Par ailleurs *R. capsulatus* possède une deuxième Amt appelé AmtY, qui, contrairement à AmtB, ne semble pas avoir des rôles spécifiques. Afin de découvrir ses fonctionnalités, AmtY a été surexprimée dans une souche d'*E. coli* manquant l'AmtB (GT1001 pRSG1) (réalisée précédemment par d'autres membres du laboratoire) et la formation des complexes AmtY-GlnK en réponse à l'addition d'ammoniac a été examinée. Il a été montré que même si AmtY est en mesure de transporter l'ammoniac lorsqu'il est exprimé dans *E. coli*, elle ne peut pass' associer à GlnK en réponse à NH_4^+.

Mots-clés: AmtB, AmtY, le transport d'ammonium, mutagenèse dirigée, ADP ribosylation, fixation de l'azote.

Abstract

Nitrogen is one of the most vital elements in the world for living creatures since it is essential for the production of the basic building blocks of the cell; amino acids, nucleic acids and other cellular constituents. The atmosphere is 78% nitrogen gas (N_2), a source of nitrogen unusable by most organisms except for those possessing the enzyme nitrogenase, such as diazotrophic bacteria species. These microorganisms are capable of converting atmospheric nitrogen to ammonia (NH_3), which is one of the most preferable nitrogen sources. This ATP demanding reaction, called nitrogen fixation, is catalysed by the nitrogenase enzyme, which is the most important enzyme in the nitrogen cycle. Some proteins are potential regulators of nitrogenase synthesis and activity; AmtB, DraT, DraG, PII proteins and etc. In this thesis I performed various experiments in order to better understand their roles in *Rhodobacter capsulatus*, in more detail.

The membrane protein AmtB, which is widespread among archaea, bacteria and eukaryotes, is a member of the MEP/Amt/Rh family. The AmtB proteins are ammonium transporters, taking up external ammonium, and have also been suggested to sense the presence of ammonium. It has been shown that in *Rhodobacter capsulatus* AmtB functions as a sensor for the presence of external ammonium in order to regulate nitrogenase. Nitrogenase consists of two metalloprotein components named MoFe-protein and Fe-protein. The addition of ammonium to *R. capsulatus* culture medium leads to a series of reactions which result in the deactivation of nitrogenase, called “nitrogenase switch-off”. A critical reaction in this process is one in which DraT adds an ADP-ribose group to the Fe-protein of nitrogenase. The entrance of ammonia through the AmtB pore is regulated by GlnK sequestration. GlnK is a PII protein and PII proteins are one of the central proteins in the regulation of nitrogen metabolism. Not only is GlnK-AmtB sequestration important in nitrogenase regulation, but binding of ammonium by AmtB or its partial transport is also necessary. AmtB-GlnK complexes are thought to bind DraG, which is responsible for removing the ADP-ribose that DraT adds to nitrogenase, to form a ternary complex.

In this thesis details of the signal transduction mechanism and ammonium transport were examined by generating and characterizing RCZC, a (D335A) site- directed mutant of AmtB. The ability of this mutant, as well as previously constructed mutants RCIA1 (D338A), RCIA2 (G344C), RCIA3 (H193E) and RCIA4 (W237A), to “switch-off”

nitrogenase activity was measured by gas chromatography. The results revealed that all the above amino acid residues have critical roles in nitrogenase regulation. Immunoblotting was also carried out to check the presence of ADP-ribosylated Fe-protein. D335, D388 and W237 seem to be crucial for NifH ADP-ribosylation, since their mutants (RCZC, RCIA1 and RCIA4 respectively) didn't show ADP-ribosylation on Fe-protein. In addition, although a slight ADP-ribosylation was observed for RCIA2 (G344C) we still consider it as an important amino acid residue in this matter whereas the remaining mutant RCIA3 (H193E) showed Fe-protein ADP-ribossylation after an ammonium shock, therefore it doesn't seem to be important in NifH ADP-ribosylation.

In addition *R. capsulatus* possesses a second Amt called AmtY, which in contrast to AmtB, doesn't appear to have any specific roles. In order to find out its functionality, AmtY was overexpressed in an *E. coli* strain lacking AmtB (GT1001 pRSG1) (which was carried out previously by other lab members) and AmtY-GlnK complex formation in response to ammonium addition was examined. It was shown that even though AmtY is able to take up ammonia when expressed in *E. coli* it fails to associate with GlnK in response to NH_4^+.

Keywords: AmtB, AmtY, ammonium transport, site directed mutagenesis, ADP ribosylation, nitrogen fixation.

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List of Acronyms and Abbreviations

ADP	Adenosine diphosphate
APS	Ammonium persulfate
ATP	Adenosine triphosphate
bp	base pairs
BSA	Bovine serum albumine
C	carbon
°C	Degrees Celsius
$CaCl_2$	Calcium chloride
C_2H_4	Acetylene
C_2H_4	Ethylene
Cm^2	Square centimeter
co	Cofactor
$Cu(No_3)_2$	Copper nitrate
D	Aspartate
DNA	Deoxy ribonucleotide acid
DMSO	Dimethyl Sulfoxide
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
F	Phenylalanine
Fe	Iron
$FeSo_4$	Ferrous sulfate
g	gram
Gln	Glutamine
Glu	Glutamate
GOGAT	Glutamate synthase
GS	Glutamine synthetase
H	Histidine
H^+	Hydron
H_2	Hydrogen

H_3BO_3	Boric acid
HCl	Hydrochloric acid
His	Histidine
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilo base pairs
kDa	Kilo daltons
KH_2PO_4	Potassium phosphate monobasic
L	Liter
LB	Luria Bertani
M	Molar
Mb	Mega base pairs
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
ml	Milliliter
mM	Millimolar
$MnSO_4$	Manganese sulfate
Mob	Mobilization
N_2	Nitrogen
NaCl	Sodium chloride
$Na_2HPO_4^-$	Disodium phosphate
$NaMoO_4$	Sodium molybdate
NaOH	Sodium hydroxide
NH_3	Ammonia
NH_4^+	Ammonium
NH_4Cl	Ammonium chloride
$(NH_4)_2SO_4^+$	Ammonium sulphate
nm	nanometer
NO_2^-	Nitrite
NO_3^-	Nitrate
O	Oxygen
OD	Optical density

P	Phosphate
PCR	Polymerase chain reaction
PVDF	polyvinyl difluoride
rpm	revolutions per minute
S	Sulfur
S	Serine
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
TAE	Tris-acetate EDTA buffer
TBS	Tris buffered saline
TBST	Tris buffered saline with tween 20
TEG	Tris-Cl EDTA glucose
Tra	Transfer
Tyr	Tyrosine
V	Vanadium
V	Volt
W	Tryptophan
xg	Times gravity
W	Watt
YPS	Yeast extract/peptone/salts
$ZnSO_4$	Zinc sulfate
α	Alpha
β	Beta
σ	Sigma
μ	Micro

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Chapter I

Literature review

1. Introduction:

Organisms need various elements for growth; C,H,N,O,P,S,Fe. Among them, nitrogen is critical since it is essential for the production of the basic building blocks such as amino acids, nucleic acids and other cellular constituents. Nitrogen exists in different forms; N_2, ammonia (NH_3), ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and organic nitrogen (1). Among them, ammonia and ammonium are the forms preferred by microorganisms for assimilation (2) while N_2 is unusable for most organisms, due to the presence of a triple bond between the two nitrogen atoms which makes it almost inert (1). Only diazotrophic species can convert dinitrogen (N_2) to ammonia, a reaction catalysed by the nitrogenase enzyme. This reaction, called nitrogen fixation, requires a large amount of energy therefore it is only carried out in the absence or deficiency of ammonia (3, 4).

$$\mathbf{N_2 + 8H^+ + 8e^- + 16ATP \longrightarrow 2NH_3 + H_2 + 16ADP + 16Pi}$$

After the conversion of atmospheric nitrogen to ammonia it is available for assimilation by plants and passed to animals in amino acid and protein forms. Ultimately molecular nitrogen is released into the atmosphere. These back to back transformations repeated in a circular manner is named the nitrogen cycle (Fig 1), which literally recycles molecular nitrogen back to the atmosphere (1).

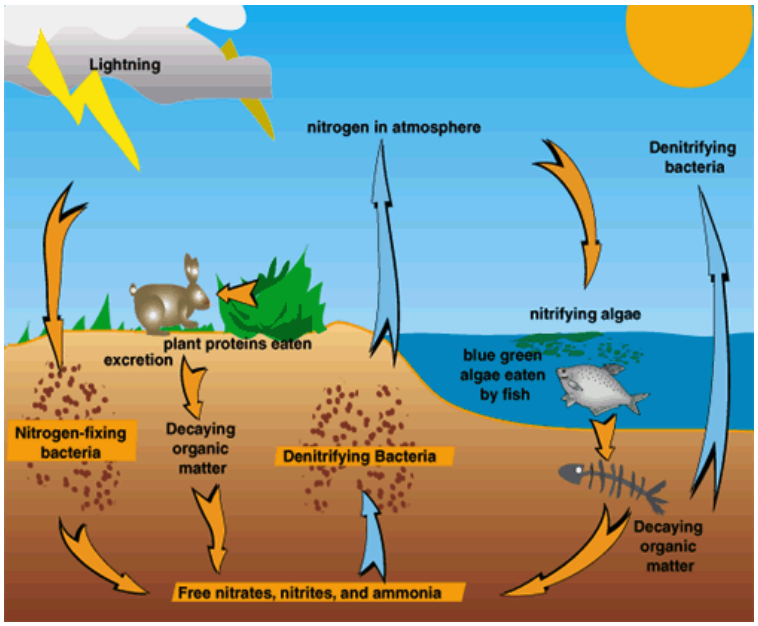


Fig 1. Nitrogen cycle (70)

1.1 *Rhodobacter capsulatus*:

This purple nonsulfur photosynthetic bacterium, previously named *Rhodopseudomonas capsulata*, has been widely used in research areas such as photosynthesis, energetics and nitrogen fixation (5). It grows very fast under either anaerobic photosynthetic conditions or aerobically in the absence of light with a doubling time of two hours and is stable in long time storage. The optimal growth temperature is 30 °C (6). *R. capsulatus* is ovoid or rod-shaped according to pH of the media. Cells are arranged as chains and angular. Their color under anaerobic conditions is brown, however in the presence of oxygen it turns red (7). It possesses a single 3.5 Mb chromosome and contains a 134 kb circular plasmid (5). *R. capsulatus* produces H_2, if grown in the presence of light under nitrogen limiting conditions (8). H_2 production is catalyzed by nitrogenase under anaerobic conditions, with the consumption of ATP and the two-electron reduction of H^+ to H_2 (9).

1.2 Nitrogen assimilation:

When organisms are in need of nitrogen, a regulatory system increases nitrogen assimilation. Assimilatory pathways are repressed under nitrogen sufficiency. In bacteria and eukaryotes PII proteins act as nitrogen sensors. In order for any nitrogen source to be assimilated, it first has to be converted to ammonium. Various metabolites are essential for its assimilation, which in turn signal nitrogen status to the regulatory system.

Two assimilatory pathways are available in prokaryotes, namely glutamine synthetase (GS)/glutamate synthase (glutamine:2-oxoglutarate aminotransferase, or GOGAT) (Fig 2) (10, used with permission) and glutamate dehydrogenase. In the former pathway, GS assimilates ammonium by converting glutamate (Glu) to glutamine (Gln). Then the amide group from Gln is transferred to 2-oxoglutarate (2-OG) which produces two Glu. In conditions where GOGAT doesn't function, glutamate dehydrogenase (GDH) makes Glu with 2-OG and ammonia. High concentrations of Glu signal ammonium sufficiency whereas high concentrations of 2-OG calls for ammonium deficiency (10).

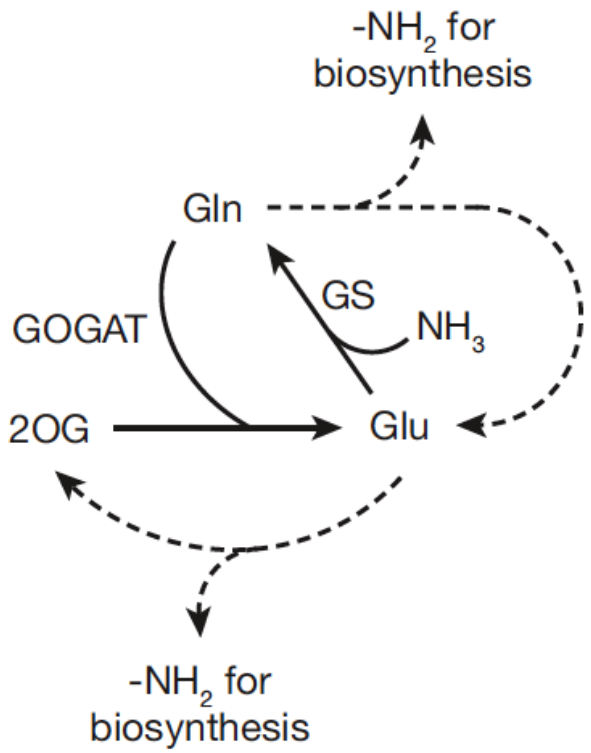


Fig 2. Glutamine synthase (GS)-glutamate synthase (GOGAT) pathway of ammonia assimilation (10)

2. Nitrogenase:

Nitrogenase is an extremely oxygen sensitive enzyme and in diazotrophic organisms, as mentioned earlier, is the enzyme responsible for the conversion of molecular nitrogen to ammonia with the consumption of 16 molecules of ATP (2 ATP per electron transferred) (11). In the photosynthetic bacteria, the required ATP and high energy electrons are provided by photosynthesis (12). Diazotrophic organisms are not found in the eukaryota while they are widely distributed in the bacteria and archaea (11).

2.1 Molybdenum-iron nitrogenase:

Nitrogenases have a conserved structure and functionality and are metalloenzymes since they contain MoFe-protein (molybdenum-iron protein) and Fe-protein (iron protein) (Fig 3) (54, used with permission), both of which are required for activity (11). Nitrogenase consists of structural genes (*nifHDK*) that encode the Fe-protein and MoFe-protein (3). This kind of nitrogenase is presesnt in all the diazotrophs. These two proteins make physical contact in

order to facilitate the electron transfer process (12).

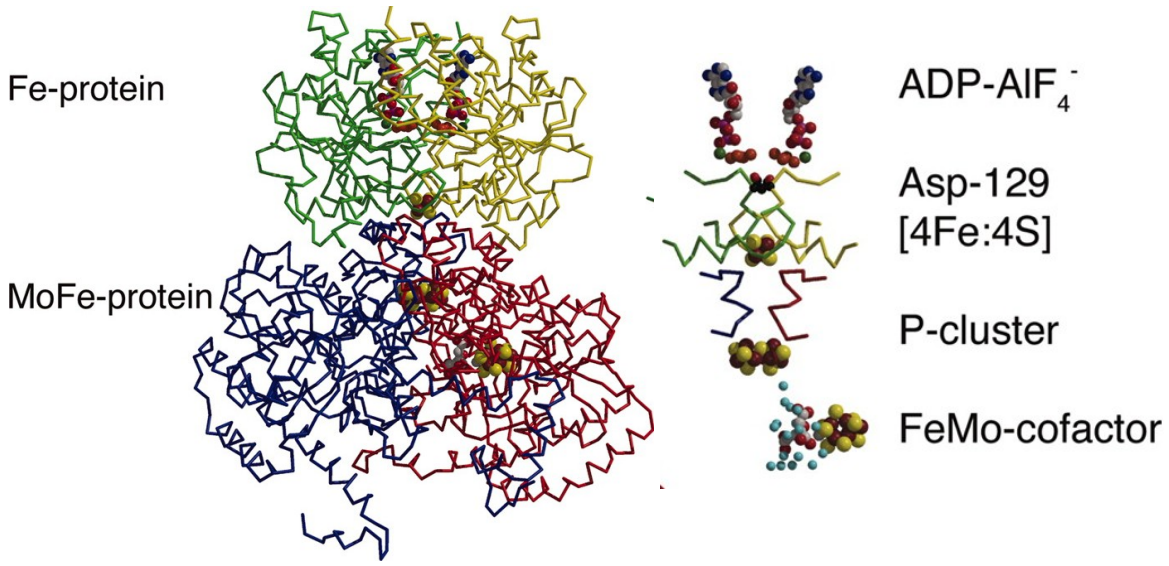


Fig 3. Structure of molybdenum-iron nitrogenase (54). The α and β subunits of MoFe protein are colored in red and blue respectively and Fe-protein subunits are shown in yellow and green.

2.1.1 FeMo-protein:

Molybdenum-iron protein is also known as dinitrogenase which is encoded by the *nifDK* nitrogenase structural genes (3). It is the larger component of nitrogenase and is heterotetrameric with α2β2 subunits; the α subunit is composed of 501 amino acids and weighs 55 kDa while the β subunit (accession number ADE84335.1) has 512 amino acids and weighs 56 kDa (11). FeMo-protein is responsible for the enzyme's activity and possesses the N_2 reduction active site (4). Dinitrogenase contains P-clusters (8Fe-7S) and the iron-molybdenum cofactor (FeMoco) which comprises the N_2 reduction active site (3, 12) (Fig 4) (11, used with permission). Dinitrogenase is extremely oxygen sensetive (11).

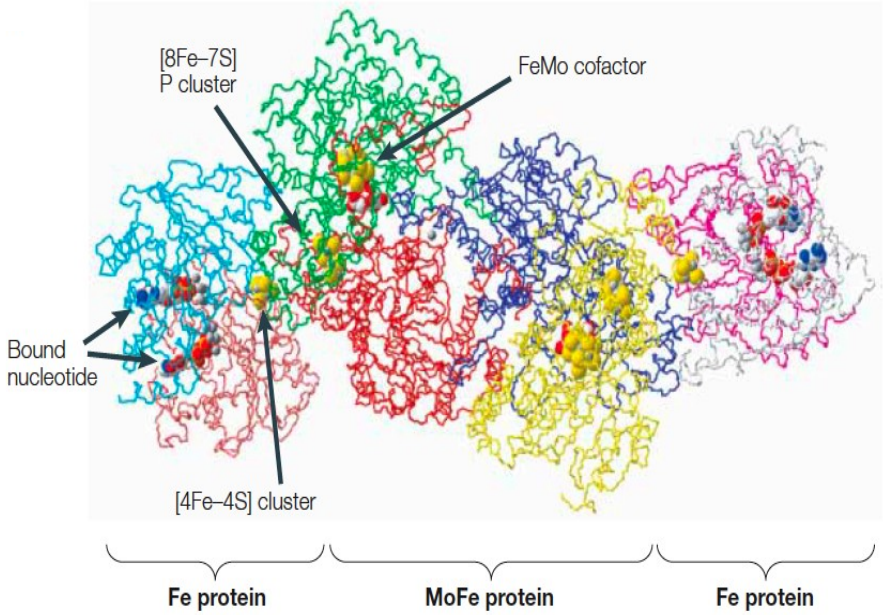


Fig 4. Structure of the complex formed between Fe-protein and MoFe-protein of nitrogenase (11). The subunits of the two Fe-protein dimers are depicted in cyan, brown, magenta and grey. The α subunits of the MoFe-protein are coloured in green and yellow, while β subunits in red and blue.

2.1.2 Fe-protein:

Iron protein, also called dinitrogenase reductase, is encoded by *nifH* (3) and is the smaller part of nitrogenase with homodimeric α2 subunits. Each α subunit (accession number ADE84336.1) contains 297 amino acids and weighs 33 kDa (11). Fe-protein contains a 4Fe-4S cluster and functions in transferring electrons to the MoFe-protein (Fig 5) (11, used with permission) in a step that requires MgATP hydrolysis (2, 12). Dinitrogenase reductase is irreversibly sensitive to oxygen (11).

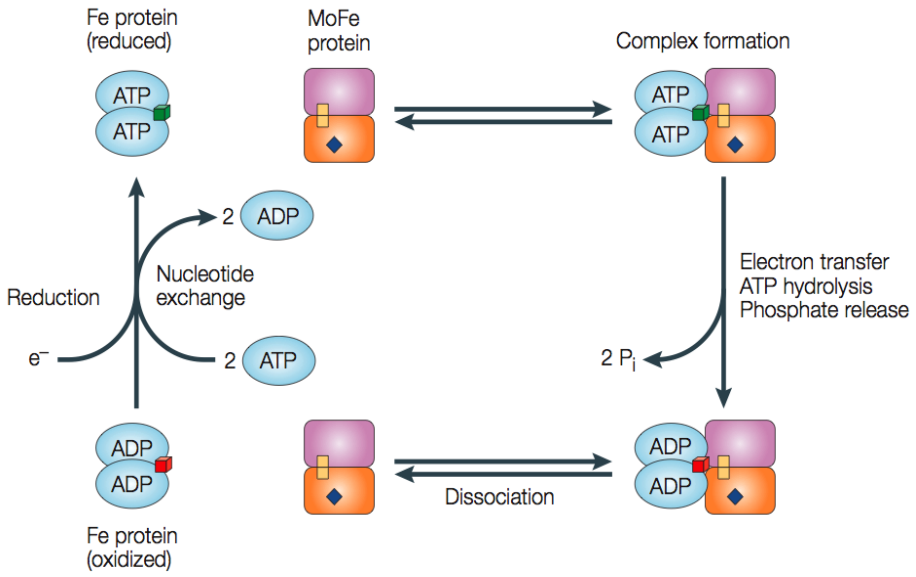


Fig 5. Depicting nitrogenase Fe-protein cycle (11).

2.2 Vanadium- dependent nitrogenase versus Iron-only nitrogenase:

Although all diazotrophs contain the molybdenum-iron nitrogenase, some, such as *R. capsulatus* can synthesize a substitute for it with vanidium-iron (FeVco) or iron-iron co-factors (FeFeco) under molybdenum-limiting conditions (11). The former belongs to the vanadium- dependent nitrogenase (V-nitrogenase) whereas the latter originates from the iron-only nitrogenase (Fe-nitrogenase). V-nitrogenase and Fe-nitrogenase are encoded by the *vnf* (vanadium-dependent nitrogen fixation) and the *anf* (alternative nitrogen fixation) genes (3). Since V-nitrogenase and Fe-nitrogenase show less specific activity in comparison to MoFe-nitrogenase, the latter is always the one used preferentially by diazotrophs (3, 13).

3. Nitrogen fixation in *R. capsulatus:*

3.1 Important Nitrogen fixation genes and proteins:

Nitrogen fixation in *R. capsulatus* requires more than 50 genes, a number of them will be briefly discussed in this section. The 3 nitrogenases are encoded by 3 different sets of genes: the molybdenum nitrogenase (*nifHDK*), the vanadium nitrogenase (*vnfH, vnfDGK*), and the alternative nitrogenase (*anfHDGK*) (14). Genes required for the biosynthesis of FeMoco are *nifU, nifS, nifB, nifQ, nifV, nifE* and *nifN*. Also *ntrB*/*C* genes are

extremely important as they trigger the first step of nitrogen fixation in *R. capsulatus* (3 and 15).

Additional genes involved in nitrogen metabolism are the two PII proteins encoded by *glnK* and *glnB, glnD*, responsible for PII modification, an ammonium transporter protein encoded by *amtB,* and the proteins responsible for the post-translational regulation of nitrogenase encoded by *draT/G* (3). These are vital elements for nitrogen fixation in *R. capsulatus,* and each will be described in detail in the following sections.

Some critical genes involved in nitrogen fixation in *R. capsulatus* are duplicated such as FeMoco biosynthesis genes *nifB1*/*nifB2*, regulatory genes *nifA1*/*n*if*A2* and the molybdenum regulatory genes *mopA*/*mopB* (3, 16).

3.2 The Mep/AmtB/Rh family:

As mentioned previously, ammonium, the preferred nitrogen source for organisms such as bacteria, fungi and plants, is conducted through the membrane via a large and ubiquitous family named the Mep/AmtB/Rh family. However ammonium, in its uncharged form ammonia, can also enter the cell through passive diffusion. However, sometimes its extracellular level decreases to a limit below which inhibits passive diffusion can no longer support cellular needs and ammonium is actively taken up by AmtB/Mep to optimize the organism's growth efficiency (11, 17). Methylamine permease (Mep) and ammonium transporter (Amt) are critical for ammonium uptake under nitrogen depleted conditions, in yeast and bacteria, eukaryotes, archaea respectively (18). The equivalent protein in animals is the Rhesus (Rh) protein involved in ammonium homeostasis that is also important for pH balance maintenance in the kidney.

The most highly conserved part of the proteins in this family is the pore's hydrophobic side chains, especially for two centrally located histidines, His193 and His342 (in *R. capsulatus*). In addition, all members of this family possess a conserved overall structure of 11 transmembrane helices. In addition, a high-affinity ion-binding site, which is specific for ammonium, is situated at the extracellular entrance of the hydrophobic pore of Mep and Amt proteins that is responsible for ammonium transport at its low concentrations. This site is not conserved in the Rh proteins (17).

3.3 Mep protein:

In yeast, Mep acts as an ammonium sensor and also conducts ammonia through its hydrophobic pore under nitrogen limited conditions. *Saccharomyces cerevisiae* possess 3 Mep proteins called Mep1, Mep2 and Mep3 (19-24). Mep2 has the highest affinity for ammonium (K_m 1–2 μM), Mep1 is second concerning ammonium affinity (K_m 5–10 μM) followed by Mep3 (K_m 1.4–2.1 μM) with a dramatic difference (24-27). In conditions of limited ammonium or amino acids a dimorphic change is induced resulting in filamentation of the diploid yeast cells which enables them to search for their prefereed conditions (24, 28). In such situations in *Saccharomyces cerevisiae* Mep2 acts as an ammonium detector (24).

3.4 Amt protein:

Similar to Mep, Amt may act as an ammonium sensor besides being an ammonia transporter channel when external ammonium concentrations are low. *R. capsulatus*, a purple nonsulfur photosynthetic bacterium, potentially has two systems for ammonium uptake. One is AmtB, which has been shown to be essential for nitrogenase switch off, the other is AmtY that is apparently silent (2).

3.4.1 AmtB:

AmtB is usually encoded in a *glnK-amtB* operon (18). GlnK plays a critical role in the regulation of ammonia transport by binding to AmtB under ammonium replete conditions and blocking its pore (25). AmtB has 453 amino acids and weighs 46.8 kDa (2). AmtB is an ammonium channel protein (Fig 6) (25, used with permission), which has been revealed to be homotrimeric with a hydrophobic pore in each subunit (4, 18, 29). AmtB is an integral membrane protein whose isolation requires detergent solubilization (17, 30). AmtB has 11 transmembarane-spanning α helices (named M1-M11 in figure 6, where the corresponding pairs are shown in the same color) with an extracytosolic N-terminus and a cytosolic C-terminus (25, 31), together forming a right handed bundle around the pore (31).

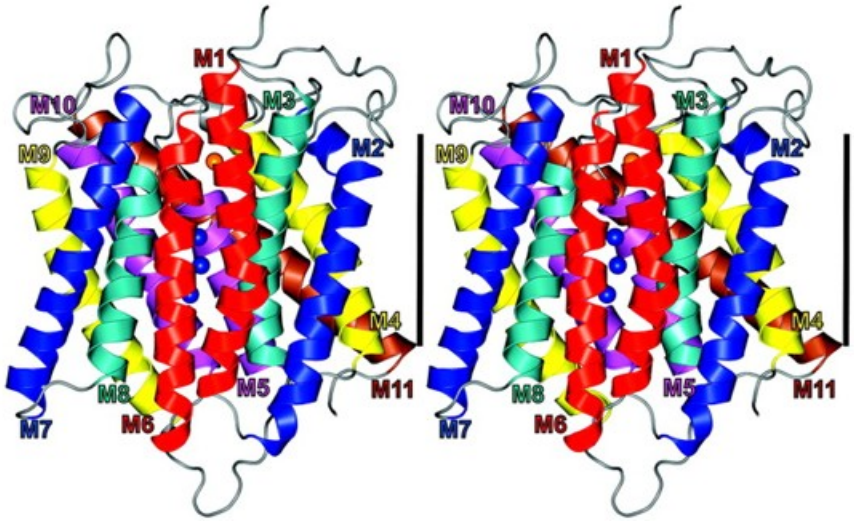


Fig 6. A 3D view of monomer ammonium transporter channel (AmtB) (25).

AmtB is a high-affinity ammonium uptake system (2) which transports ammonia through the cytoplasmic membrane (18) and is capable of methylammonium uptake. External NH_4^+ can't be conducted through the AmtB pore unless it is deprotonated at the exterior and transferred through the cytoplasmic membrane as NH_3. It is reprotonated at the cytoplasm upon its arrival (4). The presence of AmtB is essential in order for some microorganisms to have optimal growth when extracellular NH_4^+ concentrations are low (29). In other words, when the concentration of extracellular NH_4^+ decreases to a level below which passive diffusion can cope with cellular demands, ammonia can be transported through AmtB to optimize the efficiency of bacterial growth (17). In addition, AmtB can compensate for the loss of NH_4^+ from the cell through diffusion (13). Therefore, regulation of the internal ammonium concentration could also be another function for AmtB (2, 32). AmtB also acts as an ammonium sensor, under ammonium starvation conditions, which leads to a series of reactions affecting the regulation of filamentous growth in microorganisms (2, 33). In order for AmtB to sense extracellular ammonium, ammonia transportation is required through its channel (18).

Two conserved phenylalanine side chains, F131 and F240 in *R. capsulatus* (equivalent to F107 and F215 in *E. coli,* as shown in figure 7) (27, used with permission), are situated at the entrance of each hydrophobic pore at the NH_4^+ binding site and they block the access to the channel. Inside the pore two highly conserved histidines, H193 and H342 in *R. capsulatus* (equivalent to H168 and H318 in *E. coli*, as depicted in figure 7, are

thought to facilitate NH_4^+ deprotonation upon its entrance into the AmtB pore by being proton acceptors (27).

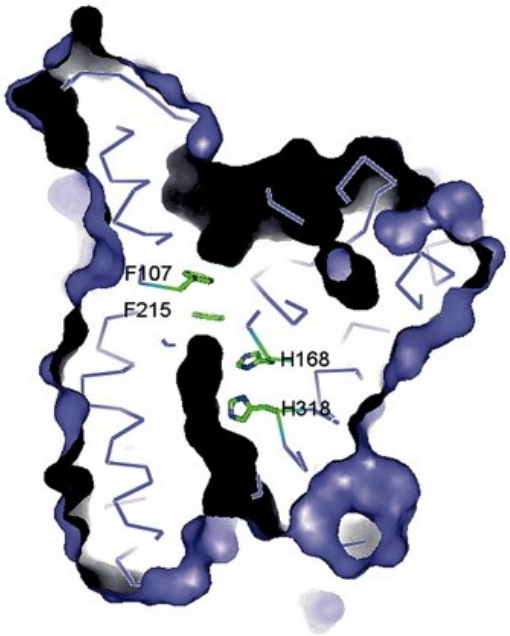


Fig 7. A cut through the AmtB monomer within the membrane, presenting a number of imoportant amino acid residues (27)

These histidines are preceded by two highly conserved aspartates, D148 and D335 in *R. capsulatus* (equivalent to D160 and D310 in *E. coli*) which appear to be involved in H-bonding interactions and have also been suggested to play structural roles. The amino acids that line the entrance of the AmtB pore in *R. capsulatus* are mostly hydrophobic and among them F131, W237 and F240 (same as F107, W212 and F215 in *E. coli*) are the most conserved ones (27). The D334 amino acid residue of *R. capsulatus*, similar to D309 of *E. coli*, participates in AmtB rearrangement upon GlnK binding. It is also a proton donor for ammonia upon its arrival at the cytoplasmic side, converting it into ammonium (34). Crystallographic studies carried out with *E. coli* AmtB suggest that the NH_4^+ binding cavity site in *R. capsulatus* is surrounded by F127, F131, W172 and S244 (similar to F103, F107,W148 and S219 in *E. coli*) and influence the specificity of the NH_4^+ binding site (27).

3.4.2 AmtY:

Unlike AmtB, which is part of the g*lnK-amtB* operon, AmtY is monocistronic and situated 2.5 kb away from *ntrBC* (genes coding for the NtrB-NtrC regulatory system which sense intracellular ammonia in *R. capsulatus*) and *nifR3* (a gene involved in nitrogen regulation in *R. capsulatus* (35)). AmtY (accession number YP_003577944.1) not very

similar to AmtB with only 42% similarity between them in *R. capsulatus* (2). They are very different in length, especially in their N-terminal region. It is not known whether AmtY plays a physiological role in ammonium uptake but it has been revealed that it is not responsible for methylammonium uptake. Since permeases that are capable of being probed by methylammonium are known as high-affinity ammonium transporters, AmtY is considered as a low-affinity one (2). AmtY is placed in the membrane and has 388 amino acids and its molecular weight is 42 kDa.

3.5 Ntr system:

Sensing ammonia is key point regulating nitrogen metabolism in diazotrophs such as *R. capsulatus*. Extracellular ammonia is sensed by the AmtB protein whereas the intracellular ammonia is detected by the Ntr system (3). This system consists of NtrB-NtrC regulatory system, the two PII proteins, GlnB and GlnK, and GlnD uridylyltransferase/UMP-removing enzyme. Unlike AmtB which senses ammonia directly, the Ntr system senses nitrogen indirectly via responding to glutamine (nitrogen assimilation product), ATP and 2-oxoglutarate. These metabolites interact with GlnD, GlnB and GlnK, but are not capable of binding to NtrB, NifA1, NifA2 and DraT (36).

3.5.1 Nitrogen regulatory protein B (NtrB):

NtrB (accession number ADE85542.1) is a bifunctional kinase/phosphatase protein playing a key role in transcriptional regulation of nitrogen fixation. This nitrogen regulator protein has a homodimeric structure (37), having 355 amino acids and weighing 38 kDa. It possesses an unique N-terminal domain and a C-terminal domain which is responsible for NtrB's kinase/phosphatase functionality. In nitrogen limiting conditions NtrB acts as a positive regulator by autophosphorylating itself in the presence of ATP and transferring the phosphate to NtrC. Autophosphorylation occurs at a highly conserved histidine, H139 in *E. coli* , which is located in a conserved region of NtrB. Phosphorylated NtrC binds at the promoters of *nifA1*, *nifA2* and *anfA*, transcriptional activators, and activates their transcription. This cascade of reactions finally leads to nitrogenase synthesis (37).

3.5.2 Nitrogen regulatory protein C (NtrC):

NtrC (accession number ADE85543.1) is a transcriptional activator via the RNA polymerase's σ^{54} holoenzyme form. It has 458 amino acids and weighs 50 kDa. NtrC consists of three domains, namely, the N-terminal domain, central output domain, and C-terminal domain. The first domain has an aspartate, D54 in *E. coli*, which is the phosphorylation site. In other words, D54 is the residue that accepts the phosphate donated from the H139 of NtrB. The second domain is responsible for the activation of transcription by RNA polymerase's σ^{54} holoenzyme form. The last domain has a helix-turn-helix motif which is responsible for NtrC DNA binding, before phosphorylazation, and its dimerization when binding to the DNA (40, 41). As discussed earlier, phosphorylated NtrC triggers transcription of nitrogen-regulated promoters (37).

3.6 NifA Protein:

R. capsulatus possess two similar *nifA* genes named *nifA1* and *nifA2* which are 97% identical to each other. Their sole difference comes from their extreme N-terminal sequence (3, 16). These genes code for NifA1 (accession number CAB53157.1), which has 579 amino acids and weighs 63.6 kDa, and NifA2 (accession number AAB91397.1), which has 582 amino acids and weighs around 64 kDa, respectively. These proteins are transcriptional activators for Mo-nitrogenase structural genes. Studies have shown that the presence of both proteins is not essential for their functionality. In other words they can substitute for each other as transcriptional activators. Under nitrogen fixing conditions, NtrC is phosphorylated by NtrB which then leads to the transcription of *nifA1* and *nifA2* genes and consequently their translation to NifA1 and NifA2. Either one of these proteins then activates the transcription of the Mo-nitrogenase structural genes *nifHDK* and other *nif* genes (3).

3.7 AnfA protein:

Under the appropriate conditions, *R. capsulatus* can also fix nitrogen using the alternative Fe-nitrogenase system. It possesses a gene involved in transcriptional activation of Fe-nitrogenase, called *anfA* which encodes the AnfA protein (accession number CAA53584.1). This protein has 538 amino acids and weighs 59.1 kDa. Like *nifA1/2*, in the

absence of ammonium, phosphorylated NtrC triggers *anfA* transcription and in turn its translation to AnfA. Unlike *nifA1*/*2*, *anfA* transcription is also dependent on the absence of molybdenum, besides the lack of ammonium. Therefore AnfA expression relies on the absence of both ammonium and molybdenum. AnfA is responsible for transcriptional activation of Fe-nitrogenase structural genes, *anfHDK*, which in turn causes the synthesis of an active Fe-nitrogenase (3, 38).

3.8. PII proteins:

PII proteins are small sized homotrimeric proteins that are normally cytoplasmic and are among the most well distributed regulatory proteins present in all 3 domains of life (3, 42, 39). They act as sensors for cellular nitrogen, carbon and energy status by binding effectors such as 2-oxoglutarate, ATP and ADP resulting in conformational changes. PII proteins also play critical roles in transcriptional and post-translational levels of Mo-nitrogenase regulation. In addition, PII proteins undergo transient uridylylaion/deuridylylation cycles via GlnD in response to intracellular ammonium (or glutamine) levels which ultimately affects carbon and nitrogen metabolism. PII proteins in *R. capsulatus* consist of 2 members. GlnB and GlnK (4, 42).

3.8.1 GlnB and GlnK:

GlnB and GlnK are PII signal transduction proteins with GlnB (accession number CAA50650.1) being expressed constitutively while GlnK (accession number AAC34722.1) is nitrogen-regulated (4, 39). They both have 112 amino acids and weigh about 12.3 kDa. They play a central role in the transcriptional regulation of Mo-nitrogenase, though GlnB is much more involved than GlnK, to such a degree that it cannot be replaced by GlnK in this regulation step. So under nitrogen replete conditions, it is GlnB, and not GlnK, that binds NtrB and inhibits the expression at Ntr promoters which in turn prevents Mo-nitrogenase expression (4). However, yeast two-hybrid experiments have shown that in the post-translational regulation of Mo-nitrogenase, both GlnK and GlnB seem to be essential (4, 36). When the ammonium concentration is low, GlnD-uridyltransferase, uridylylates PII proteins (including GlnB) and prevents GlnB from binding to NtrB which results in Mo-nitrogenase synthesis. As well, in the presence of excess ammonium, UMP-removing enzyme, deuridylylates GlnB (completely deuridylylated) and GlnK (poorly deurilylated)

which allows GlnB to interact with NifA and inactivates it, which blocks Mo-nitrogenase synthesis (4, 39).

The X-ray crystal structures of GlnK and GlnB of *E. coli* have been solved (43, 44). The monomer structures of GlnK and GlnB are similar and both contain two α-helices and six β-strands that are linked by three loops. The largest loop is called the T-loop which stretches from residues 37 to 55 and has a tyrosine, Tyr-51 (Fig 8) (39, used with permission), that is the uridylylation site. Another, smaller loop, named the B-loop, is situated between residues 82 and 88. The smallest loop, the C-loop, is at the C terminus between residues 102 to 105. In the trimeric form, the C-loop of one subunit makes clefts by lying among the T-loop and B-loop of the neighboring subunit. These three clefts seem to be important in the interaction of PII with its targets (39, 44, 45).

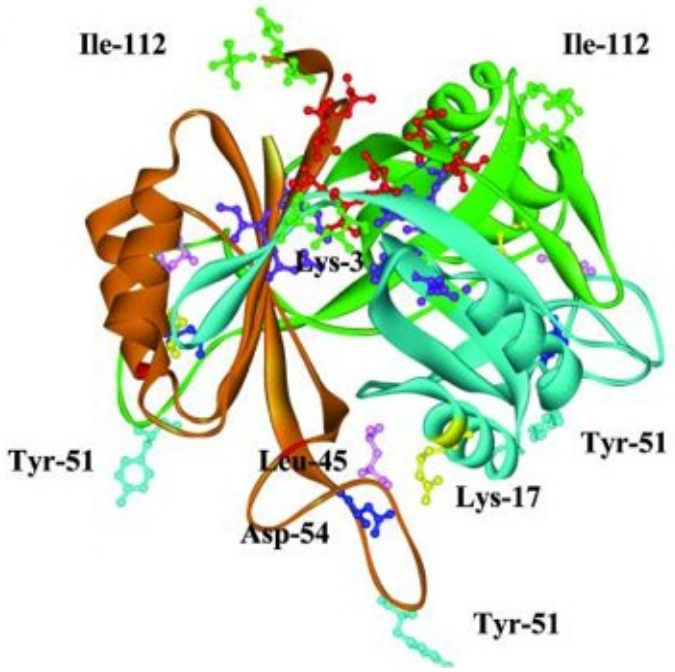


Fig 8. GlnB trimer, pointing out some critical amino acid residues (39).

3.8.2 GlnD:

GlnD (accession number YP_351859.1), which plays a central role in the Ntr regulatory system, has 930 amino acids and weighs 102.3 kDa GlnD is a uridyltransferase/UMP-removing enzyme, which acts as a nitrogen sensor by detecting the

level of glutamine. It then transmits the information to GlnK and GlnB via conformational changes due to its bifunctional uridyltransferase/UMP-removing ability. GlnK and GlnB uridylylation take place in Tyr-51 of the T-loops. As mentioned earlier, in the absence of nitrogen (or glutamine) GlnB and GlnK are uridylylated, so NtrB is able to phosphorylate NtrC which in turn activates the transcription of *nifA* genes, causing Mo-nitrogenase activity. On the other hand, in the presence of nitrogen (or glutamine) GlnK and GlnB are deuridylylated, consequently resulting in the interaction of NtrB with GlnB and blocking nitrogenase synthesis (46).

3.9. DraT & DraG:

The nitrogenase switch off/on effect, which has been seen in some photosynthetic and chemotrophic bacteria, is caused by at least two different mechanisms. One is dependent on the covalent modification/demodification of Fe-protein by ADP-ribosylation carried out via DraT and DraG, another is independent of this process and mainly relies on PII proteins (2) (these mechanisms are discussed thoroughly in the section "Post-translational regulation of nitrogenase activity").

Dinitrogenase reductase ADP-ribosyltransferase (DraT) and dinitrogenase reductase activating glycohydrolyse (DraG) are enzymes responsible for the addition of ADP-ribose to arginine 101 residue of the Mo-nitrogenase Fe-protein and for its removal respectively (47, 48). Arginine 101 and its other neighboring amino acid residues are extremely conserved in NifH proteins among various organisms which reveals their importance (48). DraT (accession number CAA50443.1) weighs around 30 kDa with 270 amino acids while DraG (accession number CAA50441) has 291 amino acids and weighs 32 kDa. Sequence analyses have revealed that *draT* and *draG* genes, coding for the corresponding proteins, are only 11 bp apart from each other, suggesting the fact that they are located in the same operon (48).

In the presence of ammonium, ATP, oxygen and darkness, DraT, ADP-ribosylates the Fe-protein of Mo-nitrogenase which consequently results in rapid inhibition of the nitrogenase enzyme or so called nitrogenase switch off. Since this is a reversible mechanism, in the absence of ammonium, oxygen and darkness, DraG removes the ADP-ribose groups from Fe-protein which reactivates the nitrogenase enzyme, also known as nitrogenase switch on (48).

3.9.1 DraT & DraG regulation via AmtB and PII proteins:

Under nitrogen fixing conditions, GlnK and GlnB are completely uridylylated and saturated with ATP and 2-OG, so they are not bound to DraG and DraT. In this situation, DraT is inactive while DraG is active and both cytoplasmically loacted (Fig 9 a) (49, used with permission). On the other hand, upon an ammonium shock, ammonium assimilation increases the level of glutamine which in turn decreases the amount of 2-OG. When a rise in glutamine is sensed, GlnD-UMP removing enzyme deuridylylates GlnB and GlnK. Also 2-OG depletion facilitates the exchange of ATP-PII to ADP-PII, so GlnK and GlnB are no longer bound to ATP. Consequently, deuridylylated GlnK-ADP binds to AmtB and DraG which block the AmtB pore and inactivate DraG. In the same situation, deuridylylated GlnB-ADP binds DraT and activates it (49) (Fig 9 b) (49, used with permission) .

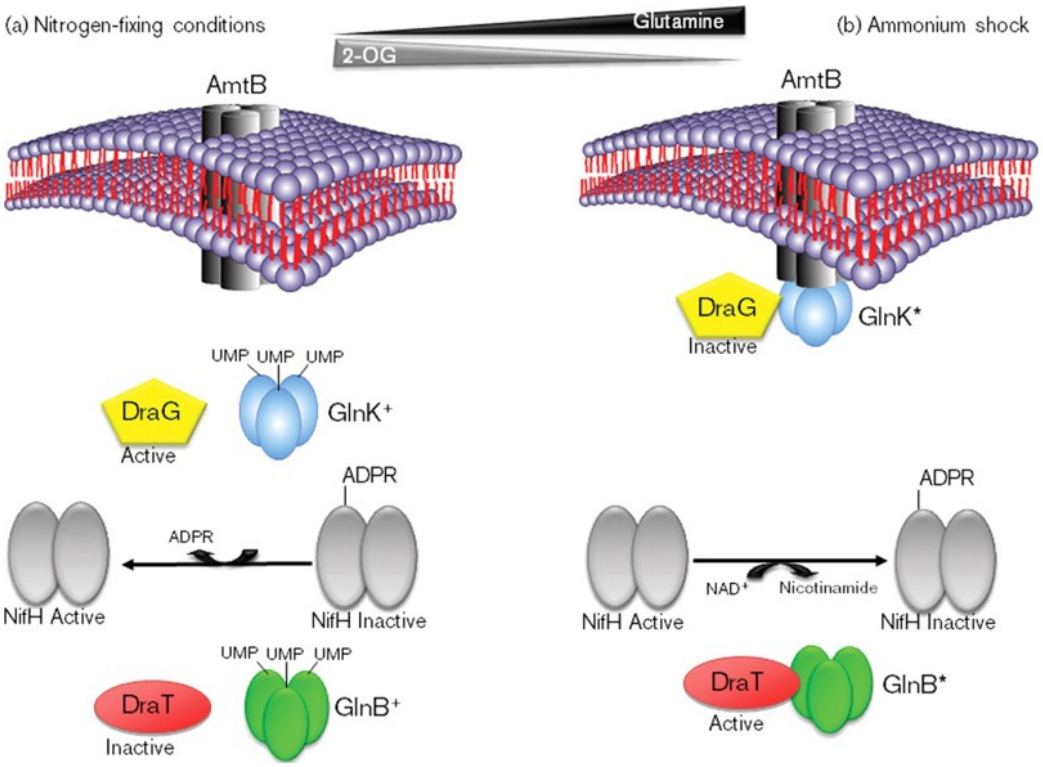


Fig 9. Role of AmtB and PII proteins in the regulation of DraT and DraG via (a) ammonium limitation and (b) ammonium repletion (49).

4. Transcriptional regulation of nitrogen fixation:

4.1 NtrB-NtrC regulatory system at low/high glutamine concentration:

When intracellular glutamine (Gln) concentration is low (equivalent to low nitrogen concentration) the bifunctional GlnD protein uridylylates GlnB at a tyrosine residue that is conserved, in the T-loop (50). Consequently GlnB is not capable of binding NtrB-kinase, therefore NtrB autophosphorylates which triggers the first step of nitrogenase synthesis cascade by donating the phoshoryl group to NtrC (3).

On the other hand at high glutamine concentration (which represents the abundance of nitrogen) the bifunctional GlnD protein removes the UMP group from GlnB. In turn deuridylylated GlnB interacts with NtrB and prevents it from autophosphorylation. As well, the NtrB-GlnB complex forces NtrC to dephosphorylate rapidly (51), which makes it unable to activate *nifA* transcription. In such a situation the process of nitrogenase synthesis is blocked from the beginning, which causes a failure in nitrogen fixation (3).

4.2 NifA and AnfA transcriptional activators system:

4.2.1 Activation of nitrogen fixation genes under nitrogen depletion:

Upon a lack of fixed nitrogen, NtrB protein (a sensor kinase) is phosphorylated automatically and acts as a phosphodonor for NtrC protein. NtrC binds to *nifA1*, *nifA2* and *anfA* genes, which leads to their transcription and the production of NifA1, NifA2 and AnfA proteins respectively (52). NifA1/2 transcribes Mo-nitrogenase's structural genes (*nifHDK*) and any other *nif* genes. On the other hand AnfA transcribes Fe-nitrogenase's structural genes (*anfHDGK*) (3). Therefore Mo-nitrogenase and alternative nitrogenase are synthesized resulting in the fixation of atmospheric nitrogen (Fig 10) (3, use with permission).

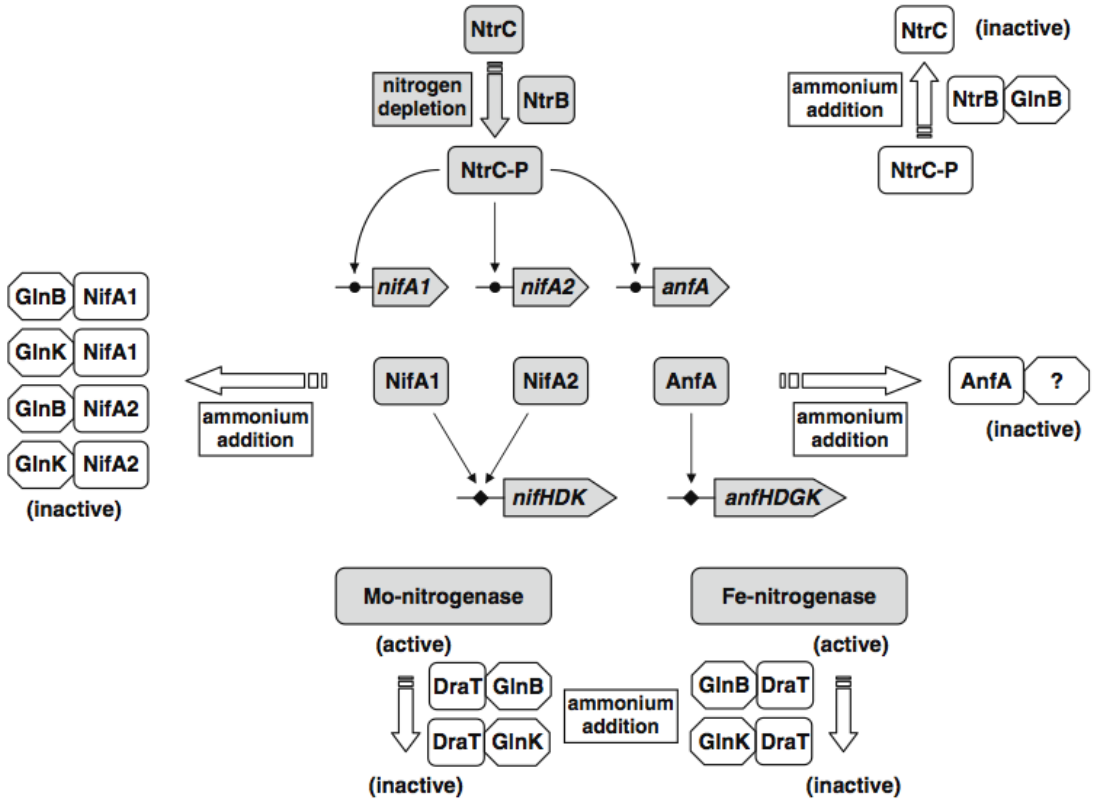


Fig 10. Nitrogen control of nitrogen fixation by GlnB and GlnK (mainly focusing on nitrogen depletion conditions) (3).

4.2.2 Deactivation of nitrogen fixation genes under nitrogen repletion:

The addition of fixed nitrogen to a nitrogen-fixing culture shows a 3 level effect (Fig 11) (71, used with permission). The first step is at the pre-transcriptional level and causes an interaction between NtrB and GlnB that leads to the dephosphorylation of NtrC, therefore, *nifA1*, *nifA2* and *anfA* genes are not transcribed. At the transcriptional level an increase in the amount of fixed nitrogen results in interaction of GlnK and GlnB with NifA1 and NifA2 which consequently can no longer activate *nif* gene expression. As well, AnfA will be also inactivated by a rise in the level of fixed nitrogen, however its interaction partner is still unclear. The last level influenced by the addition of fixed nitrogen is the activation of DraT through an interaction with GlnK or GlnB which in turn will add ADP-ribose groups to the Fe-protein of nitrogenase enzyme and results in its transient inactivation (3). Previous studies have shown that either GlnB or GlnK are sufficient to

interact with NifA1 and NifA2 to block nitrogenase synthesis which in turn inactivates nitrogen fixation (3, 53).

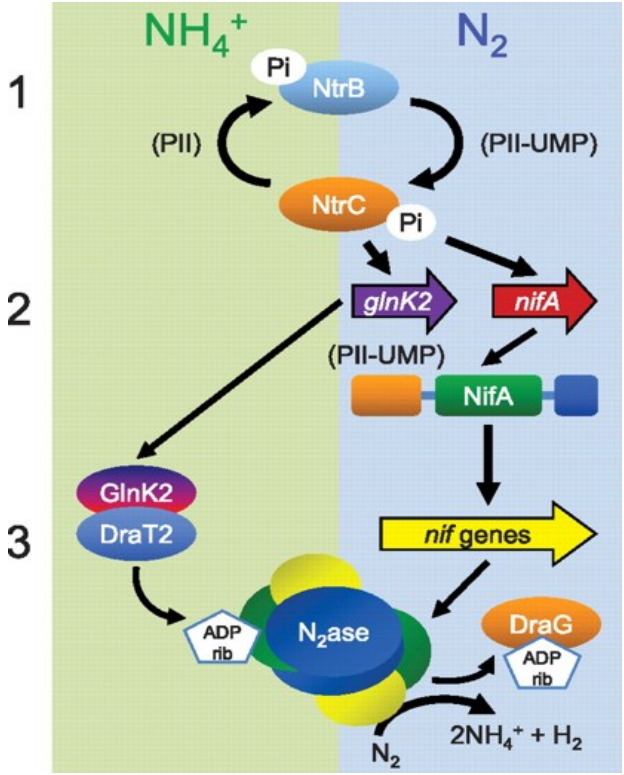


Fig 11. Three stages of deactivation of nitrogen fixation genes under nitrogen repletion (71).

5. Post-translational regulation of nitrogenase activity:

A number of purple nonsulfur photosynthetic bacteria, such as *R. capsulatus*, possess a modification/demodification system as a post-translational regulation system for nitrogenase (Fig 12) (29, used with permission) DraT (dinitrogenase reductase ADP-ribosyltransferase) and DraG (dinitrogenase reductase-activating glycohydrolase) play a significant role in this nitrogenase control system (3). Under high extracellular NH_4^+ concentrations (equivalent to low 2-oxoglutarate and ATP/ADP) or in the dark, GlnD-UMP removing enzyme deuridylylates Glnk. This is concomitant with the membrane sequestration of GlnK-DraG and the formation of the ternary complex (GlnK-DraG-AmtB), so ammonia is not able to pass through the AmtB pore (18, 29, 54). Also DraT doesn't interact with GlnK and/or GlnB so DraT is able to add ADP-ribose groups to the Arg101 of an α subunit of the Mo-nitrogenase's Fe-protein (NifH) and consequently, reversibly,

inhibits the MoFe-protein (NifDK) from interacting with it. This ADP-ribosylation leads to the inactivation of nitrogenase enzyme that is known as nitrogenase switch off (3).

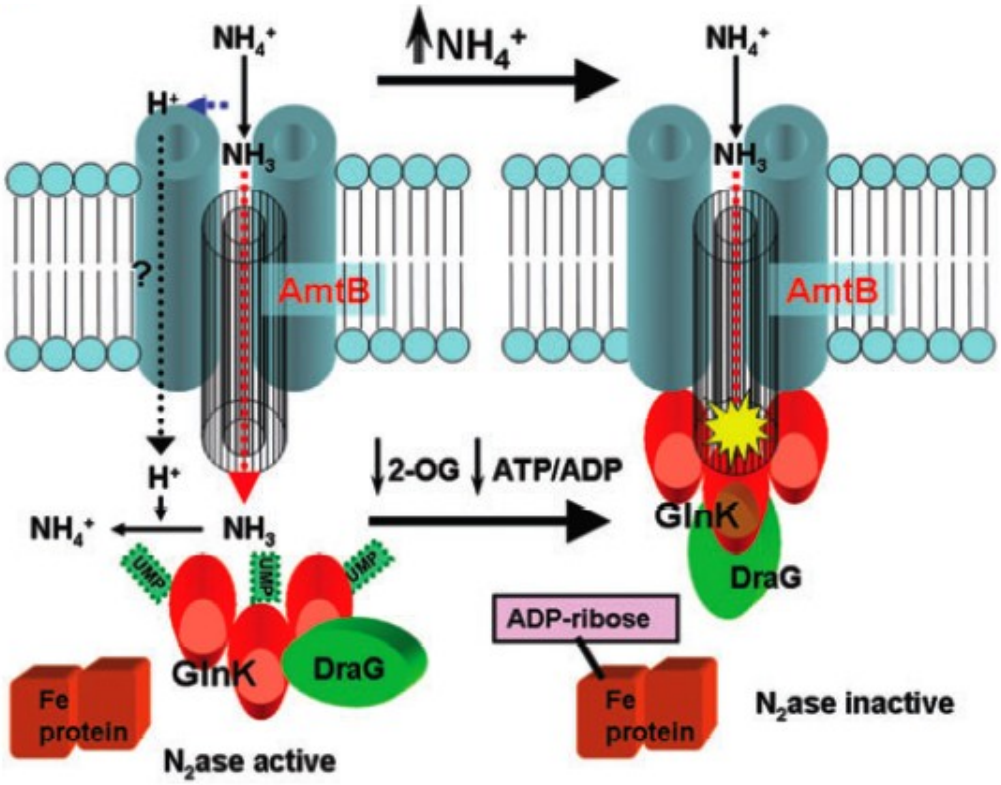


Fig 12. Nitrogenase post-translational regulation system, ADP-ribosylation-dependent (29)

Whereas in nitrogen fixing conditions (equivalent to high 2-oxoglutarate and ATP/ADP) or in the presence of light, GlnK is uridylylated by GlnD-uridyltransferase, which then makes a binary complex with DraG in the cytoplasm (18, 29, 54). In addition DraG removes the ADP-ribose added by DraT which is now bound to GlnK and/or GlnB, therefore Fe-protein and MoFe-protein bind together resulting in the activation of nitrogenase enzyme, also called nitrogenase switch on (3). However, previous studies have showed that under partial nitrogen limitation conditions, a fraction of Fe-protein is kept in its ADP-ribosylated form (3, 55).

R. capsulatus has a second nitrogenase post-translational regulation system, besides the one discussed above, which is independent of ADP-ribosylation (Fig 13) (3, used with permission). The exact function of this control system is as yet unclear, however it is known that it has some elements in common with the first regulation system, such as AmtB

and PII proteins (GlnB/GlnK) (3). The presence of this regulatory system was proven by the construction of mutants lacking DraG/DraT activity. These mutants, although lacking DraT/DraG activity, demonstrated nitrogenase switch off under nitrogen repletion, indicating the presence of a second nitrogenase regulation system independent of ADP-ribosylation (56). Another study proving the existence of a second nitrogenase post-translational regulation system was carried out by making strains with mutated *nifH* allels that couldn't be ADP-ribosylated at the Arg101, which nevertheless showed nitrogenase switch of upon addition of ammonia (57).

The mechanism of switch off independent of ADP-ribosylation is extremely dependent on PII proteins (GlnK and GlnB) as well as AmtB. The hypothesis behind this regulation system is that Mo-nitrogenase is controlled by the influences on the Rnf complex, which is a specific membrane-bound complex that is necessary for transferring electrons to nitrogenase under photoheterotrophic conditions. However, supporting evidence for this have not yet been published (3).

Upon the addition of ammonia, which consequently leads to a decrease in the 2-oxoglutarate level and ATP/ADP level, deuridylylated GlnK is sequestered at the membrane in a binary complex with AmtB. The AmtB-GlnK complex recruits one or more parts of the Rnf complex which results in inactivation of electron transfer and nitrogenase switch off (3). On the other hand, under nitrogen fixing conditions GlnD-uridylyltransferase uridylylates GlnK and the Rnf complex is able to transport electrons to the Fe-protein (NifH), therefore nitrogenase is active which is called nitrogenase switch on (3).

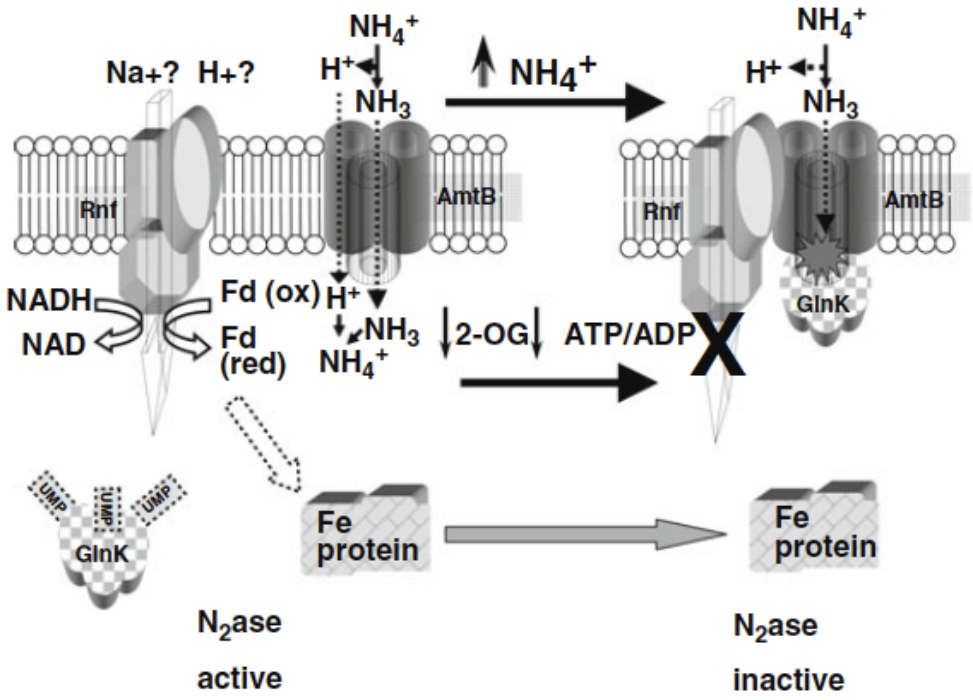


Fig 13. Nitrogenase post-translational regulation system independentf of ADP-ribosylation (3).

Chapter II

Materials and methods

Strains	Relevant characteristics	References
E. coli		
DH5α	*F⁻ φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk⁻, mk⁺) phoA supE44 λ- thi-1 gyrA96 relA1*	Invitrogen
S17.1	RP4-2-*Tc::*Mu-*Km::*Tn*7,pro,res⁻,mod⁺*,Tp^r,Sm^r	Simon et al.,1983
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [*F′ *proAB* $lacI^q$*ZΔM15* Tn*10* (Tet^r)*].*	Agilent technologies
GT1001 pRSG1	*ΔamtB* complemented *Rc-amtY*	Blakey et al.,2002
R. capsulatus		
SB1003	wild type, Rif^r	Cullen et al.,1997
RCAY63	*amtB::*Km^r	Yakunin et al.,2002
RCZC	RCAY63 carrying pZC (D335A)	This study
RCIA1	RCAY63 carrying pIA1 (D338A)	This study
RCIA2	RCAY63 carrying pIA2 (G367C)	This study
RCIA3	RCAY63 carrying pIA3 (H193E)	This study

Table 1. Relevant characteristics of the strains used in this study.

Plasmids	**Relevant characteristics**	**References**
pMECA	Ap^r, MCS in *lacZ* at *EcoRI/HindIII*, 2860 bp	Thomson J.M. & Parrott W.M.,1998
pAY98	Ap^r, Rc*glnKamtB* (2191 bp), in pMECA at *EcoRI/PstI*,4891 bp	Tremblay P.L, & Hallenbeck P.C., 2007
pJB3TC20	Ap^r , Tc^r, broad-host range vector, 7069 bp	Blatny J.M. et al,1997
pZC	Ap^r , Tc^r, Rc*amtB*D335A (2191 bp), in pJB3TC20 at *EcoRI/PstI*, 7026 bp	This study
pIA1	Ap^r , Tc^r, Rc*amtB*D338A (2191 bp), in pJB3TC20 at *EcoRI/PstI*, 7026 bp	This study
pIA2	Ap^r , Tc^r, Rc*amtB*G367C (2191 bp), in pJB3TC20 at *EcoRI/PstI*, 7026 bp	This study
pIA3	Ap^r , Tc^r, Rc*amtB*H193E (2191 bp), in pJB3TC20 at *EcoRI/PstI*, 7026 bp	This study
pIA4	Ap^r , Tc^r, Rc*amtB*W237A (2191 bp), in pJB3TC20 at *EcoRI/PstI*, 7026 bp	This study
pIMI	Ap^r , *draG* (895 bp) in pET22B (5493 bp)	This study
pPLT60	Ap^r , *draG* (895 bp) in pQE31 (3463 bp) at *BamHI/HindIII*, 3421 bp	This study

Table 2. Relevant characteristics of the plasmids used in this study.

1. Bacterial culture:

E. coli cells were grown aerobically overnight in 5 ml LB (Luria-Bertani) with ampicillin (100 µg/ml) and incubated at 37°C in a shaker (Thermo scientific, model MAXQ 5000). LB plates, also used for culturing *E. coli*, were incubated in a 37°C incubator (Canlab) overnight. Liquid LB medium consists of 10 g bacto-tryptone, 5g yeast extract and 10 g NaCl, which are mixed in 1 L of distilled water and autoclaved (58). If LB plates are needed, 15 g agar is added before autoclaving. Appropriate antibiotics are added after cooling down. Around 25 ml of LB-agar is poured into Petri dishes (Fisher brand) in a laminar flow hood (Environmental air control, INC) and left for 30 minutes to solidify and then kept at 4°C for storage.

For *E. coli* cell fractionation (which will be discussed in detail later) purposes, M9Gln (M9 glucose, 1mM glutamine) was used, which lacks ammonia and may or may not contain IPTG. In this medium, cells were incubated aerobically overnight at 30°C in a shaker (Minitron). Briefly the medium contains 200 ml of sterile M9 salts (6.4% $Na_2HPO_4^-$ $7H_2O$, 1.5%KH_2PO_4, 0.25% NaCl) mixed with 2 ml of 1 M sterile $MgSO_4$, 40 ml of 10% sterile glucose, 1 ml of 0.1M sterile $CaCl_2$, 10 ml of 100 mM sterile glutamine and finally adjusted to 1 L with distilled water (59).

For culturing *R. capsulatus*, inocolum is from a 1 ml glycerol stock which is transferred to a 17 ml screw-cap tube filled with 16 ml of YPS with appropriate antibiotics. YPS is made of 2 mM $CaCl_2$, 2 mM $MgSO_4$, 0.3% yeast extract and 0.3% peptone dissolved in distilled water and autoclaved (60, 61). This could also be done in 8.5ml screw-cap tubes. Tubes are incubated 24 hours anaerobically at 30°C in the presence of light, three 150W light bulbs, in the Biotronette Mark III Environmental Chamber (Labline instruments). In case of YPS plates, 15 g agar was added to the liquid YPS before sterilization. After cooling, antibiotics were added when necessary. The plates were poured as mentioned above for LB-agar. After growth in YPS, 1 ml of cells were transferred to screw-cap tubes filled completely with RCV + 30 mM $(NH_4)_2SO_4$+ with the necessary antibiotics (Fig 14 A). Liquid RCV consists of 6.6 ml of 60% sodium-lactate, as the carbon source, 30 ml of 0.64 M phosphate buffer pH 6.8 (sterilized separately and then added to the rest of the components, since it precipitates during autoclaving if combined earlier), mixed with 50 ml of super salts which contains 40 ml 1% EDTA, 48 ml 0.5% $FeSO_4$ $7H_2O$,

20 ml 20% $MgSO_4$, 20 ml 7.5% $CaCl_2$, Thiamine-HCl (0.1 g/100 ml) and 20ml trace elements (which contains 0.39 g $MnSO_4$ H_2O (final conc 10mM), 0.7g H_3Bo_3 (final conc 45mM) , 0.01 g $Cu(No_3)2$ $3H_2O$ (final conc 0.16mM), 0.06 g $ZnSO_4$ $7H_2O$ (final conc 0.83 mM) and 0.18 g NaMoO4 $2H_2O$ (final conc 3.28 mM) in 250 ml deionized water) (60), then it is autoclaved. If RCV plates are required, 15 g agar is added to 1 L liquid RCV, before autoclaving and the plates are poured as described earlier. Growth conditions for RCV were as mentioned for YPS. For some experiments, which will be explained later, one more step was performed, consisting of transferring 1 ml of RCV+30 mM $(NH_4)_2SO_{4+}$ to a screw-cap tube, filled to the top with RCV lacking ammonia, with similar incubation conditions. RCV tubes could be kept in the environmental chamber for renewal or future usage. In case of RCV plates with streaked *R. capsulatus* (Fig 14 B), they were kept in an anaerobic jar in the presence of a gas pack (BD BBL GasPack Plus Anaerobic system Envelope with palladium catalyst) to remove the oxygen and optimize growth conditions.

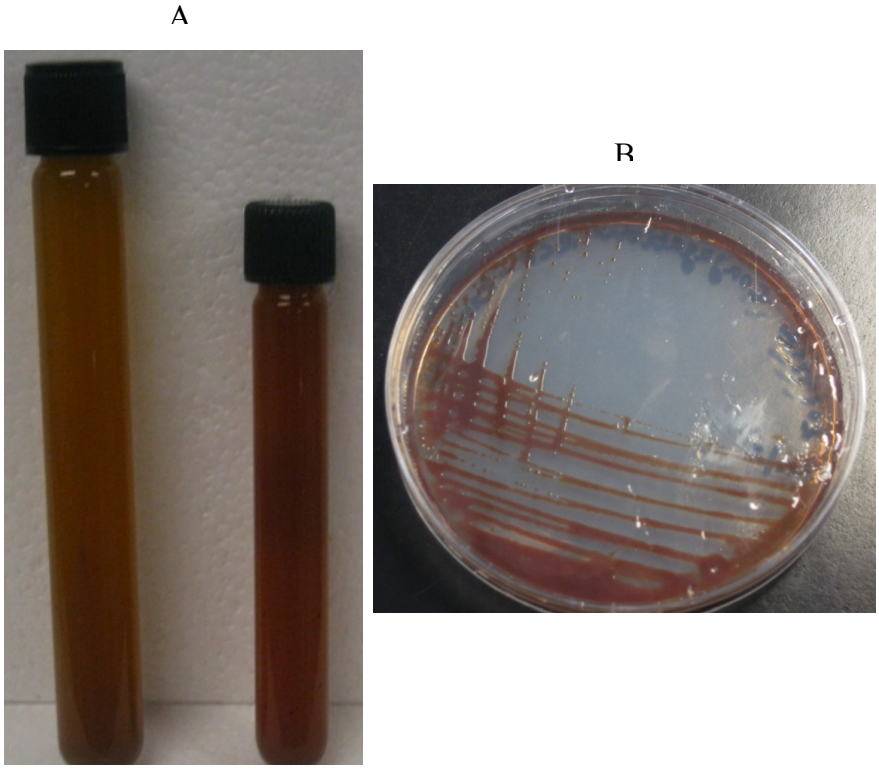


Fig 14. *Rhodobacter capsulatus* culture in (A) RCV liquid and (B) RCV plate

2. Small-scale plasmid preparation:

A chemical protocol (explained later) or two different mini-prep kits, QIAprep spin

miniprep kit and Spinsmart Smart™ Plasmid miniprep DNA purification kit, were used to isolate plasmid DNA. It has to be mentioned that they all showed about the same efficiency. The concentration of DNA was measured using a NanoDrop spectrophotometer (ND-1000).

The chemical Mini-prep protocol starts with inoculating the desired colony into LB containing the appropriate antibiotics and incubating overnight at 37°C with agitation. The cells were harvested by centrifugation (Adams dynamic centrifuge), 10,000 rpm for 5 min, and the supernatant discarded. The cell pellet was resuspended in 100 µl TEG (25 mM Tris-Cl pH 8, 10 mM EDTA pH 8 and 50 mM glucose) which contains 2 mg/ml lysozyme, and transferred to a sterile eppendorf tube and kept on ice for 10 min. 200 µl SDS/NaOH (0.2 M NaOH and 1% SDS) was added to the eppendorf and mixed gently and incubated on ice for 10 min. 150 µl of 3 M sodium acetate pH 4.8 was added while mixing gently followed by incubation at -20°C which causes a white precipitate to form. It was then centrifuged (Sigma, rotor 80301) for 20 min at 12,000 rpm at 4°C. The supernatant was poured into new eppendorf tubes, previously filled with cold 100% ethanol, and incubated at -20°C for 10 min. DNA was pelleted by centrifugation for 15 min, 15,000 rpm at 4°C. Ethanol is removed and the DNA pellet is rinsed with 0.5 ml cold 70% ethanol. In the next step, ethanol was removed by inversion and the DNA pellet was dried by leaving the eppendorf inverted for 5 min. DNA was dried in an additional step under vacuum, using a Speed-Vac (UNIEQUIP, model UNIVAPO 100H) for 10 min. Finally the pellet was resuspended in 25µl of deionized water containing 100µg/ml DNase free Rnase A and stored at -20°C.

3. Site directed mutagenesis by PCR:

In order to better understand the importance of a number of conserved amino acid residues in AmtB pore, several mutants were constructed in our lab by site-directed mutagenesis. Two primers were designed in such a way that they anneal back to back on the template plasmid, with the forward primer containing the desired mutation and a reverse complement primer with no mutations involved. In the PCR reaction, pAY98 (Table 2) was used as the template which was amplified with the Phusion high-fidelity DNA polymerase (New Englands Biolabs). This enzyme was used for PCR reactions because of its low error rate. This enzyme has 5′→ 3′ polymerase activity, 3′→ 5′ exonuclease activity and its final product is blunt-ended. I constructed RcD335A mutant via site directed mutagenesis, as

explained below.

The primers (from Alpha DNA) used for site directed mutagenesis are shown in below, with the bold letter being the mutation. The AmtB sequence of pAY98 is seen in figure 15, indicating the positions of forward and reverse primers In this mutant, the aspartate residue 335 in *R. capsulatus* AmtB was converted to alanine, which is underlined in the forward primer, to investigate its importance in AmtB functionality in transporting ammonia and nitrogenase switch off/on effect and making a complex with GlnK.

Forward primer:
5' GTTCAAATACGAC<u>G**C**C</u>AGCCTGGACG 3'
Reverse complement primer:
5' ATCGCCTTCACCTTGGTGACGAA 3'

The PCR reaction mixture contained; 10µl of 5X Phusion GC Buffer, with 0.2 mM dNTP, 0.5 µM of each primer (forward and reverse), 50 ng DNA (in this case pAY98), 1.5 µl DMSO, 27.5 µl deionized water and 1 µl of the Phusion enzyme, in a sterile PCR tube. The PCR machine (PERKIN ELMER, Gene Amp, PCR system 2400) was programmed for 30 cycles with an initial denaturation step at 98°C for 30 seconds, followed by an additional denaturation at the same temperature for 10 seconds. The program continues with a 65°C annealing step for 30 seconds followed by an extension step at 72°C for 3 minutes and a final extension stage at the same temperature but for 10 minutes (as recommended in the phusion high-fidelity DNA polymerase catalog).

5'ATGAACAATCTGACCAAACTGACGGGTCTTGTGGCGGCGCTGGCTGCTGCGGCCCTGC
CTGCCTTTGCGCAGGAAGCGGCCGCTCCGGTCGCCGAAGCCGTTGCCACCGTCACCGA
AGCCGCGGCGCCGATCGTCGACAAGGGCGATGTCGCCTGGATGATGACCTCGACGCTTC
TTGTGCTGTTCATGATCATTCCGGGCCTGGCGCTTTTCTACGGCGGTCTGGTGCGCAGCC
AGAACATGCTCTCCGTGCTGATGCAGACGACGATGATCACCTCGGTGGTGATGATCGTCT
GGGTGCTTTGGGGCTATTCCTTCGCCTTTGGCGGCGGCACCAACCCGTTCTGGGGCGGT
CTGGGCAAGGTCTTCCTGGCCGGCGTGACCGGCGACAGCCTGGCGGCGACCTTCACCG
ATGGCGTGATGCTGCCGGAATATGTGTTCATCGCCTTCCAGATGACCTTTGCCGCGATCA
CGCCCGCGCTTTACGTGGGCGCCTTTGCCGAGCGGATGAAATTCTCGGCGGTGATCCTC
TTCACCGTGCTTTGGGTCACCGTGGTCTATTTCCCGATCGCCCACATGGTCTGGGATGCC
TCGGGTCTGATCTTCAACTGGGGCGCCATCGACTTTGCCGGCGGCACCGTGGTGCATATC
AACGCCGGGATCACCGGTCTGATGGCGGCGATCGTTCTGGGGCCGCGCGTCGGTTTCGG
CCGCGAGAACATGGCCCCGCATTCGATGACGCTGACCATGGTGGGCGCGATGATGCTCT
GGGTCGGCTGGTTCGGCTTCAACGCCGGGTCCAACCTTGAGGCGACCTCGGGCGCGAC
GCTGGCGATGCTGAACACCTTTGTTGCCACCGCCGCGGCCGTCGTCAGCTGGTCGGCCA
CCGAAGCGCTGTTCCGCGGCAAGGCCTCGGGTCTGGGCGCGGCTTCGGGCATGGTCGC
CGGTCTGGTGGCGATCACCCCGGCCTGCGGCACCTCGGGCCCGGTCGGCGCGATCCTGC
TTGGCCTGATCGTCTCGCCGGTGTGCTACTTCTTCGTCACCAAGGTGAAGGCGATGTTCA
AATACGACGACAGCCTGGACGTGTTCGGCGTGCATGGCATCGGCGGGATCGTCGGCGCG
GTGATGACGGGCGTTCTGATGGCCCCCGGCTTCGGCGGCACCGGCGGCGACGATTTCTC
GATCGTGTCGCAGGTGATCATCCAGATCAAGGCGGTCGTCGTGACCATCGCCTGGGCGG
GGATCGGCTCGATCATCCTTCTGTACATCGTCAAGGCCGTCACCGGCCTGCGCGTGGCC
ACCGATGACGAACGTCAGGGCCTTGACCTGACGACCCATGGCGAAAGCGCCTACCACT
CGTAA3'

Fig 15. The original sequence of AmtB protein related to pAY98 (PCR template) and the positions of the forward and reverse primers. The position of forward primer, which hybridizes to the antisense strand, is highlighted in green (the nucleotide to be mutated is shown in bold) whereas the position of reverse primer, which hybridizes to the sense strand, is highlighted in blue. The arrows indicate the direction of polymerization for each primer.

The PCR product was digested with DpnI and migrated on agarose gel. The use of DpnI, which cuts methylated DNA, is advantageous in the PCR site-directed mutagenesis protocol used here, where the parental pAY98 should be digested while the newly synthesized mutated strands remain intact. The correct band was excised, extracted, and then ligated. The product of ligation was introduced into DH5α (Table 1) (competent cell preparation is explained in section 8 of materials and methods), and plasmid DNA was extracted from transformed colonies, followed by sequencing to verify the mutation. Purified plasmid went underwent double digestion with PstI and EcoRI in order to recover the mutated Rc*glnKamtB*D335A fragment and cloning it into PstI and EcoRI sites of PJB3TC20 (Table 2). Two final steps were transformation into S17.1 (Table 1) and conjugation in RCAY63.

4. Digestion:

Double digestion with with PstI and EcoRI was performed by transferring 5µl of 10X NEBuffer 3 to an eppendorf adding deionized water (calculated to bring final volume to 50µl), along with 0.5µl 100X BSA from NEB **,** 1µg DNA and finally 1µl of each EcoRI (NEB) and PstI (NEB). The eppendorf is incubated at 37°C for 1 hour in order and then placed at 80°C for 20 minutes to inactivate the enzymes.

In case of DpnI digestion, for each 25µl of PCR product, 3µl 10X NEBuffer 4 and 1µl DpnI (NEB) were mixed in an eppendorf and incubated at 37°C for 1 hour and inactivated for 20 minutes at 80°C.

5. Agarose gel electrophoresis:

Agarose gel electrophoresis was performed after mini preps and restriction enzyme digestions for verifying their accuracy. Also it was carried out as a prerequisite step in purifying DNA from agarose gel, which will be explained in the following section.

0.8% agarose gels were made by weighing the appropriate amount of agarose (Multicell agarose D1-LE, WISENT INC) and mixing it with 1X TAE buffer (0.48% Tris-

base, 1.14 ml glacial acetic acid, 0.037% EDTA and the volume is adjusted to 1L to have 50X Tris-Acetate-EDTA buffer). The mixture was microwaved for 1 minute to disssolve the gel. Meanwhile, the gel casting tray was sealed with two special rubber gaskets and placed in the electrophoresis chamber (Thermo scientific, OWL, Easy Cast B1A) and a comb was also put in the tray. When the melted gel cooled, ethidium bromide (0.5 µg/ml) was added to the agarose-TAE mixture (only in case of verification, but in case of purification the gel was stained with ethidium bromide after migration). Ethidium bromide is used, since it is fluorescent when exposed to UV, especially when attached to DNA. Then agarose gel was poured in the gel casting tray and left until polymerized. The comb was then removed and 1X TAE buffer was poured in the electrophoresis chamber till it covered the gel. DNA samples were mixed with 6X blue loading dye, before loading in the wells. Along with the samples, a marker (1 kb DNA ladder from NEB) was loaded in a separate well. Finally the electrophoresis box was connected to 100V current via two electrodes, which moves the DNA towards the positive electrode. After migration, gels were exposed to UV (by MultiImage light cabinet, Alpha Innotech corporation) in order to take a picture or to be cut for DNA extraction.

6. DNA extraction from agarose gel:

When pure DNA was needed, such as when the PCR product was digested with DpnI and had to be purified from agarose gel (in order to get rid of impurities and excess of oligonucleotide primers) prior to its transformation in DH5α, also before ligating the mutated Rc*glnKamtB*D335A in EcoRI and PstI sites of PJB3TC20, the DNA samples were run on 0.8% agarose gels. Ethidium bromide wasn't added to the gel prior to migration, but after migration, the gel was soaked in an ethidium bromide bath (0.5µg/ml ethidium bromide in 100ml water) for 5 minutes and rinsed with water for an additional 5 minutes to wash out the excess ethidium bromide. The gel was visualized on a UV transilluminator (fotodyne) and the corresponding band, according to the marker, was cut and placed in an eppendorf. DNA was extracted from the gel by the QIAquick gel extraction kit according to

the manufactere's instructions and stored at -20°C. The concentration was measured using a Nanodrop spectrophotometer.

7. Ligation:

After each digestion comes a ligation step, since it has been proven that circular DNA is much better taken in by competent cells than linear ones during transformation. Here two kinds of ligation were performed, one to relegate the DpnI digested PCR product before transformation into DH5α, the other to ligate the mutated Rc*glnKamtB*D335A into the EcoRI and PstI sites of PJB3TC20 prior to transformation in S17.1. For the former reaction, T4 ligase (NEB) along with its 10X buffer (NEB) was used overnight in a cooler (Microcooler, BOEKEL industries. INC, model 260011) at 16°C. Whereas for the latter, a 4:1 molar ratio of insert to vector (~10 ng vector) was mixed with T4 ligase and its appropriate buffer and incubated under the same conditions.

8. Competent cell preparation:

In order to make chemically competent cells, 100 µl of glycerol stock of the desired cells, from -80°C, were inoculated in 500 ml of LB without antibiotic. Cells were incubated in a 37°C shaker until they reached an OD_{600} of 0.3 to 0.4. Cells were harvested by centrifugation at 5000 rpm at 4°C for 10 minutes in a Sorval GSA rotor, in 250 ml centrifuge bottles. The supernatant was discarded and the pellets were placed on ice and resuspended in 1/4 volume of ice cold 100 mM $MgCl_2$ (125ml). Cells were centrifuged at 4000 rpm for 10 minutes in the same conditions. The supernatant was decanted and the cell pellets were placed on ice and resuspended in 1/20 volume of ice cold 100mM $CaCl_2$ (25 ml). An additional 9/20 volume of $CaCl_2$ (225 ml) was added to the suspension and kept on ice for 20 minutes. The cell suspension was centrifuged at 4000 rpm and the cell pellets were resuspended in 1/50 volume of ice cold sterile 85 mM $CaCl_2$ in 15% glycerol W/V (10 ml), dispensed in 100 µl aliquots, and frozen at -80°C.

9. Transformation:

DpnI digested PCR product (pAY98-RcAmtBD335A) was introduced into DH5α. The *glnKamtB*D335A fragment, ligated in PJB3TC20 (making a new plasmid called pZC, described in table 2), was introduced into DH5α before transformation into S17.1. In addition, pPLT60 (Table 2) and pIMI (Table 2) were introduced into XL1-Blue (Table 1). For these transformations, appropriate competent cells were removed from -80°C and kept on ice to thaw. The cells were briefly mixed, and 100µl of cells were removed. 1-50ng of DNA (no greater than 10µl) was added to the competent cell tube and mixed gently. Tubes were then placed on ice for at least 10 minutes. Afterwards the cells were heat-shocked for 45-90 seconds at 42°C and then placed on ice for 2 minutes. 800µl LB was then added to the tube which was incubated for 1 hour at 37°C with shaking followed by pelleting the cells by centrifugation (Sigma, rotor 80301) at 1400 rpm for 1 minute. The cells were then resuspended in 100 µl of LB liquid and plated on LB plates containing the appropriate antibiotics. The plates were incubated overnight at 37°C and stored at 4°C afterwards.

10. Sequencing:

Sequencing was performed after pJB3TC20-RcAmtBD335A, pZC, had been introduced into DH5α, to verify the correct introduction of the desired mutation. For this purpose, a mini-prep was carried out from the transformation plate and sent for sequencing to the IRIC Genomics platform. Two forward primers (made by Alpha DNA), which anneal to the *amtBglnK* sequence, were used to sequence *amtB* in pZC:

First forward primer:
5' TGT AAG AAC CAC AGG GGA AAC 3'
Second forward primer:
5' TTC GAT GAC GCT GAC CAT GG 3'

11. Cloning Rc*glnKamtB*D335A in PJB3TC20:

In order to clone Rc*glnKamtB*D335A in pJB3TC20, to have pZC (pJB3TC20-RcAmtBD335A), pAY98- RcAmtBD335A was digested with EcoRI and PstI, and the Rc*glnKamtB*D335A fragment was excised from an agarose gel, purified and ligated into the same restriction enzyme sites in pJB3TC20. This plasmid possesses the transfer gene (*tra*) which is responsible for the production of the pili required for the physical contact between cells for conjugal transfers (63, 64). Introduction of pZC into S17.1, which possesses a mobilization gene (*mob*) a critical gene required for plasmid mobilization and its involved in the transfer to *R. capsulatus*. Both *tra* and *mob* are essential for a plasmid transfer by conjugation (64).

12. Conjugation:

S17.1 containing pZC was conjugated with RCAY63. S17.1/ pZC was inoculated in 5 ml LB containing tetracycline (10µg/ml) and incubated overnight at 37°C aerobically while shaking. In addition, strain RCAY63 (Table 1), a *R. capsulatus* strain, was cultured in YPS containing kanamycin (10 µg/ml) over night at 30°C in 17ml screw-cap tubes phototrophically. After growth, 50 µl of each culture was mixed together and spread on 2 cm^2 of the center of a YPS plate with no antibiotics and incubated overnight in the dark at 30°C. A bacterial suspension was then recovered with 1 ml RCV and no antibiotics. Serial dilutions were made and were spread on RCV plates with kanamycin (10µg/ml) and tetracycline (1.5 µg/ml). Plates were incubated anaerobically (gas pack) at 30°C and incubated in the presence of light in the Biotronette Mark III Environmental Chamber (Labline instruments) until the appearance of red colonies on the plates. Afterwards, these colonies were grown in YPS tubes with tetracycline (1.5µg/ml). After growth, the culture was transferred to RCV+30mM $(NH_4)_2SO_{4+}$ tubes with tetracycline and kept at 30°C with light for further experiments.

13. Determination of nitrogenase activity, switch on/off effect:

This experiment was carried out to observe the effect of the site directed mutations on nitrogenase activity, which was measured using gas chromatography (GC). In this technique, acetylene (C_2H_2) reduction leads to the production of ethylene (C_2H_4) which is an indicator for nitrogen fixation and in turn nitrogenase activity (66). SB1003 (Table 1) strain used as a positive control for the switch off effect and RCAY63 was the negative control. Mutants (Table 1) RCZC (D335A), RCIA1 (D338A), RCIA2 (G344C), RCIA3 (H193E) and RCIA4 (W237A) were investigated for nitrogenase activity by gas chromatography.

Cells were grown in 17 ml screw cap tubes in RCV+30mM $(NH_4)_2SO_4^+$. One ml of the culture was transferred to another screw cap tube filled with RCV without NH_4^+ for 18-24 hours, in order to induce nitrogenase. To anaerobic 25 ml vials that were previously sealed and filled with argon, using a stream of argon for 10 minutes, 5 ml of the culture (RCV lacking NH_4^+) was transferred without introducing oxygen (Fig 16). This was achieved by using a 10 ml glass syringe with a long 16 or 18 gauge needle and filling it from the bottom portion of the culture, which was less exposed to oxygen.

Fig 16. 25 ml anaerobic vials containing 5 ml liquid cultures

These vials were preincubated at 30°C in the light for 15 minutes along with agitation. Afterwards, 2 ml of acetylene was added to the vials and the amount of produced ethylene was measured by injecting 50 µl of the gas phase in the gas chromatograph (GC-8A (Shimadzu)), every 5 minutes. Liquid samples were also taken at the same time points for Western-blotting to check ADP-ribosylation of nitrogenase Fe-protein. After 20 minutes, 300 µM of NH_4Cl was added to the vials and sampling was continued every 5 minutes for the next 30 minutes, liquid samples were also taken at each time point. Liquid samples were kept in 3X SDS PAGE sample buffer and stored at -20°C. The total amount of ethylene was calculated at each time point and the state of nitrogenase modification was determined by western- blotting of the liquid samples.

14. Cell fractionation:

Cell fractionation was carried out in order to check the presence of GlnK in the membrane after an ammonium shock in RCIA3, RCZC, SB1003, RCAY63 and *E. coli* GT1001 pRSG1 (Table 1). The detection of GlnK in cytoplasmic and membrane fractions was done by western blotting via anti-GlnK antibody.

R. capsulatus strains were each grown in duplicate photoheterotrophically in the absence of oxygen, in 320 ml of RCV with appropriate antibiotics except for SB1003, lacking ammonium source (NH_4Cl) to their early stationary phase. To one of the two cultures, 1 mM NH_4Cl was added and both were incubated an additional 15 minutes under the same conditions. Cells were then harvested by centrifugation at 10,000 rpm at 4°C for 30 minutes in a Sorval GSA rotor. The supernatant was discarded and the cell pellets were resuspended in 10 ml of sodium-phosphate buffer pH 7. The suspension was sonicated on ice 5X 30 seconds with 30 seconds pause between each burst. The extract was centrifuged at 10,000 rpm at 4°C for 10 minutes and the supernatant was centrifuged by ultracentrifugation in a Beckman (70,000 rpm, type 70.1 TI) rotor at 250,000 xg for 45 minutes. Keeping the first 1 ml of the supernatant gave the cytoplasmic fraction, which was then frozen at -80°C. The remaining supernatant was removed. The pellet containing the

membrane was resuspended in 10ml of sodium-phosphate buffer pH 7 and recentrifuged at 4°C for 45 minutes at 250,000 xg. The pellet contains the membrane portion which was resuspended in 1ml of sodium-phosphate buffer pH 7 and frozen at -80°C (59).

E. coli GT1001 pRSG1 was precultured in 5 ml LB with ampicillin by incubating at 37 °C overnight. And used to inoculate in 500 ml (1/100 dilution) M9Gln (M9 glucose, 1mM glutamine) lacking ammonia with and without IPTG (59). The cells were incubated on a shaker at 30°C overnight until they reached to an OD_{650} of 1.3-1.4. The culture was then divided to two 250 ml and 30 mM NH_4Cl was added to one of them, while incubating both for another 15 minutes. Cells were harvested by centrifugation at 5000 rpm at 4°C for 10 minutes in a Sorval GSA rotor. The supernatant was decanted and the pellet resuspended in 10 ml of sodium-phosphate buffer pH 7. From this stage on, cells were treated as for *R. capsulatus* strains (59).

15. Bradford assay:

The total protein concentration of samples (membrane and cytoplasmic fractions, DraG induced and non-induced samples) was measured by Thermo Scientific Coomassie Plus (Bradford) protein assay reagent and Bradford reagent Biotechnology grade from Bioshop. Bovine serum albumin (BSA) was used as a standard. Standards and samples were read at 595 nm (UV-VIS UV-2101PC, Shimadzu).

16. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE):

This technique is widely used to separate proteins due to their molecular weight and not the electrical charge. Since the SDS, which is an ionic detergent, used in this process binds to proteins and denatures them and imparts a negative charge to all, they would all be negatively charged. The required gels in order to separate protein samples are 15% resolving and 4% stacking gel. The former is constituted of 2.3 ml distilled water, 5 ml

acrylamide/bis-acrylamide (30:0.8), 2.5 ml Tris 1.5 M pH8.8, 100 µl SDS 10%, 100µl ammonium persulfate (APS) 10% and 10 µl tetramethylethylenediamine (TEMED), while the latter consists of 3 ml distilled water, 0.67 ml acrylamide/bis-acrylamide (30:0.8), 1.25ml Tris 0.5 M pH6.8, 50 µl SDS 10%, 50 µl ammonium persulfate (APS) 10% and 5 µl tetramethylethylenediamine (TEMED). The migration buffer used for running the SDS PAGE electrophoresis is made of 4 mM Tris, 39 mM glycine, SDS 0.1% and distilled water. Sample preparation was done by adding 3X SDS PAGE sample buffer (3 ml of 0.63 M Tris pH 6.8, 3 ml SDS 20%, 3 ml glycerol 100%, 30µl bromophenol blue 1% and 1.5 ml Mercaptoethanol) and incubating at 100°C for 10 minutes. A "PageRuler unstained protein ladder" from Thermo scientific or in some cases protein ladder (10-250 kDa from NEB was used as markers, However a "prestained protein marker broad range" from NEB, was used specially when SDS PAGE was followed by a western blot. Samples were loaded on the gel and migrated at 200V for around 1 hour. The Bio-Rad SDS PAGE apparatus "Mini protein II electrophoresis cell" was used for SDS PAGE.

17. Western blotting:

After protein separation by SDS PAGE, the desired protein bands such as *E. coli* GlnK, *R. capsulatus* GlnK, DraG and Fe-protein, were visualized by western blotting and the appropriate antisera; anti EcGlnK, anti RcGlnK, anti DraG, anti 6X histidine, and anti Fe-protein. For this purpose, separated proteins were transferred to a PVDF-plus transfer membrane (GE water & process technologies) which was soaked in methanol, distilled water and transfer buffer (4 mM Tris, 39 mM glycine, SDS 0.1%, Methanol 20% and distilled water), with a 16V electrical current over night. The transfer process was carried out by using a "Mini trans blot cell" apparatus of Bio-Rad.

When the prestained protein marker was observed on the membrane, the transfer process had come to an end. The membrane was then incubated in "Tris buffered saline" (TBS), 25 mM Tris pH 7.4, 0.15M NaCl and distilled water, for 5 minutes along with agitation. This was followed by two 5 minutes incubation in "Tris buffered saline with

Tween20" (TBST), TBS+0.1% Tween20, on a shaking platform. The membrane was incubated in blocking solution (TBST+ 5% milk powder) for 30 minutes with agitation. It was then washed twice for 5 minutes by incubation in TBST with agitation. This was followed by a 2 hour incubation with the appropriate primary antibody (rabbit IgG anti-EcGlnK, anti-RcGlnK, anti-DraG or anti-6X histidine, and anti-Fe-protein antibody), diluted 1/5000, in blocking solution (TBST+ 0.5% milk powder) along with shaking. This step was followed by two 5 minutes TBST washes and then an hour and a half of incubation with the secondary antibody (antibody against rabbit IgG coupled with peroxidase), diluted 1/25000, in blocking solution (TBST+ 0.5% milk powder) along with agitation. This final incubation was followed by three washing steps, twice with TBST and once with TBS. The fixed proteins on the membrane were visualized using the Mandel Scientific LumiGlo Chemiluminescent substrate system and exposure to Classic blue autoradiography BX film.

18. Overexpression of DraG protein:

In order to produce proteins to make anti-DraG antibodies, plasmids containing a His-tagged DraG (pIMI and pPLT60) were introduced into XL1-Blue competent cells and expression of DraG was induced with IPTG. Attempts were made to purify DraG using the 6x His-tag in order to make antibody against it to be used for further investigations. (67), 10ml LB with Amp (100 µg/ml), in a sterile 50ml flask, was inoculated with a single colony and incubated at 37°C overnight on a shaking platform. To 100ml of prewarmed LB with antibiotic, in a 500ml flask, 5ml of the overnight culture was inoculated and grown at 37°C with vigorous shaking until an OD_{600} of 0.6 was obtained. Then 1ml of that culture was centrifuged and the cell pellet was resuspended in 3X SDS PAGE sample buffer and stored at -20°C for later SDS PAGE analysis. To the rest of the culture, 1 mM of IPTG was added to overexpress DraG protein. The culture was incubated for 4 to 5 hours at 37°C on a shaking platform. Following incubation, another 1ml sample was taken and pelleted, then

resuspended in 3X SDS PAGE sample buffer an stored at -20°C for future SDS PAGE analysis. Cells were harvested by centrifugation in a Sorval GSA rotor at 5000 rpm for 20 minutes at 4°C. Cell pellets were frozen at -20°C, in order to be purified later.

19. Standard sample preparation to isolate native protein (DraG):

Samples were prepared for protein purification using the protocol of Talon metal affinity resins user manual (68). The cell pellet was resuspended in 2ml of chilled 1X equilibration/wash buffer pH 7 (50 mM sodium phosphate and 300 mM NaCl) and incubated it at room temperature for 20 to 30 minutes. The suspension was then sonicated (3X 30 seconds with 30 seconds pause between each burst) and centrifuged in a Sorval GSA rotor at 11,500 rpm for 20 minutes at 4°C, to pellet any insoluble material. The clarified sample was transferred to a clean tube after reserving a small portion of it at 4°C for SDS PAGE analysis.

20. Batch/gravity-flow column purification of DraG protein, using TALON Resin:

TALON Resin (TALON metal affinity resin, Clontech) was used for protein purification (68). In this method of purification, the desired protein, which contains a 6X-His tag, binds to the nickel ions present in the resin. During the washes, untagged proteins are washed from the column while the tagged ones remain bound to the column. To elute the desired protein, imidazole elution buffer is added to the column. Imidazole competes with the polyhistidine tag in binding to the column, so the tagged proteins elute. For protein purification purposes, the resin was throughly resuspended and the required amount was transferred to a sterile centrifuge tube. It was then centrifuged in a Sorval GSA rotor at 3000 rpm for 5 minutes at 4°C to pellet the resin and the supernatant was discarded. The resin was resuspended in 10 bed volumes of chilled 1X equilibration/wash buffer pH 7 and recentrifuged under the same conditions. Once more the resin was resuspended and

centrifuged. The clarified sample was added to the resin and gently agitated at room temperature for 20 minutes on a platform shaker to let the polyhistidine-tagged protein bind to the resin. The supernatant was removed after centrifugation at 3000 rpm for 5 minutes at 4°C. The resin was then washed with 10-20 bed volumes of 1X equilibration/wash buffer pH 7 with agitation at room temperature for 10 minutes at room temperature. Afterwards, the suspension was centrifuged at 3000 rpm for 5 minutes and the supernatant was discarded. The last washing and centrifugation was repeated. The pellet was resuspended by vortexing in one bed volume of 1X equilibration/wash buffer pH 7. The suspension was transferred to a gravity-flow column with an end-cap in place, and the resin was allowed to settle. Then the end-cap was removed and the buffer was drained until it reached the top of the resin bed. The column was washed with 5 bed volumes of 1X equilibration/wash buffer pH 7. The polyhistidine-tagged protein (DraG) was eluted by the addition of 5 bed volumes of 1X elution buffer pH 7 (50 mM sodium phosphate, 300 mM NaCl and 150 mM imidazole) to the column. The eluted fractions were collected in 500µl portions and stored at -20°C for SDS PAGE and western blot analyzes.

Chapter III

Results

1. Probing the role of AmtB in nitrogenase regulation in *R. capsulatus* through site-directed mutagenesis:

1.1 Creating AmtB site directed mutant D335A (RCZC):

In order to better understand the importance of some conserved amino acid residues in AmtB protein's functionality in the regulation of nitrogenase enzyme, a number of mutants were constructed in our laboratory via site directed mutagenesis. Mutant RCZC has a point mutation in aspartic acid residue 335 (D335) of *R. capsulatus* AmtB, that is encoded by the *glnKamtB* operon. This mutation converts the aspartic acid residue (GAC) to alanine (GCC), by changing the middle adenine (A) nucleotide to cytosine (C), which was done by "site directed mutagenesis" achieved by PCR.

The DNA used as a template for PCR was pAY98 (4897 bp), a plasmid carrying the *R. capsulatus glnKamtB* operon (2181 bp) between EcoRI and PstI restriction sites. Two primers were used for the PCR reaction, one with the point mutation which would anneal to the non-coding DNA strand (Forward primer) and the other without any mutation that anneals to the coding strand (Reverse complement primer). After PCR and digestion with DpnI (To reduce the number of non-recombinant clones, since DpnI only cuts dam methylated DNA which would be in the parental DNA plasmid) it was electrophoresed on an agarose gel in order to check the correctness of the size of the PCR product and to extract the DNA for transformation into DH5α (on LB+Ampicillin plates). As depicted in figure 17 and compared to the 1 kb NEB DNA ladder, the PCR product (pAY98-RcAmtBD335A) is the size expected, almost 5 kb.

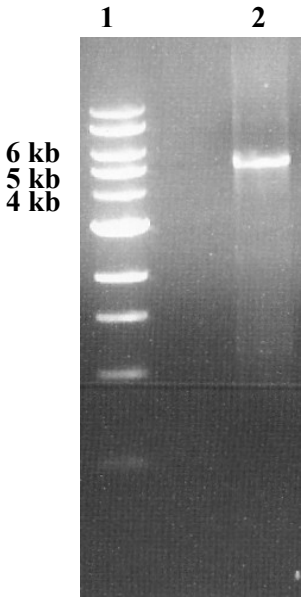


Figure 17. DpnI-digested PCR product on 0.8% agarose gel.
Lane number 1 is 1 Kb NEB DNA ladder and number 2 represents pZC

After transformation, DNA plasmid was extracted from the transformances. An aliquot of the purified plasmid was sequenced to verify that the mutation was in one correct location. Part of the *amtB* sequencing result containing the mutated nucleotide is shown in below, with the underlined codon coding for alanine and the letter in bold representing the mutated nucleotide. The wild type sequence is also given for comparison purposes. The results confirmed the change of adenine (A) to cytosine (C) and consequently the conversion of aspartate residue 335 (D335) to alanine.

Wild type:
TTCGTCACCAAGGTGAAGGCGATGTTCAAATACGAC<u>G**A**C</u>AGCCTG
D335A mutation:
TTCGTCACCAAGGTGAAGGCGATGTTCAAATACGAC<u>G**C**C</u>AGCCTG

After sequence confirmation, the DNA was double digested with EcoRI and PstI and subjected to gel electgrophoresis. This gave two closely situated bands (Fig 18). The top band was around 2.8 kb (precisely 2706 bp) while the lower one was nearly 2.2 kb (exactly 2191 bp), representing part of the pAY98 plasmid and the Rc*amtBglnK*D335A portion of the plasmid respectively. The smaller band (2191 bp) was cut and extracted for ligation into pJB3TC20.

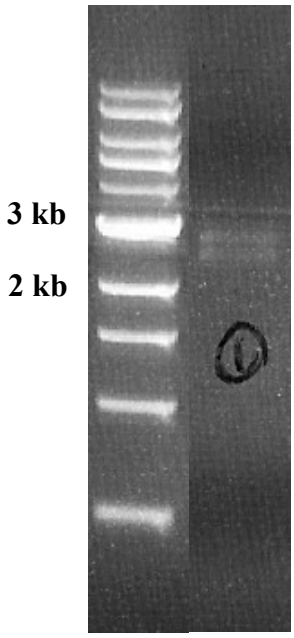


Figure 18. Double digested pAY98-RcAmtBD335A sample on 0.8% agarose gel.
well number 1 is 1 kb NEB DNA ladder whereas number 2 shows the digested sample

Ligation required the double digestion of plasmid pJB3TC20 with the same restriction enzymes. To verify the size of plasmid pJB3TC20 and its proper digestions, the sample was run on agarose gel (Fig 19) prior to ligation with the mutated AmtB fragment. This plasmid is 7069 bp and when digested with EcoRI and PstI only one band at 7026 bp was observed since the other band is so small (43 bp) that it can't be visualized on the gel.

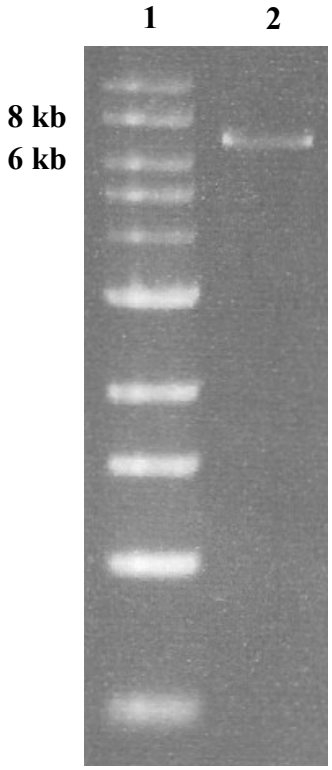


Figure 19. Double digested pJB3TC20 sample on 0.8% agarose gel.
well number 1 is 1 kb NEB DNA ladder while number 2 shows double digested pJB3TC20

Double digested pJB3TC20 and the extracted 2191 bp AmtB fragment were ligated together with T4 ligase overnight and then introduced in DH5α (on LB+tetracycline or ampicillin plates) before another transformation in S17.1 (on LB+tetracycline plates). To confirm that the transformed colonies contained both vector and insert, mini prep were made, with EcoRI and PstI, and run on agarose gels. As observed in figure 20, two bands were visualized on the gel, one being the size of the vector (7026 bp) and the other presenting the right size for the AmtB fragment (2191 bp). Therefore we proceeded to the final step which was conjugation in *R. capsulatus* RCAY63 which lacks AmtB.

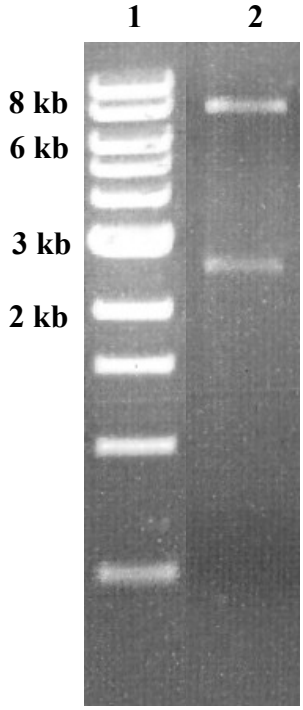


Figure 20. double digested pZC sample on 0.8% agarose gel.
Lane number 1 is 1 kb NEB DNA ladder and number 2 shows double digested pZC.

1.2 Regulation of nitrogenase activity in RCZC, RCIA1, RCIA2, RCIA3 and RCIA4 mutants:

Regulation of nitrogenase activity in *R. capsulatus* strains SB1003 (wild type, positive control), RCAY63 (lacking AmtB, negative control), RCZC (D335A), RCIA1 (D338A), RCIA2 (G344C), RCIA3 (H193E) and RCIA4 (W237A) was studied by gas chromatography, as described in section 13 of methods and materials. Cells were grown in RCV+30mM $(NH_4)_2SO_4^{+}$ overnight and transferred to RCV without ammonium source in order to induce nitrogenase expression, until the next day when hydrogen bubbles appear in the culture tube. Then 5 ml of the cultures were injected into an anaerobic vial, pre-filled with argon. The absence of oxygen is critical, since nitrogenase enzyme is extremely oxygen sensitive. Vials were preincubated for 15 minutes and after the 15th minute 2ml of acetylene were injected into the vials and the amount of ethylene produced was measured every 5 minutes. An ammonium shock was given at the 20th minute and its influence on nitrogenase activity was followed by continuing to measure the amount of ethylene every 5 minutes. An effect, i.e. nitrogenase switch-off would be seen as an inhibition in ethylene

production.

The nitrogenase assay carried out with the wild type strain SB1003 (Fig 21) shows an linear increase in the amount of ethylene produced with time before the addition of ammonium. However after ammonium addition, the total amount of ethylene remains almost constant, starting from the 25th minute. This pattern is caused by nitrogenase inactivation or so called nitrogenase switch off, which proves the presence of nitrogenase regulation in SB1003 (positive control), as expected.

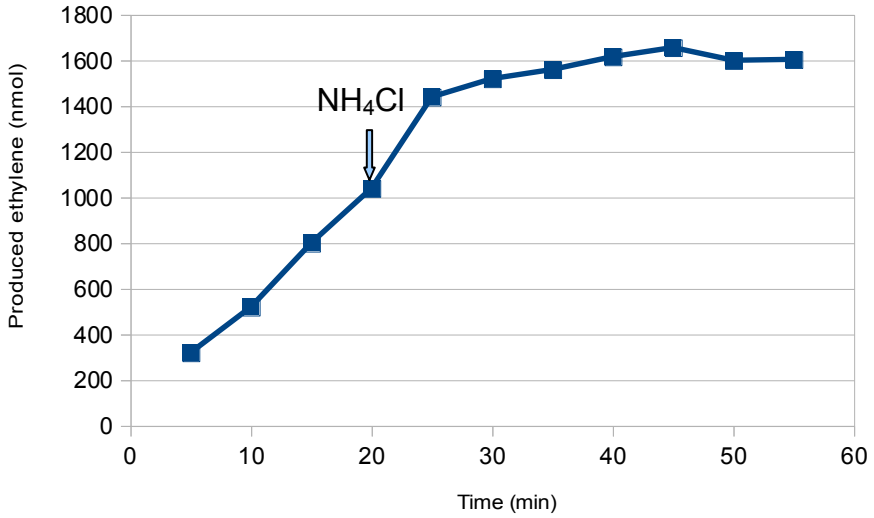


Figure 21. Nitrogenase regulation in wild type strain SB1003

The same experiment was performed for RCAY63, which lacks AmtB. As seen in figure 22, the amount of ethylene produced is linear with time, and change is observed after ammonium addition. This is a typical pattern in strains with no nitrogenase regulation, as expected for RCAY63 since switch off can't take place.

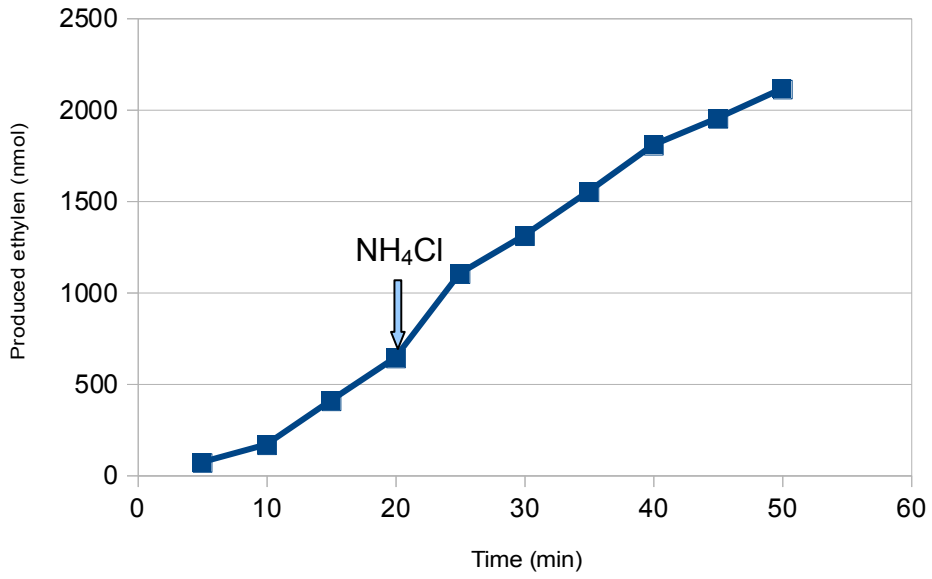


Figure 22. Nitrogenase regulation in RCAY63

The constructed site directed mutants, RCZC (D335A), RCIA1 (D338A), RCIA2 (G344C), RCIA3 (H193E) and RCIA4 (W237A), were also tested for nitrogenase regulation and their ability to switch off after the addition of ammonium. These mutants all have had a mutation in one or two of the AmtB amino acid nucleotides, which seemed to be highly conserved between *R. capsulatus* and *E. coli*. As depicted in figure 23, all the above mentioned mutants gave a pattern similar to RCAY63, which leads to the fact that none of them are capable of switch off, and therefore nitrogenase regulation must be absent.

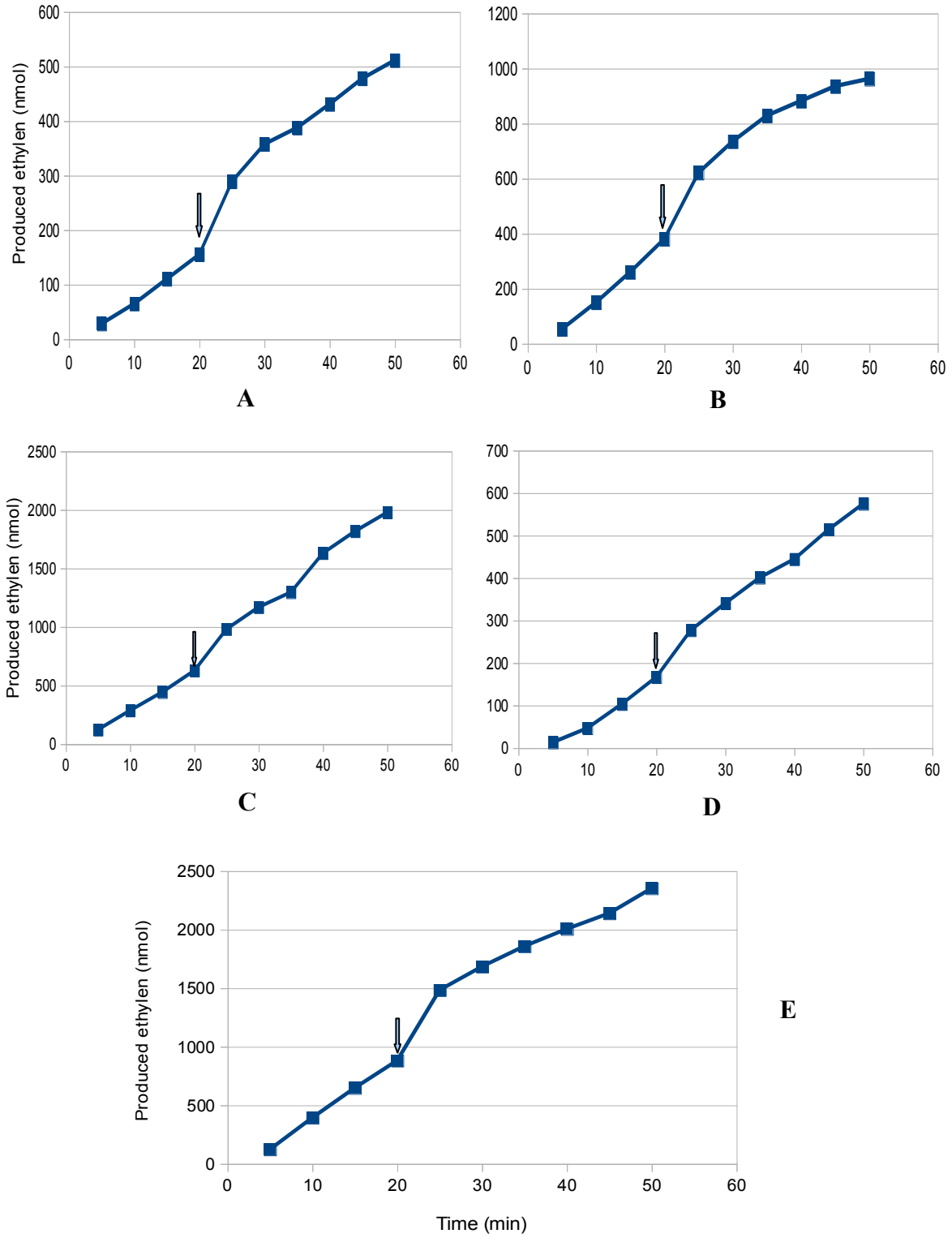
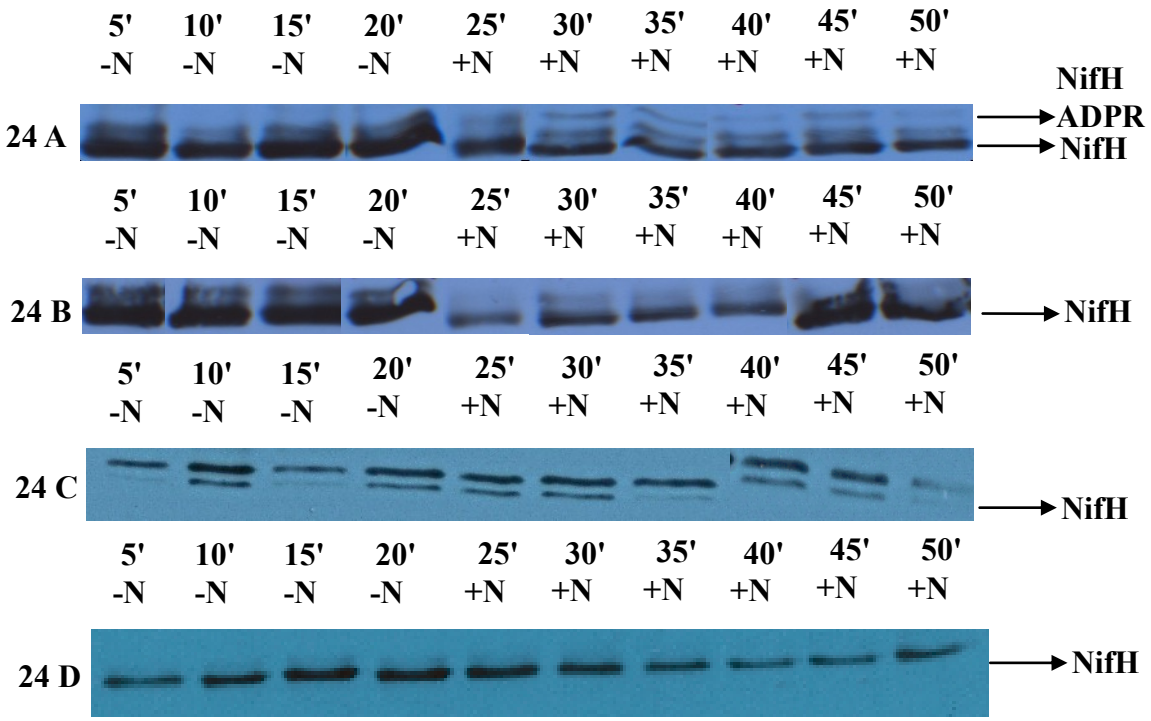


Figure 23. Nitrogenase regulation in site directed mutants RCZC (A), RCIA1 (B), RCIA2 (C), RCIA3 (D) and RCIA4 (E). Arrows show the moment that NH_4Cl was added to the samples.

1.3 Fe-protein ADP-ribosylation in RCZC, RCIA1, RCIA2, RCIA3 and RCIA4 mutants:

Liquid samples were taken every 5 minutes during the nitrogenase assay, kept in SDS PAGE sample buffer and stored at -20°C prior to performing SDS PAGE followed by western-blotting with anti Fe-protein antibody. This step allowed the determination of the state of Fe-protein modification in RCZC (D335A), RCIA1 (D338A), RCIA2 (G344C), RCIA3 (H193E) and RCIA4 (W237A) mutants after the addition of ammonium. SB1003 and RCAY63 were used as positive and negative controls. As observed in figure 24 A, SB1003 showed Fe-protein ADP-ribosylation starting from the 30th minute, 10 minutes after the ammonium injection, whereas the Fe-protein in RCAY63 (Fig 24 B) didn't undergo ADP-ribosylation after the addition of ammonium. Among the mutants, RCZC (D335A), RCIA1 (D338A) and RCIA4 (W237A) (Fig 24 C, E and G respectively) showed no ADP-ribosylation, while RCIA2 (G344C) (Fig 24 E) presented a slight form of ADP-ribosylated Fe-protein from the 25th minute onwards (however it is barely visible in this picture). On the other hand RCIA3 (H193E) (Fig 24 F) is the only mutant that has Fe-protein ADP-ribosylation at the same time points as SB1003, although no switch-off took place for this mutant after ammonium addition.



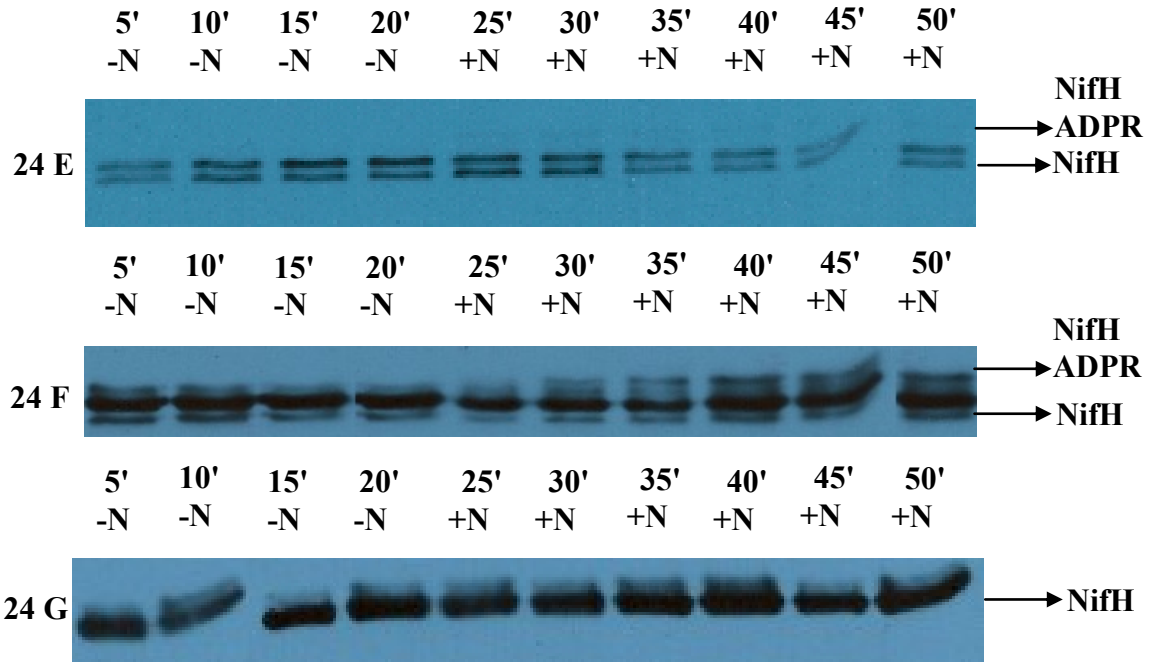


Figure 24. Modification of Fe-protein by ADP-riboylation for SB1003 (A), RCAY63 (B), RCZC (C), RCIA1 (D), RCIA2 (E), RCIA3 (F) and RCIA4 (G)

1.4 AmtB-GlnK sequestration in response to ammonium shock in RCIA3 mutant:

In order to find out whether GlnK (which weighs 12.3 kDa) binds to AmtB in the membrane in response to an ammonium shock, cytoplasmic and membrane portions of RCIA3 were separated by the cell fractionation method described in section 14 of materials and methods. Two RCIA3 cultures of RCV-$(NH_4)_2SO_4^+$ were cultured overnight. One of the cultures was then treated with ammonium while the other was kept without ammonium. The cytoplasmic and membrane fractions were prepared and their proteins separated on SDS PAGE (Fig. 25). 4µg of each sample was loaded per well, then transferred to a PVDF membrane and blotted with anti RC-GlnK antibody. However, nothing was detected on the film, although this experiment was repeated several times without success. To determine if the problem was due to the antibodies used, a dot blot was performed, which revealed that the primary and secondary antibodies bind together and function properly.

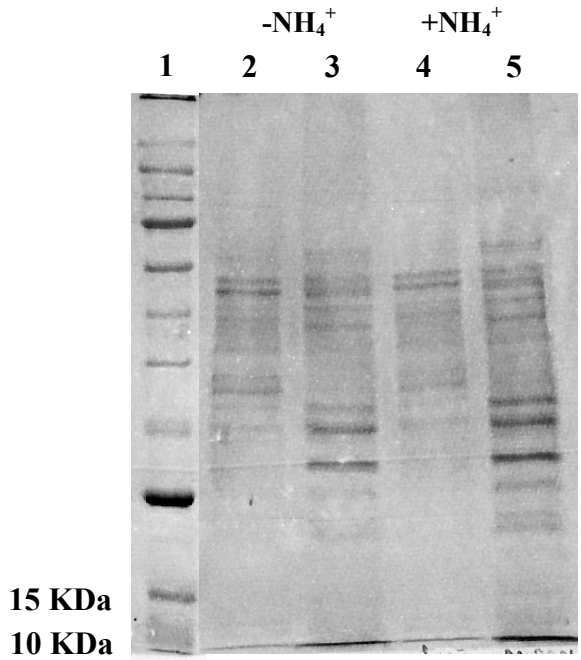


Figure 25. SDS PAGE photo of RCIA3 cytoplasmic and membrane fractions. Lane 1 is protein ladder (10-250 kDa) from NEB, lane 2 and 3 represent RCIA3 cytoplasmic and membrane portions respectively in the absence of ammonium, lane 4 and 5 are RCIA3 cytoplasmic and membrane portions in the presence of ammonium.

The same procedure was carried out for RCZC along with RCAY63 (negative control) and SB1003 (positive control) (Fig 26). As it was expected the results for SB1003 revealed that the level of cytoplasmic GlnK was greater in the absence of NH_4^+ than its presence. While in the membrane portion in the lack of nitrogen source nothing was detected but with the addition of nitrogen source, GlnK was detected. As expected, this revealed GlnK-AmtB sequestration in the membrane with an ammonium shock. On the other hand for RCAY63 the amount of GlnK in the cytoplasmic portion was unaffected by the addition of NH_4^+. Whereas in the membrane fraction, no GlnK was detected neither before nor after ammonium addition which is because this strain lacks AmtB. Therefore GlnK-AmtB binding in the membrane does not happen in response to ammonium despite the presence of GlnK in the cytoplasm. RCZC showed a similar pattern to SB1003, which indicates the sequestration of GlnK to AmtB in the membrane level after an ammonium shock.

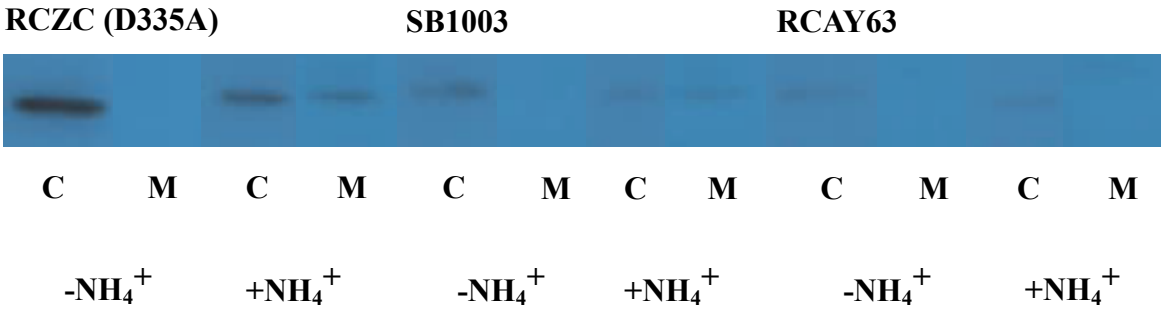


Fig 26. GlnK-AmtB sequestration in response to ammonium shock in RCZC (D335A), SB1003 and RCAY63. Cytoplasmic fractions (C) and membrane fractions (M) were blotted with anti RC-GlnK antibody in the presence ($+NH_4^+$) and absence ($-NH_4^+$) of ammonium

2. Probing the role of *R. capsulatus* AmtY expressed in *Escherchia coli:*

2.1 AmtY-GlnK sequestration in response to ammonium shock in *E. coli* (GT1001 pRSG1):

Since the function of AmtY in *R. capsulatus* is unclear, it was expressed in *E. coli* (GT1001 pRSG1) to test its functionality in that species. Possible sequestration of GlnK by AmtY after an ammonia shock was examined by carrying out a cell fractionation assay. The presence of GlnK in the extracted membrane and cytoplasmic fractions was verified by first separating both portions before and after NH_4^+ addition (with and without IPTG) using SDS-PAGE (15%), with loading 3 µg of samples per well after the determination of the protein concentration in the fractions.

This step was followed by western blotting, using anti-EcGlnK antibody. The results revealed that for the cytoplasmic portion the amount of GlnK didn't change after NH_4^+ shock (in the absence of IPTG) (Fig 27 A). As well, the same situation was observed for the membrane fraction. The amount of GlnK in the cytoplasmic fraction was greater than that in the membrane fraction. The analysis was also performed for cultures which had been treated with IPTG. The amount of GlnK in the cytoplasmic fraction was unaffected by the addition of NH_4^+ (Fig 27 B). On the other hand, a small difference was observed in the membrane fraction after ammonia shock, which indicates a slight increase in GlnK sequestration by AmtY. Therefore, it seems that AmtY doesn't bind GlnK in *E.coli* (GT1001

pRSG1) in response to NH_4^+.

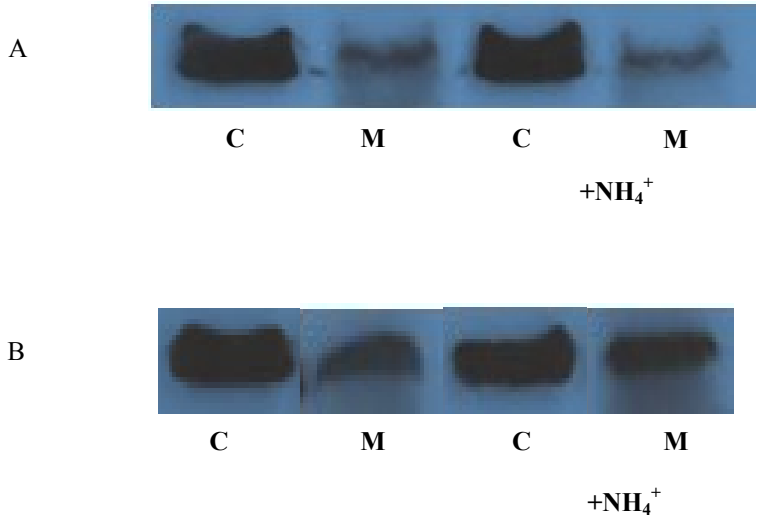


Figure 27. GlnK sequestration. Two parallel cultures of GT1001 pRSG1 were grown in M9Gln under nitrogen limited conditions, once -IPTG (A) and another time +IPTG (B). 30mM NH_4Cl was added to one of the parallel cultures and incubated for 15 minutes. Cells were then harvested and with further centrifugations cytoplasmic (C) and membrane (M) portions were separated. SDS-PAGE electrophoresis was carried out for the cytoplasmic and membrane fractions, before and after the addition of NH_4Cl. This was continued by western blotting using anti-GlnK antibody.

3. Probing the formation of ternary complex, GlnK-AmtB-DraG, by generating anti-DraG antibody:

3.1 Over-expression and purification of DraG protein:

Plasmids pPLT60 and pIMI were introduced into XL1-Blue competent cells on LB+ampicillin plates, followed by over-expression of DraG by the addition of IPTG to the growth medium (as described in section 18 of materials and methods). After protein induction and purification steps (explained in sections 19 and 20 of materials and methods), samples before and after induction and the eluted fractions (from purification step) were run on 15% SDS PAGE to separate the protein bands (Fig 28 A), The SDS PAGE gels were then subjected to a western blot analysis (Fig 28 B) with anti-6X His antibody. As shown in

figure 34, DraG (30 kDa) carried on pIMI appeared to be overexpressed. However, its purification was not successful. The same procedure was repeated several times for pPLT60 plasmid, even using culture volumes as large as 1L and different incubation times with IPTG, but overexpression of DraG was never detected. In addition, attempted purification of DraG from pIMI was repeated several times with freshly made buffers, but for unknown reasons, the DraG protein band was lost during purification, while other protein bands seemed to be collected in the eluted fractions instead. The above mentioned problems might be due to a problem with plasmid construction (pPLT60) or the production of insoluble inclusion bodies(pIMI).

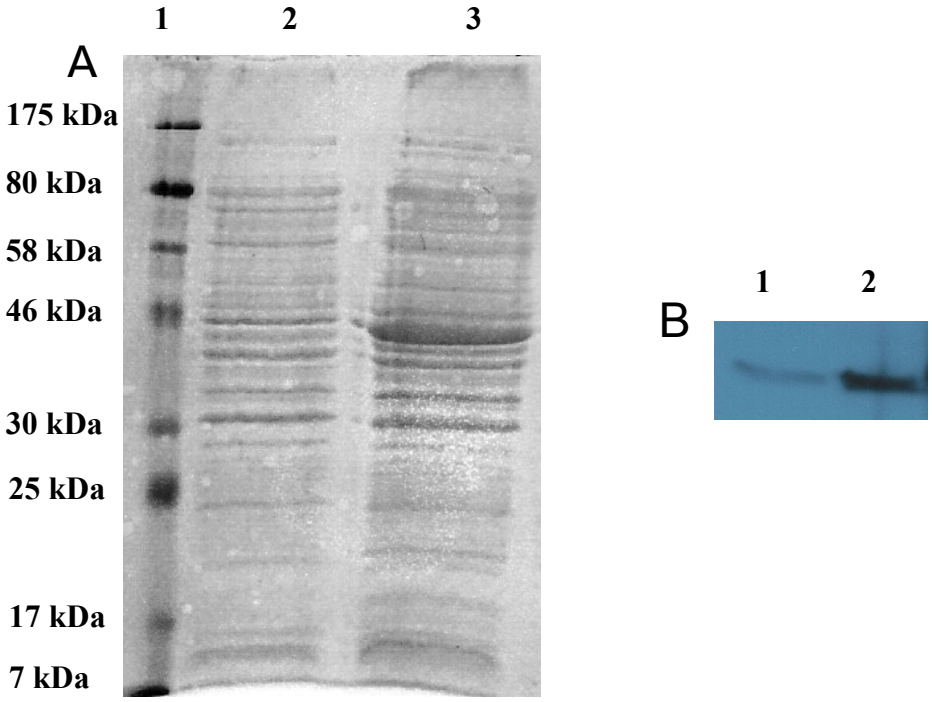


Figure 28. DraG over-expression in pIMI. (A) SDS PAGE picture which lane 1 is "prestained protein marker broad range" from NEB, lane 2 is before induction and lane 3 is after induction. (B) Western blot photo which lane 1 is the non-induced form and number 2 presents it after induction.

Chapter IV

Discussion

Amt, an ammonium transport protein, plays an important role in some bacteria in the regulation of nitrogenase. It has been shown that AmtB, one of the Amt proteins of *R. capsulatus*, is necessary for ammonium-induced nitrogenase switch-off and ADP-ribosylation in this species (2). Two different mechanisms have been shown to affect nitrogenase activity in *R. capsulatus* in response to exogenous ammonium; ADP-ribosylation of nitrogenase Fe-protein and the ADP-ribosylation-independent switch-off effect (2). AmtB appears to be involved in both responses and the working hypothesis is that AmtB regulates ADP-ribosylation by forming a complex with GlnK and probably DraG at the membrane in the presence of excess extracellular ammonium. This ternary complex removes DraG from the cytoplasm causing nitrogenase switch-off (18).

In order to better understand the mechanistic details on the molecular level of the role of AmtB in ammonia sensing and metabolic regulation, a series of site-directed *R. capsulatus* AmtB with changes in amino acids thought to be important in various aspects of the interaction with ammonia, were constructed in our laboratory. The mutants were also characterized in terms of nitrogenase switch-off and AmtB-GlnK formation. Here we have investigated D335, D338, G344, H193 and W237 highly conserved amino acid residues by making their appropriate mutants RCZC (D335A), RCIA1 (D338A), RCIA2 (G344C), RCIA3 (H193E) and RCIA4 (W237A).

Among the above mentioned amino acid residues, the highly conserved H193 has been previously studied by P. Tremblay et al 2008, (18) and proven to be absolutely critical for ammonium transport by *R. capsulatus* AmtB as well as *E. coli*. It was also shown in the same work that a mutation in that residue disables the nitrogenase switch off. This indicates the importance of this histidine residue in nitrogenase regulation. In our study on RCIA3 (H193E), no switch-off was seen even after ammonium addition (Fig 28 D), which matched the results obtained previously, supporting its critical role in ammonium transport and nitrogenase regulation. However, here it has been shown, against expectations, that this mutant shows some NifH ADP-ribosylation in response to ammonium addition (Fig 29 F). One explanation for this is that nitrogenase is in excess and a small decrease in active nitrogenase brought about by ADP-ribosylation is not enough to cause a decrease in the measured nitrogenase activity. This idea is supported by a previous study which found that even in highly active nitrogen fixing *R. capsulatus* cultures some of the nitrogenase is in

the ADP-ribosylated form (56). The amino acid D335 has also been previously studied by P. Tremblay et al 2008, (18) along with D334 in a *R. capsulatus* in a AmtB double mutant construction. Their results revealed that this double mutant was incapable of ammonium transport and nitrogenase switch off. However, it was unclear if this effect was due to one or both of the aspartic acid mutations. To study the role of D335 alone, the single mutant RCZC (D335A) was constructed here and its response to an ammonium shock investigated. RCZC didn't show switch-off (Fig 28 A), suggesting that the results obtained by P. Tremblay et al 2008 were due to this mutation in the double mutant and strongly suggesting an important role for this residue in nitrogenase regulation and ammonium transport. In addition, here it has been shown that there is no modification of nitrogenase Fe-protein in the single mutant (Fig 29C). Therefore the D335 mutation blocks both kinds of nitrogenase regulation, ADP-ribosylation of Fe-protein and the ADP-ribosylation-independent switch-off effect. Thus, this aspartate residue seems to be extremely crucial in AmtB function and nitrogenase regulation.

The 3 remaining mutants, RCIA1 (D338A), RCIA2 (G344C) and RCIA4 (W237A), had never been subjected to detailed investigations. When a nitrogenase assay was conducted all three mutants showed a linear rate of acetylene reduction despite ammonium addition after the 20th minute which in turn means that nitrogenase switch-off is absent (Fig 28 B, C and E). This experiment reveals the crucial role of aspartate 338 (D338), glycine 344 (G344) and tryptophan 237 (W237) in *R. capsulatus* in nitrogenase regulation and ammonium transport. These mutants were also examined to check the occurrence of Fe-protein ADP-ribosylation. Results with RCIA2 (G344C) mutant indicated a slight modification of NifH protein via ADP-ribosylation (Fig 29 D). Again this suggests that active nitrogenase is in excess. On the other hand with mutants RCIA1(D338A) and RCIA4 (W237A), no NifH ADP-ribosylation was seen even after ammonium addition at the end of the 20th minute (Fig 29 G). No modification means that D388 and W237 in *R. capsulatus* are required for nitrogenase regulation via Fe-protein ADP-ribosylation.

The AmtB family is almost invariably encoded in a *glnKamtB* operon (18), suggesting that GlnK and AmtB have a tightly conserved genetic linkage in bacteria (59). Previous studies have suggested that upon addition of NH_4^+, GlnK sequesters AmtB to the membrane which causes switch-off. It has been shown that AmtB is essential for proper

nitrogenase regulation in *R. capsulatus* and the ammonium-induced membrane sequestration of GlnK with AmtB seems to be vital for this process. Previous studies have also shown that GlnK sequestration after ammonium shock appears not to be enough in order to ensure nitrogenase switch-off and ADP-ribosylation but that binding of ammonium by AmtB or its partial transport is also needed (18). However the amount of membrane-sequestered GlnK seemed to be not important in this process. To check AmtB-GlnK sequestration in response to ammonium shock in the RCIA3 mutant, cell fractionation was carried out to separate cytoplasmic and membrane fractions, which were then separated by SDS PAGE and subsequently transferred to a PVDF membrane and blotted with anti RC-GlnK antibody. However nothing was detected on the film, although this experiment was repeated several times. Dot blot analysis revealed that both primary and secondary antibodies were functional. On the other hand, when this experiment was carried out for RCZC, GlnK was detected. Its level in the cytoplasmic portion was higher in the lack of ammonium in comparison to when ammonium was added. Because in the former case, almost all the GlnK is accumulated in the cytoplasm but after the the addition of ammonium, some sequester to AmtB in the membrane to block this channel. While in the membrane fraction and in the absence of ammonium, GlnK was not seen but visualized after an ammonium shock. This confirms the binding of GlnK to AmtB under nitrogen repletion conditions, which indicates the fact that the point mutation (D335A) in RCZC did not affect GlnK-AmtB sequestration to the membrane. Therefore aspartate 335 (D335) does not have a critical role in the sequestration of GlnK to AmtB in the membrane level.

R. capsulatus possesses two genes coding for ammonium transport systems, called *amtB* and *amtY.* AmtB is an ammonium transporter that is also capable of methylammonium uptake and which acts as an ammonium sensor involved in nitrogenase switch-off, whereas AmtY's functionality is unclear, although it has been shown to not be competent for methylammonium uptake (1, 2, 69). As well, previous studies showed that AmtY didn't appear to participate in nitrogenase switch-off or in ammonia transport in (2). In this work AmtY was expressed in *E. coli* (GT1001 pRSG1) to be tested for its functionality in that species. To detect possible GlnK-AmtY sequestration after ammonia shock a cell fractionation assay was carried out. The presence of the GlnK was verified using SDS-PAGE (15%) and western blotting of both cytoplasmic and membrane fractions

before and after NH_4^+ addition (with and without IPTG). The results revealed that, in the absence of IPTG, the amount of GlnK in the cytoplasmic or membrane fractions did not appear to change after the NH_4^+ shock (Fig. 33A). The same experiment was performed in the presence of IPTG. The levels of GlnK in the cytoplasmic portion was unaffected before and after the increase of NH_4^+ (Fig. 33B). On the other hand, a small difference was observed in the membrane fraction after ammonia shock, which indicates a slight increase in the AmtY-GlnK sequestration when over-expression is induced by IPTG. Thus, the results obtained here suggest that AmtY doesn't associate with GlnK in *E. coli* (GT1001 pRSG1) in response to NH_4^+.

Previous investigations on AmtY functionality carried out in our laboratory had revealed that AmtY was transcribed in *R. capsulatus* under N-limiting conditions. It was also shown that AmtY could transport methylammonium in *E. coli* in addition to its capability in correcting the growth defect of an *amtB*$^-$ strain. However, a full understanding of the function of AmtY in *R. capsulatus* obviously requires further investigations.

In *R. capsulatus* at high extracellular ammonium concentrations, GlnK is sequestered to the membrane by making a complex with AmtB in order to regulate nitriogenase activity, which consequently leads to its switch-off. However, as discussed previously, the formation of a GlnK-AmtB complex is not sufficient for nitrogenase regulation. It is suggested in other species that DraG binds this binary complex, forming a ternary complex, GlnK-AmtB-DraG, which affects nitrogenase switch off. However, details of this process are unclear and it has never been shown if this applies to *R. capsulatus*. Therefore, an attempt was made to generate an anti-DraG antibody to be used for probing the formation of ternary complexes containing DraG. Several repeated attempts were made to over-express His-tagged DraG using pPLT60 and pIMI, carried by *E. coli* XL1-Blue cells. Induction of overexpression with IPTG was only successful for pIMI, however it was not possible to purify the induced His-tagged DraG in order to generate antibody against it, possibly due to protein instability or inclusion bodies. In the future, specialized *E. coli* strains and expression systems ,which reduce inclusion body build ups, could be used to over express Drag. Also the incubation during the induction with IPTG could be carried out at lower temperatures instead of 37°C, in order to decrease inclusion bodies. Another solution to this problem might be purifying DraG under denaturing conditions to overcome

the appearance of inclusion bodies. In addition, other affinity tags could be used as an alternative to 6x His-tag.

Conclusion

In order to better understand the role of a series of highly conserved amino acids in *R. capsulatus* AmtB and their influence on nitrogenase regulation, a number of site directed mutants (RCIA1, RCIA2, RCIA3 and RCIA4) were created by previous lab members besides an extra one (RCZC) constructed in this study. Mutations were done at D338, G344, H193E, W237A and D335 amino acid residues of *R. capsulatus* AmtB. These mutants were assayed for nitrogenase activity to determine if nitrogenase switch-off was present upon addition of ammonium. Other possible analyses include, determining nitrogenase NifH ADP-ribosylation and GlnK-AmtB complex formation. Results revealed that all 5 residues are essential for nitrogenase regulation and ammonium transport as they all lost the ability to switch-off nitrogenase activity even after ammonium addition. W237 and D335 and D338 amino acid residues were absolutely critical for Fe-protein modification whereas H193 residue does not seem to be important for this function. Although the G344 mutant showed NifH modification, it was only slight, suggesting that it is an important amino acid involved in Fe-protein modification. No results were obtained for GlnK-AmtB sequestration in response to ammonium shock in RCIA3, whereas RCZC showed GlnK-AmtB sequestration in the membrane in response to ammonium repletion.

A series of other mutations, such as W237L/F, F131L, W172A/L and F240A/L, could be generated in the future to further probe the role of AmtB in nitrogenase regulation in *R. capsulatus*. In addition, mutants will be assayed for methylammonium uptake with [14C] in the future.

R. capsulatus has two Amt proteins namely AmtB and AmtY. AmtB is an ammonium transporter and a sensor for it which plays an important role in nitrogenase regulation, while AmtY seems to have none of these functions in this species. To find out AmtY function, it was expressed in *E. coli* (GT1001 pRSG1) and was assayed for GlnK-AmtY formation before and after ammonium shock. The results showed that the amount of GlnK wasn't changed before and after the addition of ammonium neither for the cytoplasmic portion nor for the membrane fraction. It can be concluded from the results that AmtY does not associate with GlnK in *E. coli* in response to NH_4^+.

In order to make anti-DraG antibody ,to be used for probing the formation of ternary complexes (GlnK-AmtB-DraG), further works need to be done to over-express DraG and to purify it. To optimize the conditions, some changes should be taken into account. For instance, DraG over expression could be done with specialized *E. coli* strains and

expression systems. Incubation temperature could also be decreased during induction with IPTG. Purifying DraG under denaturing conditions could be another solution to this problem. Using other tags instead of 6x His-tag might be helpful too.

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