STUDIES ON THE REGULATION OF GENES RELATED TO NITROGEN FIXATION AND N-ASSIMILATION IN *Azoarcus* sp. strain BH72: THE ROLE OF NtrBC

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2003

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN (NATURAL) SCIENCE

(Dr. rer. nat.)

THE FACULTY OF BIOLOGY AND CHEMISTRY
UNIVERSITY OF BREMEN
GERMANY

Untersuchungen zur Regulation von Genen, die in der N_2-fixierung und N-Assimilation von *Azoarcus* sp. Stamm BH72 involviert sind: Die Rolle von NtrBC

DISSERTATION

Zur Erlangung des Doktorgrades
der Naturwissenschaften
(Dr. rer. nat.)

Dem Fachbereich Biologie/Chemie der
Universität Bremen
vorgelegt von

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aus Indien

Bremen 2003

The studies of the presented work have been carried out from February 2000 till March 2003 at the department of Biology/Chemistry of Bremen University, Germany, under the guidance of Prof. Dr. Barbara Reinhold-Hurek.

Die Untersuchungen zur folgenden Arbeit wurden von Februar 2000 bis März 2003 am Fachbereich Biologie/Chemie der Universität Bremen unter der Leitung von Prof. Dr. Barbara Reinhold-Hurek durchgeführt.

Vom Fachbereich Biologie/Chemie der Universität Bremen als Dissertation angenommen am:

Datum der Disputation:

1. Erstgutachterin: Prof. Dr. Barbara Reinhold-Hurek
2. Zweitgutachterin: Prof. Dr. Friederike Koenig

Dedicated to My Parents

…….Their dreams are my strength

Parts of the results presented in the following work have been published in:

Egener, T., Martin, D.E., Sarkar, A., and Reinhold-Hurek, B. (2001). Role of a Ferredoxin Gene Cotranscribed with the *nifHDK* Operon in N_2 Fixation and Nitrogenase "Switch-Off" of *Azoarcus* sp. Strain BH72. *J. Bacteriol.* **183,** 3752 – 3760

Egener, T., Sarkar, A., Martin, D.E., and Reinhold-Hurek, B. (2002). Identification of a NifL-like protein in a diazotroph of the *ß*-subgroup of the *Proteobacteria*, *Azoarcus* sp. strain BH72. *Microbiol* **148**, 3202 – 3212.

Sarkar, A. and Reinhold-Hurek, B. (2002). Characterization of *ntrBC* of *Azoarcus* sp. strain BH72. *In* Book of Abstracts, 5th European Nitrogen Fixation Conference, Norwich, Great Britain.

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Abbreviations

σ^{54}-promoter	RpoN dependent promoters having the –12/-24 consensus and responsive to nitrogen
2D	two dimensional
amtB, amtY	genes encoding for putative proteins AmtB and AmtY respectively, having similarity to membrane bound ammonium transport proteins.
APS	Ammonium persulphate
EDTA	Ethylene diamine tetra acetic acid
fdxN	gene encoding for ferredoxin
glnB	gene encoding for the signal transmitter protein P_{II} or GlnB
glnIII	gene encoding for glutamine synthetase three (GSIII).
glnK	gene encoding for the P_{II} paralogue, GlnK signal transmitter protein
glnY	gene encoding for the third P_{II} paralogue, GlnY signal transmitter protein
glt	gene encoding for glutamate synthetase (GOGAT)
GOGAT	glutamine-2-oxoglutarate aminotransferase
GS	glutamine synthetase
gusA	gene encoding for β-glucuronidase
nif	nitrogen fixation, gene encoding for nitrogen fixation
NifA/NifL	transcription activator of *nif* gene / inhibitor of NifA
NifH	Dinitrogenase reductase
nifHDK	structural genes for the nitrogenase enzyme complex
nifLA	gene encoding for the NifL and NifA
nir	gene encoding for assimilatory nitrate reductase
ntrBC	genes encoding for NtrBC two component system
NtrBC	two component regulatory system of N-metabolism
OD_{578}	Optical density measured at a wavelength of 578 nm
PAGE	polyacrylamide gel electrophoresis
P_{II}-proteins	signal transmitter protein of N-metabolism
SDS	sodium dodecylsulphate
SSC	standard saline citrate
TBS	Tris-buffered saline
TEMED	N,N,N', N'- tetramethylethylene-diamine
Tris	N-tris-(hydroxymethyl)-amino methane
ugk	ORF upstream of *glnK*

Summary

The aim of this work is to study the regulation of genes related to nitrogen fixation and ammonium assimilation in *Azoarcus* sp. strain BH72 and elucidate the role of NtrBC in this regulatory process. The regulation has mostly been studied at the level of transcription.

Characterization of the transcripts for *nifHDK,* encoding for the structural genes of nitrogenase and *nifLA*, encoding for its transcription activator, revealed unusual as well as common features in *Azoarcus* sp. BH72. Although both *nifHDK* and *nifLA* utilized their respective upstream sigma 54 promoters for transcription (transcriptional start sites verified by primer extension), *nifHDK* was unusually found to be cotranscribed with its downstream *fdxN* (ferredoxin) gene. For these experiments a protocol has been successfully developed for the first time by which all the primer extensions were carried out non-radioactively, using an automated sequencing machine. Also *nifA* was found to be transcriptionally linked to its upstream *nifL*, a feature similar to the γ-subgroup of *Proteobacteria,* and its expression in strain BH72 was found to be responsive to nitrogen.

Success has also been made to clone and characterize *ntrBC* from strain BH72, which act as an important two-component system in sensing and initialising the N-regulatory cascade. *ntrB* and *ntrC* from strain BH72 were also found to be transcriptionally linked. Surprisingly, no standard promoter consensus could be detected upstream of the *ntrB* transcriptional start site. Along with this, putative Shine- Dalgarno sequences, representing translational initiation sites, were also lacking upstream of their respective start codons. However, *ntrB* itself was found to be expressed in an N-responsive manner and unusually found to be independent from autoregulation.

A marker exchange deletion mutant of *ntrBC* (strain BntrBsp) verified that these genes were not cryptic since the mutant had a phenotype and that several genes were under its direct or indirect control. The mutation affected nitrogen metabolism in several ways. Although *nif*+ in phenotype, the *ntrBC* mutant was affected in N_2 fixation, with reduced *nifH::gusA* expression. The expression of its essential transcriptional activator *nifLA* on N_2, was also found to be NtrBC regulated. Nitrate had a severe effect on the *ntrBC* mutant, manifested by prolonged generation time of growth, opaque rounded colonies, and upregulation of PHB biosynthesis in BntrBsp. Even nitrogenase genes were not completely repressed in BntrBsp on nitrate under microaerobiosis. This may be explained in terms of poor nitrate

assimilation, further substantiated by poor expression of the assimilatory nitrate reductase in BntrBsp. However the *ntrB* non-polar mutant was not affected under these conditions, suggesting the existence of an NtrB independent NtrC phosphorylation (activation) mechanism. Unusually the “twitching motility” was found to be upregulated in BntrBsp compared to that of the wild type, a phenotype which was up to now not known to be linked to these genes.

Looking at the level of P_{II} proteins in strain BH72, *glnK* was unexpectedly found to be transcriptionally linked to its upstream *ugk* gene, encoding for a protein putatively exported into the periplasm, and harbouring a signal peptide. Although a putative sigma 54 promoter consensus is present upstream of *ugk*, a common transcriptional start of *glnK* and *ugk* could not be mapped by primer extension, possibly due to active processing of the single major transcript. Interestingly *glnK* expression was also found to be down regulated in the *ntrBC* mutant in a similar manner as that of *glnB*. The nitrogenase activity can be regulated at the posttranslational level by ammonium, referred as “ammonium switch off”, which may involve two different mechanisms. In strain BH72, GlnK and AmtB have been shown to be essential for these responses. Strangely enough, ammonium “switch off” response and posttranslational modification of nitrogenase still occurred in *ntrBC* mutant, under low GlnK expression. Interestingly, for the first time, GlnY was found to be expressed (albeit at low level) in the *ntrBC* mutant along with other P_{II} proteins. Thus studies on NtrBC may help in understanding the regulatory cascades of the three paralogues, which is still not very clear in *Azoarcus* sp. BH72.

Analyses of the expression of nitrogen assimilatory genes encoding for glutamine synthetase, GS and glutamate synthetase, GOGAT (using unreleased sequence information from the *Azoarcus* genome project, Bielefeld) in strain BH72 revealed that the transcription of these assimilatory genes was affected by nitrogen. Surprisingly, *glnIII* expression, encoding for GS, was found to be independent from NtrBC control whereas *glt* encoding for putative GOGAT was found to be moderately regulated by NtrBC. Thus it appears that NtrBC of *Azoarcus* has a significant contribution on the N-metabolism, regulating nitrogenase expression via *nifLA*, differentially regulating the three P_{II} paralogues at least at the level of transcription and affecting the nitrate assimilatory cascade. However, presence of several NtrBC independent N-responses like physiological “switch off”, posttranslational nitrogenase modification, and glutamine synthetase transcription provide hints for the existence of another novel N-regulatory circuit apart from NtrBC in *Azoarcus* sp. BH72, which makes it a more interesting model for the β-subgroup of *Proteobacteria*.

Introduction:

Today it is widely recognized that there is probably no ecological niche on earth where bacteria have not evolved to exploit whatever nutrients are available to support life. Despite this enormous versatility of bacterial metabolism, certain fundamental mechanisms have to exist to regulate and integrate enzyme synthesis and enzyme activity. At the centre of this metabolic control is the need to coordinate the catabolism and assimilation of carbon and nitrogen sources so as to maximize potential growth rates under any particular nutritional regime. Nitrogen is one of the most important elements required for life as it is necessary for the production of amino acids, nucleotides, amino sugars (required for the synthesis of lipopolysaccharides and peptidoglycans), NAD, and *p*-aminobenzoate (a precursor in folate biosynthesis). Consequently bacteria have developed a number of mechanisms by which nitrogen can be assimilated from a variety of sources, ranging from ammonium to atmospheric dinitrogen (N_2). Ammonium is almost always the preferred nitrogen source, as it can be assimilated directly into glutamine and glutamate, the key donors for biosynthetic reactions and is therefore the least energetically expensive substrate to process. By contrast organic sources such as amino acids must first be degraded to ammonium and inorganic sources such as N_2, NO_3^- or NO_2^- must be reduced before assimilation (Reitzer, 1996).

The process of biological nitrogen fixation is strictly restricted to prokaryotes. The most spectacular highlight in this field before the 20th century was the discovery by Hellriegel and Wilfarth that symbiotic N_2 fixation by legumes was dependent upon “ferments” in the soil, which are responsible for nodulation of roots. The nodulating bacteria from *Pisum sativum* were isolated by Beijerinck. The process of nitrogen fixation is highly energy demanding as nitrogenase (the key enzyme for N_2 fixation) requires 16 moles of ATP for the reduction of 1 mole of N_2 to ammonium. So it is quite likely that the synthesis of nitrogenase and its subsequent activity is tightly regulated in organisms. Owing to high oxygen sensitivity of nitrogenase, the environmental oxygen tension is a major regulatory factor in diazotrophs. Likewise the availability of fixed nitrogen is a significant regulatory effector in free-living diazotrophs but is of less importance for symbiotic organisms that are adapted to export fixed nitrogen to their host. The structural organization of nitrogenase, reveals the following salient features. The enzyme protein of nitrogenase is organized in a complex of two proteins: dinitrogenase (or MoFe protein) and dinitrogenase reductase (or Fe protein) (Burris, 1991). This nitrogenase is commonly referred to as molybdenum nitrogenase (*nif*-encoded). A second nitrogenase with Vanadium as a

cofactor and encoded by *vnf* had been reported from *Azotobacter vinelandii* (Benemann *et al*., 1972); a third alternative heterometal-free nitrogenase (*anf*-encoded) has also been reported for the first time in *Azotobacter vinelandii* and later in *Rhodobacter capsulatus* (Masepohl *et al*., 2002). Nitrogenase MoFe protein is an $\alpha_2\beta_2$ tetramer containing the cofactor FeMoco, believed to be the site for nitrogen reduction while nitrogenase Fe-protein is a homodimer, containing an (Fe_4S_4) cluster and acting as an obligate electron donor to MoFe protein (Howard *et al*., 1994).

The main enzymes for ammonium assimilation are glutamine synthetase (GS) encoded by *glnA* and glutamine-2-oxoglutarate amino transferase (GOGAT). Ammonium is assimilated to glutamine from glutamate in an ATP dependent reaction catalysed by glutamine synthetase. Glutamine is reduced by NADPH and reacts with cellular 2-ketoglutarate to form glutamate as product, the reaction being catalysed by glutamate synthetase (GOGAT). Bacterial GS molecules are reported to be dodecamers formed from two face-to-face hexameric rings of subunits, with 12 active sites formed between the monomers in which the ATP and glutamate bind (Almassy *et al*., 1986). The counterpart of glutamine synthetase is the GOGAT enzyme or glutamate synthetase. In *Corynebacterium glutamicum*, *gltB* and *gltD* are the putative genes coding for the large and small subunit respectively, and the transcription of this operon depends on N-starvation (Beckers *et al*., 2001). NAD(P)H assimilary nitrate reductase catalyzes the rate limiting and regulated step, the two electron reduction of NO_3^- to NO_2^-, in the pathway of inorganic nitrate assimilation, reported for *Arabidopsis* sp. (Solomonson *et al*., 1990; Campbell *et al*., 1999). In most of the cases it has been shown to be a multimeric protein composed of identical subunits each of which contain FAD, a *b*-type cytochrome (cytochrome b_{557}) and a molybdenum cofactor (Solomonson *et al*., 1984). In this context it is important to mention that respiratory, dissimilatory or “anaerobic” nitrate reductase is membrane bound and closely associated with formate dehydrogenase. Absence of oxygen and presence of nitrate / nitrite induce its activity to reduce and dissimilate nitrate to nitrite and so the dissimilatory nitrate reductase is strictly different from assimilatory nitrate reductase mentioned earlier (Fedtke *et al*., 2002).

The process of biological N_2 fixation as well as the pathways of nitrogen assimilation are well coordinated and regulated in organisms. In most prokaryotes studied, the control is exerted at the level of transcription of the N_2 fixation genes (*nif*) or N-assimilation genes (*glnA*). In certain organisms, including *Rhodospirillum*, regulation can occur posttranslationally by covalent modification and consequent inactivation of the nitrogenase Fe protein (Fitzmaurice *et al*., 1989). In other case, the nitrogenase

can be subjected to a reversible conformational protection from oxygen damage; nitrogenase in crude extracts of *Azotobacter vinelandii,* sediments as an air-tolerant complex that contains a third redox protein called the Shethna, FeS II, or protective protein (Shethna, 1970). The expression of the *nif* genes can also be a developmentally regulated process as exemplified in heterocystous cyanobacteria, where nitrogenase is synthesized and is most active within certain specialized cells (heterocysts), where the oxygen evolving photosystem II is inactive and is thereby spatially separated from the O_2-evolving process of photosynthesis (Bottomeley *et al*., 1979).

Typically the promoters of the *nif* genes share a unique consensus sequence atypical in terms of general bacterial promoter sequences (-10/-35 consensus). The *K. pneumoniae nif* promoters have a highly conserved sequence between positions –11 and –26 with respect to the point of transcription initiation (Fig 2.1). This sequence, which has a consensus of TGG-N8-TTGCA is quite unlike the consensus TTGACA-N17-TATACA found in –35, -10 regions of most prokaryotic promoters. Within this consensus, three residues remain invariant namely, the GG pairs at –25, -24 and the G at –13. The –12 position is more or less conserved as C with exception of a few rhizobial promoters where it is replaced by A. This difference suggested that these promoters could be recognized by a modified form of RNA polymerase. It was found indeed, that an alternative novel sigma factor of RNA polymerase (σ^{54}), encoded by the *rpoN* (*ntrA*) gene is needed to initiate transcription from these promoters (Hirschmann *et al*., 1985). Furthermore, this sigma factor was found to be totally dependent on an activator protein such as NifA or NtrC, for transcription initiation. Interestingly, many non-*nif* genes such as *E. coli glnAp2* (for N-assimilation) and *fdhF*, *Pseudomonas putida xylCAB* are known to be RpoN dependent (Kustu *et al*., 1986).

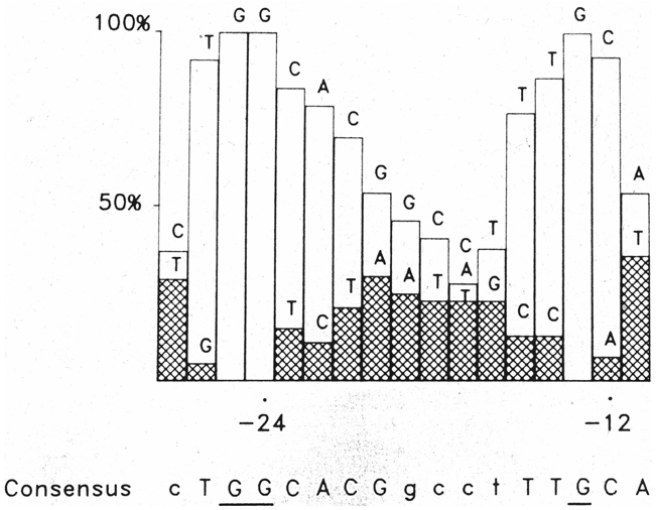


Fig 2.1 Bar diagram summarizing the sequence of 64 σ^{54}-dependent promoters from 22 species (Morett and Buck, 1989). The frequency of each base is plotted at each position between -27 and –11 with respect to the transcription start site. The derived consensus is given below the histogram

In enteric and many other bacteria, there is a general nitrogen regulation system (*ntr*) that controls the expression of many genes concerned with nitrogen metabolism. It comprises of four gene products: a uridylyltransferase (UTase) encoded by *glnD*, trimeric effector proteins (P_{II}) encoded by *glnB* and a pair of regulatory proteins encoded by a single operon *ntrBC* which regulates σ^{54} dependent transcription of many nitrogen-regulated genes (Ninfa *et al*.,1995).

The *ntrBC* forms a part of bacterial two component systems of phosphotransfer by histidine-aspartate signalling (Hoch and Silhavy, 1995). In its simplest, the transmitter module of a sensor protein autophosphorylates at a conserved histidine residue, then transfers the phosphoryl group to a conserved aspartate in the receiver domain of a response regulator, which is often a transcription activator. The primary structures of the receiver domains are relatively well conserved and they fold as single units whose tertiary structure has been determined in a number of cases (Stock *et al*., 1989; Volkman *et al*.,1995). Less well conserved, the transmitter module has characteristic sequence motifs called the H, N, G1, F and G2 boxes. Tertiary structures have been determined for histidine kinases EnvZ (Tanaka *et al*., 1998) and CheA (Bilwes *et al*., 1999) revealing the presence of separated phosphotransfer and kinase domains. NtrB, the histidine kinase for nitrogen regulation, has a poorly characterized N- terminal sensor domain that has homology to PAS domains (Taylor *et al*., 1999) and is joined to the transmitter module by a Q-linker (Drummond *et* al., 1986). NtrB generally exists as dimer (Ninfa *et al*., 1993). The response regulator NtrC is composed of three domains: an N-terminal receiver domain which contains the site for phosphorylation at aspartate position 54, a central output domain which is directly responsible for activation of transcription by the σ^{54} holoenzyme form of RNA polymerase and a C-terminal DNA binding domain which carry the major dimerization determinants for the protein and mediates binding to transcriptional enhancers. The receiver domain of NtrC is connected to the central output domain by a flexible protease sensitive linker (Keener *et al.*, 1988). Phosphorylation–dependent interdomain interactions between the receiver domain of one subunit and the output domain of its partner subunit in an NtrC dimer precede and give rise to oligomerization needed for transcriptional activation (Lee *et al*., 2000). Moreover yeast two hybrid systems have been used to show protein contacts between two component regulators (Martinez-Argudo *et al*., 2001).

A cascade of regulatory interactions is generated to initiate the process of N_2 fixation or N-assimilation (Fig 2.2). The UTase (uridylyltransferaes/uridylyl-removing) enzyme, product of *glnD*, is considered to be the primary sensor to the cellular N-status and responds to the ratio of the α-ketglutarate to glutamine pools. In *Azospirillum* sp. the N-regulated activity of the main N-assimilating enzyme (GS) is not altered in *glnD* insertion mutant (Van Dommelen *et al*., 2002). In enteric bacteria, the activity of GS is regulated by adenylation/deadenylation, depending on the nitrogen availability. This modification is catalyzed by the bifunctional enzyme adenyltransferase/ATase, the acitivity of which is in turn controlled by P_{II}. So P_{II} occupies a pivotal position in the nitrogen regulation network. The nitrogen status of the cell as sensed by uridylyltransferase, is signalled to P_{II} by adjusting the degree of uridylylation of the latter. Native P_{II} indicates a nitrogen rich status, whereas P_{II}-UMP flags a nitrogen-poor status of the cell. Usually the GlnD senses glutamine as a nitrogen signal. Glutamine inhibits uridylylation of P_{II} by affecting the rate of UMP transfer and in presence of $Mg^{+2,}$ stimulates deuridylylation of P_{II}-UMP. Low levels of oxoglutarate (sign of N-excess) stimulate the interaction of unmodified P_{II} and NtrB, leading to an inhibition of kinase activity and activation of phosphatase activity of NtrB so that its response regulator NtrC is inactivated by dephosphorylation.

Under N-limitation, interaction of P_{II} with NtrB is inhibited by uridylylation. At the same time, binding of oxoglutarate to P_{II} under N-deficiency inhibits P_{II}-NtrB interaction further. Under such a situation, histidine autokinase activity of NtrB is stimulated and phosphorylates its response regulator NtrC. Phosphorylated NtrC (active form) acts as a transcriptional activator of different *ntr* regulated operons. It usually binds to enhancer sequences, upstream of the promoter and interacts with the promoter-bound σ^{54} by means of a DNA loop to activate the formation of the transcription open complex at these promoters (Popham *et al*., 1989). On one hand it activates transcription of its own operon along with *glnA* (where *glnA* is encoded along with *ntrBC*, as in enterics). Side by side with an upregulated transcription of *glnA*, the covalent modification of GS-AMP is removed by the P_{II}-UMP stimulated ATase (*glnE*) activity forming more of unmodified GS (active), subsequently enhancing N-assimilation. The two other potential targets of phosphorylated NtrC are the *nifLA* and *glnKamtB* where it acts as transcriptional activators for these N-regulated genes. The *nifA* is positively regulated by phosphorylated NtrC as reported in *Herbaspirillum seropedicae* (Wassem *et al*., 2002) or in *Klebsiella pneumoniae* (Soupene *et al*., 1997). In a similar way, *E. coli glnA, glnK,* and *nac* promoters have been found to be positively regulated by NtrC (Atkinson *et al*., 2002).

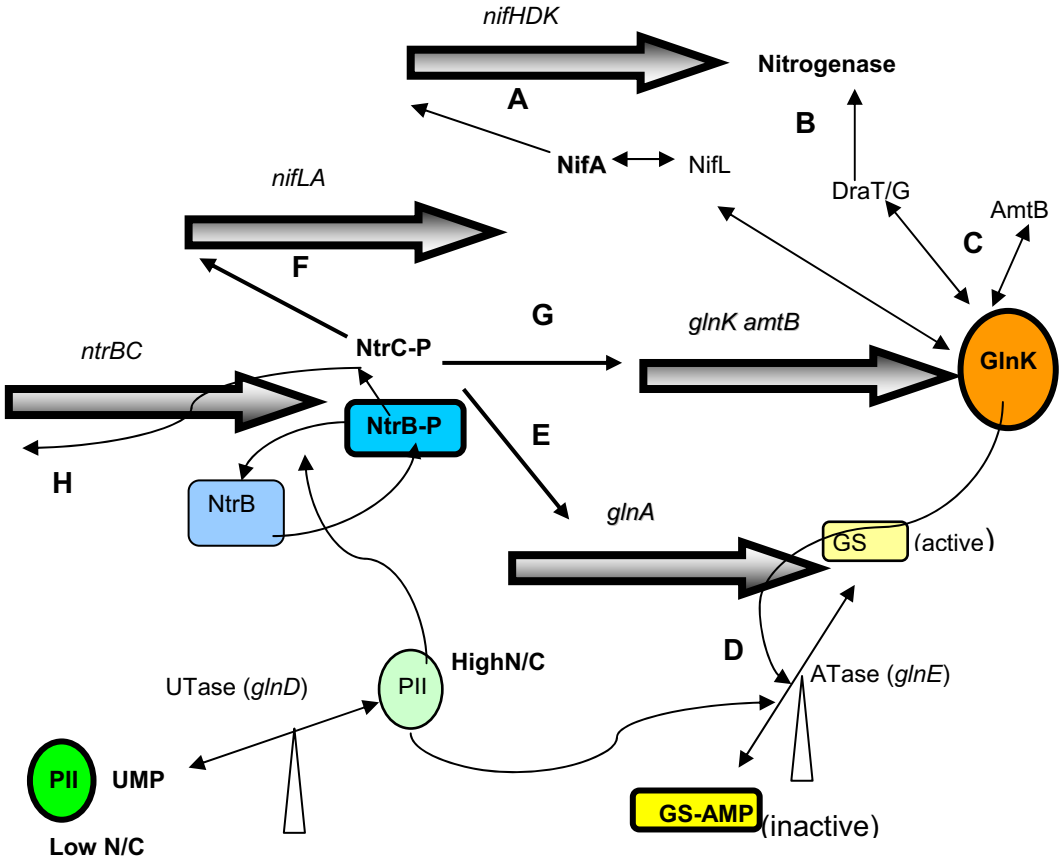


Fig 2.2 Schematic model illustrating different regulatory interactions generated during N_2 fixation and ammonium assimilation in *Proteobacteria*. The grey block arrows represent transcriptional activation of the mentioned genes. Transcriptional activation for *nifH* gene expression (**A**) or *nifLA* expression (**F**), posttranslational nitrogenase activity control (**B**), ammonium uptake (**C**), posttranslational activity control of GS (**D**) or its transcriptional activation (**E**). Transcriptional activation of *glnK-amtB* operon (**G**) or expression of *ntrBC* (**H**). The scheme shows compilation of known signal transduction processes and may vary among different species.

The NifA protein acts as an essential proteobacterial transcription activator of *nifHDK*, the structural genes for nitrogenase. In diazotrophs such as *Proteobacteria* of the α-subgroup or *Herbaspirillum seropedicae* belonging to β subgroup (Souza *et al.,* 1991), the NifA proteins show a conserved cysteine motif in the central domain, (not present in γ-*Proteobacteria*) which is probably the site of a redox sensitive Fe-S cluster (Dixon, 1998; Fischer *et al.,* 1988). Diazotrophs belonging to γ-*Proteobacteria* such as *Azotobacter vinelandii* and *Klebsiella pneumoniae* are characterized by a

NifL/A two-component regulatory system, with NifL being the sensor inhibiting the NifA activity in response to O_2 (Dixon, 1998). Stoichiometric amounts of both proteins are needed to ensure proper transcription regulation (Dixon, 1998; Govantes *et al*., 1996). For O_2 sensing, the flavoprotein NifL inhibits NifA activity in the oxidized form (Dixon, 1998; Hill *et al*., 1996). The mechanism by which the cellular N-status is sensed and the signal transmitted is more complex and may vary considerably in different diazotrophs. One level of control is the transcriptional regulation of the *nifA* itself, which may be nitrogen regulated via the two component regulatory system NtrBC as in *Klebsiella pneumoniae* (Drummond *et al.,* 1983) or *Herbaspirillum seropedicae* (Souza *et al*., 2000). At another level, the activity of NifA is modulated, P_{II} like proteins being the central signal transmitter proteins. The activity of nitrogenase itself is posttranslationally regulated in some organisms like *Rhodospirillum rubrum*, or *Azospirillum brasilense* by reversible mono-ADP riobosylation of nitrogenase (Zhang *et al*., 1997; Ludden *et al.*, 1989). In *Azospirillum brasilense* it has been reported that *ntrBC* mutations have no effect on *nif* expression but do alter the regulation of *draT/G* (genes responsible for ADP ribosylation) and alter nitrogenase activity in response to ammonium (Zhang *et al.*, 1994).

The small trimeric signal transmitter protein P_{II}, product of the *glnB* gene, plays an important role in regulation of N_2 fixation and assimilation in several species of *Proteobacteria*, cyanobacteria, Gram positives and *Archea* (reviewed by Merrick and Edwards, 1995). Recently *glnB* like genes have also been identified in plants, namely *Porphyra sp*., a red algae, in which they are encoded in the chloroplast DNA (Reith *et al*., 1995), and in *Arabidopsis thaliana*, in which they are encoded in the nuclear DNA (Hsieh *et al*., 1998). As mentioned earlier, GlnD (UTase/UR enzyme, product of *glnD*) uridylylates GlnB under N-limiting conditions and deuridylylates it under conditions of nitrogen excess, thereby stimulating NtrB-dependent dephosphorylation of NtrC, resulting in prevention of transcription of *ntr*-dependent operons. Moreover identification of a second P_{II} like protein (named GlnK or GlnZ) in several proteobacteria raised the question as to whether these proteins are also involved in the control of nitrogen metabolism. Interestingly in many *Bacteria* and *Archea*, the P_{II} paralogue *glnK* occurs in an operon with an *amtB* gene coding for an integral membrane protein, a homologue was identified as high affinity ammonium transporter in *Saccharamyces cerevisiae* (Marini *et al*., 1994) and *Arabidopsis thaliana* (Ninnemann *et al.*, 1994). An involvement in ammonium transport is also assumed for bacteria (De Zamaroczy *et al*., 1998, Michel-Reydellet *et al.,* 1997, Soupene *et al*., 1998). It has been proposed that the physical linkage of *glnK* and *amtB* reflects a functional relationship and physical interaction of these proteins

(Thomas *et al*., 2000). Not only is the *glnKamtB* operon a putative target of NtrC; in *E. coli*, GlnK participates in the regulation of NtrC phosphorylation and GS adenylation through adenyltransferase (ATase, *glnE* product) (Atkinson *et al.*, 1998). GlnK is also necessary to relieve NifL-dependent inactivation of NifA under conditions of nitrogen limitation (He *et al.*, 1998; Jack *et al.*, 1999). Furthermore (methyl)ammonium uptake appears to be regulated by GlnZ, a GlnK homologue in *Azospirillum brasilense* (De Zamaroczy, 1998). A finer tuning to these regulatory interactions is added by heterotrimer formation by GlnK and P_{II} in vivo as has been reported in *E. coli* (van Heeswijk *et al.*, 2000), making the situation more interesting to study.

The aim of this work is to find out how the genes related to nitrogen fixation and N-assimilation are regulated in *Azoarcus* sp. strain BH72 with special emphasis on NtrBC. So at this point, it is important to get introduced to *Azoarcus.* To reclaim the salt-affected soils of the arid regions of Punjab of Pakistan, Kallar grass, a C_4 plant (*Leptochloa fusca* (L.) Kunth), was introduced as a pioneer plant in the Punjab region (Sandhu *et al*., 1975). Kallar grass has a high tolerance of waterlogged conditions, soil salinity and alkalinity (Khan, 1966). Luxuriant growth of these plants without nitrogen fertilizers along with acetylene reduction activity in its rhizosphere (Malik *et al*., 1980) indicated a possible occurrence of nitrogen fixation. Analysis of the microbial population in different zones of the rhizosphere showed that the population of diazotrophic bacteria on the rhizoplane was found to be 35 fold higher than in nonrhizospheric soil (Reinhold et al., 1986). Interestingly the rhizoplane was dominated by *Azospirillum* and the endorhizospheric population was predominated by unidentified diazotrophic rods of which one of them was later assigned to the genus *Azoarcus* spp. (Reinhold-Hurek *et al*., 1993b). DNA-rRNA hybridization studies placed them in separate rRNA branch in the β-subdivision of *Proteobacteria*. These strains showed a surprising diversity: they formed five groups distinct at species level according to DNA-DNA hybridisation studies although they were very similar to each other in morphology and nutritional requirements. Therefore instead of five, only two named species, *A. indigens* and *A. communis* were proposed while strain BH72 was included into the genus *Azoarcus*. The strains S5b2 (formerly *Azoarcus* sp. Group C) and 6a3 (formerly *Azoarcus* sp. Group D) have recently been placed under new genera, *Azovibrio restrictus* and *Azospira oryzae* respectively (Reinhold-Hurek et al., 2002). Recently, two new species of *Azoarcus* have been described: *A. tolulyticus* (Zhou *et al.*, 1995) and *A. evansii* (Anders *et al*., 1995). They are non-plant associated and originate from polluted sediments of US and South

America. In contrast to plant-associated species, they are capable of degrading the aromatic hydrocarbon anaerobically with nitrate as electron acceptor.

Azoarcus sp. strain BH72, is also capable of infecting rice seedlings in the laboratory (Hurek *et al*., 1994). It can infect the roots of rice and spread systemically into the shoot without causing symptoms of plant disease. Reporter gene studies have shown that nitrogenase (*nif*) genes of *Azoarcus* spp. can be expressed endophytically in the arenchyma of these seedlings, suggesting that the interior of the rice roots provides a microenvironment suitable for N_2 fixation (Egener *et al*., 1999). Strain BH72 is unusual in that it can shift into a state of "hyperinduction" under certain growth conditions that include extremely low oxygen concentrations (30 nM). This state of strain BH72 is characterized by increased activity and efficiency of N_2 fixation (Hurek *et al*., 1994), appearance of intracellular membrane stacks (diazosomes), and association of the iron-protein of nitrogenase with diazosome membranes (Hurek *et al*., 1995). Diazosome formation can be induced reproducibly in the laboratory by cocultivating strain BH72 with the ascomycete *Acremonium alternatum* which was isolated from the root interior of Kallar grass as well (Hurek *et al*., 1998). The cells attach to the fungal mycelium, and the fungal respiration may provide sufficient microaerobic niches for diazosome formation. The association of nitrogenase with these membranes suggests that they are involved in efficient N_2 fixation, possibly by providing a more efficient electron flux to nitrogenase. Interestingly strain BH72 harbours unusual short type IV pili, encoded by the *pilAB* locus which are involved in the colonization of both plant and fungal surfaces, indicating that there may be common traits in the initial steps of interactions with eukaryotic organisms of their habitat (Dörr *et al*., 1998).

At this point it is important to mention some of the characteristic features of strain BH72 in context to genes related to nitrogen fixation, which have already been reported for this organism. In contrast to most other bacteria, strain BH72 was found to cotranscribe a ferredoxin gene with the structural *nifHDK* genes of nitrogenase. Mutational analyses revealed that ferredoxin is not essential for N_2 fixation but for the rapid "switch off" of nitrogenase activity in response to ammonium addition (Egener *et al.*, 2001). The NifA in strain BH72 acts as an essential transcription activator for *nifHDK*. Surprisingly a NifL like protein functionally similar to *γ-Proteobacteria* exists in this *β*-proteobacterial member and is encoded in the *nifLA* operon (Egener *et al*., 2002). P_{II} like proteins are the central signal transmitter proteins in sensing the N-status of cells. Strain BH72 posses' two paralogous gene copies, the *glnB* and the *glnK* as other *Proteobacteria*; a third copy, the *glnY*, has recently been identified in

this β subgroup *Proteobacterium*. GlnY can only be detected in a *glnB*$^-$*glnK*$^-$ double knockout mutant and occurs only in the uridylylated state, irrespective of the N-status of the cell (Martin *et al.*, 2000). Moreover it has already been reported that P_{II} like proteins in strain BH72 have distinct roles for the physiological "switch off" and post translational covalent modification of dinitrogenase reductase upon ammonium addition or anaerobiosis. Moreover the AmtB protein (encoded from a *glnK-amtB* operon in strain BH72) was found to be essential for ammonium induced switch-off, serving probably as an ammonium sensor transmitting the signal to membrane associated GlnK (Martin *et al*., 2002).

In this study, an attempt has been made on one hand, to map transcription start points of some characterized *nif* operons (*nifHDK* and *nifLA*) from strain BH72 and verify their cotranscription status (*nifHDK* with *fdxN* and *nifL* with *nifA*). Side by side, the effect of nitrogen on *nifA* transcription has been investigated by RT-PCR. On the other hand, an effort has been made to clone and characterize the *ntrBC*-like genes from strain BH72, map its transcription start by primer extension, check the effect of nitrogen on its expression and find out whether *ntrBC* in strain BH72 is auto regulated. A marker exchange *ntrBC* deletion mutant of strain BH72 was generated to answer several questions related to its regulatory aspect. Examining such diverse functions as "twitching motility" and trying to find out any role of NtrBC in this aspect, studies were addressed to check the effect of nitrate on the *ntrBC* mutant, not only on its growth and colony morphology but also on the expression of assimilatory nitrate reductase. The effect of nitrate together with nitrogen has also been studied to explore whether NtrBC mediated regulation of *nifHDK* and *nifLA* expression exists in strain BH72. In context of N_2 fixation, the P_{II} proteins play an important role. So attempt has been made on one hand, to study the effect of different nitrogen sources on the expression of *glnB* and *glnK* by reporter gene fusion and RT-PCR and on the other hand to check whether NtrBC from strain BH72 has any effect in modulating their expression. Along with this, it is very interesting to check whether NtrBC has any effect on the expression of the third P_{II} paralogue GlnY, which is otherwise expressed only in a *glnB*$^-$*K*$^-$ background. Some of the other approaches made in the following work include amplification of N-assimilatory genes encoding for GS and GOGAT by RT-PCR using the information from the unreleased data of *Azoarcus* genome project, Bielefeld, check the effect of nitrogen on their expression by RT-PCR and find out whether NtrBC from strain BH72 exert a regulatory function on their expression.

3 Material and methods

3.1 Material

3.1.1 Chemicals

As long as not mentioned specifically, chemicals and reagents were mostly purchased from the following companies: Fluka (Buchs, Switzerland), Riedel de Haen (Seelze), Serva (Heidelberg) and Sigma (Deisenhofen). Complex Media and Agar were from Difco (Detroit, Michigan, USA). Restriction enzymes and DNA dependent DNA polymerases were usually from New England Biolabs (Schwalbach) and Amersham Biosciences. AMV Reverse transcriptase was purchased from Roche. Agarose used for gel electrophoresis was from Biozym (Hess, Oldendorf) and Serva (Heidelberg).

3.1.2 Gases

N_2 and H_2 (purity of each 99.993%), C_2H_2-acetylene (99.6%) and C_2H_4-ethylene (51ppmv $\pm$ 2% in N_2) were purchased from Messer Griesheim (Siegen).

3.1.3 Strains and plasmids

The bacterial strains used in this work are listed in Table 1. and plasmids used are listed in Table 2.

Table 1. Bacterial strains used in this work with description.

Strain	Description	Source or reference
E. coli		
DH5α	*F' recA1 endA1 hsdR17*(r_k-, m_k+) *supE44* (λ^- *thi*-1*relA1*ϕ80d*lacZ*Δ*M15*Δ*(lacZYA-argF)*$_{U169}$	Hanahan (1983)
MC1061	*hsdR araD139* Δ(*araABC-leu*)7679 Δ(*lac*)× 74*galU galK rpsL thi*	Meisnner *et al*., (1987)
Azoarcus sp.		
BH72	wild type	Reinhold *et al*., (1986)
BntrBsp	Sm/Sp^R, BH72 *ntrBC*::Sm/Sp^R	This study
BntrBKan	Km^R, BH72 *ntrB*::Km^R	This study
BGLK-GUS	Ap^R, BH72 *glnK::gusA* (chromosomal fusion)	pSGLK-GUS
BGLY-GUS	Ap^R, BH72 *glnY::gusA* (chromosomal fusion)	pSGLY-GUS
BGLB-GUS	Ap^R, BH72 *glnB::gusA* (chromosomal fusion)	pSGLB-GUS
BNtrBspGLK-GUS	Ap^R, BntrBsp *glnK::gusA* -chromosomal fusion	pSGLK-GUS
BNtrBspGLY-GUS	Ap^R, BntrBsp *glnY::gusA* -chromosomal fusion	pSGLY-GUS
BH72(pNHGUS)	Tet^R, Transconjugant BH72 with pNHGUS	This study
BH72(pLGUS)	Tet^R, Transconjugant BH72 with pNLGUS	This study
BntrBsp(pNHGUS)	Tet^R, Transconjugant BntrBsp with pNHGUS	This study
BntrBsp(pLGUS)	Tet^R, Transconjugant BntrBsp with pNLGUS	This study
BHNTR-GUS	Ap^R, BH72 *ntrB::gusA* (chromosomal fusion)	pSNTR-GUS

TABLE 2. Plasmids used in this work with description.

Plasmid	Description	Source or Reference
Cloning vectors		
pBK-CMV	Km^R, Neo^R, ColE1-Replicon, f1(-)origin, SV40ori.	Stratagene
pBSKII	Ap^R, ColE1-Replicon	Stratagene
pBKSII	Ap^R, ColE1-Replicon	Stratagene
pUC4K	Ap^R, Km^R, Km-resistance cassette	Pharmacia

pHP45Ω	Sp/Sm^R, Sp/Sm-resistance cassette	(Prentki and Krisch, 1984)
pLAFR3	Tet^R, Cosmid vector	(Staskawicz *et al*., 1987)
Constructs used		
pDZD3	Km^R, 3.7 kb chromosomal *Sau*3AI fragment having *glnB* locus in pBK-CMV	Martin *et al*., 2000
pDZD6	Km^R, *glnY-amtY* locus on a 5.5 kb chromosomal *Sau*3A fragment in pBK-CMV	Martin *et al*., 2000
pDZD17	Km^R, *glnK-amtB* locus on a 5.8 kb chromosomal *Sau*3A fragment in pBK-CMV	Martin *et al*., 2000
pDZD41	Km^R, *Xho*I subclone of pDZD4 having upstream of *ugk* in pBK-CMV	Martin,1996
pNTRC2	Km^R, 6.5 kb phage bank clone bearing the *ntrC* region in pBK-CMV	Egener, 1998
pSNTR2.1	Km^R, 4.5 kb subclone from pNTRC2; *Hin*dIII digested and religated in pBK-CMV.	This study
pSNTR2.7	Ap^R, 1.2 kb *Sma*I fragment from pSNTR2.1; subcloned in pBKSII	This study
pSNTR2.8	Ap^R, 1.87 kb *Sma*I fragment from pSNTR2.1; subcloned in pBKSII	This study.
pSNTR2.9	Ap^R, 0.54 kb *Pst*I subclone from pSNTR2.1 in pBKSII	This study
pSNTR2.6	Ap^R, 0.76 kb *Pst*I subclone of pSNTR2.1 in pBSKSII	This study
pSNTR2.5	Ap^R, 0.51 kb *Pst*I subclone of pSNTR2.1 in pBKSII	This study
pSNTR2.10	Ap^R, 0.3 kb *Pst*I subclone of pSNTR2.1 in pBKSII	This study
pSNTR2.2	Km^R, *Pst*I digested pNTRC2, and religated	This study.
pSNTR2.3	Km^R, *Xho*I digested pNTRC2, and religated	This study
pSNTR2.1Ω	Km^R, Sp/Sm^R, 1.55 kb region between the two extreme *Nru*I sites (after complete digest) deleted from pSNTR2.1 and replaced by Ω cassette.	This study
pBKSNTR2.1	Ap^R, *Asp*718-*Sst*I fragment from pSNTR2.1 (*ntrBC* region) cloned in pBKSII	This study
pBKSNTR2.1kan	Ap^R, Km^R, Kanamycin cassette from pUC4K inserted in the *ntrB* region at *Nru*I site.	This study
pNHGUS	Tet^R, *nifH::gusA* fusion in pLAFR3	Egener, 1998

pLGUS	Tet^R, *Hind*III-*Bsr*BI-fragment of a part *nifL* gene along with its upstream, cloned in pLAFR3-GUS	Egener, 1998.
pSGLK-GUS	Ap^R, 2.4 kb *Kpn*I-*Sal*I fragment from pDZD17 (*glnK*-region) cloned in pBluescript II SK; *gusA* being inserted at *Eco*RI site in the vector at right orientation.	This study
pSGLY-GUS	Ap^R, 1.5 kb *Sal*I fragment from pDZD18 (*glnY*-region) cloned in pBluescript II SK; *gusA* being inserted at *Eco*RI site in the vector at right orientation.	This study
pSGLB-GUS	Ap^R, 0.9 kb *Sst*I-*Bgl*II fragment from pDZD3 (*glnB*-region) ligated to *Sst*I-*Bam*HI digested pBKSII; *gusA* being inserted at *Eco*RV site in the vector at right orientation.	This study

3.2 Culture media and growth conditions.

All culture media were normally autoclaved at 121°C for 30 minutes. Non-autoclavable stock solutions were usually sterile filtered using filters of pore size 0.2 μm (Nr.FP 030/3 from Schleicher und Schuell, Dassel). For making solid media, I.5% Agar was usually added to the medium.

3.2.1 Media for *E. coli*

LB medium (Luria-Bertani medium, *Sambrook et al.*, 1989)

10g/l Bacto-Tryptone

5g/l Yeast Extract

10g/l NaCl

pH : 7 (adjusted with NaOH).

LB+ medium (Kushner, 1978)

10g/l Bacto-Tryptone

5g/l Yeast extract

10g/l NaCl
10mM $MgSO_4$
10mM $MgCl_2$
2.5mM KCl
pH 7.0 (adjusted with NaOH)

SOC medium
Bacto-Tryptone 20g/l
Yeast extract 5g/l
After autoclaving, the following solutions were added sterile to 1l of the medium
20mM Glucose
2.5mM KCl
10mM $MgSO_4$
10mM NaCl
10mM $MgCl_2$

3.2.2 Media for *Azoarcus* sp. BH72

VM-Ethanol medium (Reinhold-Hurek *et al.,* 1993)
0.4g/l KH_2PO_4
0.6g/l K_2HPO_4
1.1g/l NaCl
0.5g/l NH_4Cl
0.2g/l $MgSO_4.7H_2O$
26.4mg $CaCl_2$
10mg/l $MnSO_4.7H_2O$
2mg/l $Na_2MoO_4.2H_2O$
66mg/l Fe(III)-EDTA
1mg/l Yeast extract
3g/l Bacto-Tryptone
6ml/l Ethanol (sterile filtered and added after autoclaving)
pH 6.8

VM-Malate medium (Reinhold-Hurek *et al.*, 1993)
Equivalent to VM-Ethanol medium; only 6ml/l Ethanol replaced by 5g/l Malic acid and 4.5g/l KOH.

SM medium (Reinhold *et al.*, 1986)

0.4g/l KH_2PO_4

0.6g/l K_2HPO_4

0.1g/l NaCl

0.2g/l $MgSO_4.7H_2O$

26.4mg/l $CaCl_2.2H_2O$

10mg/l $MnSO_4.7H_2O$

2mg/l $Na_2MoO_4.2H_2O$

66mg/l Fe(III)-EDTA

5g/l Malic acid

4.5g/l KOH

pH 6.8

SM medium with Nitrogen-Source (Reinhold *et al.*, 1985)

Equivalent to SM medium: However each time, a Nitrogen source in form of 10mM NH_4Cl or KNO_3 was added and called SM+N or SM+NO_3 respectively.

KON medium

Similar to SM medium but supplemented with 5 g / l Yeast Extract and 1 g / l NaCl

Selection (SEL) medium.

SM medium with 6 ml / l ethanol instead of potassium malate and (1 g / l) KNO_3

3.2.3 Antibiotic and other supplements

While growing a strain resistant against a particular antibiotic, the medium was supplemented with sterile filtered antibiotic which was added after the medium was autoclaved and cooled down. Following antibiotic concentrations (μg/l) were used for growing *E. coli* or *Azoarcus* sp. BH72 respectively: Ampicillin (150/50), Kanamycin (50/50), Streptomycinsulphate (20/20), Spectinomycin (40/20), and Tetracyclin (12/12).

3.2.4 Cultures for *E. coli*

Unless not stated, *E. coli* cultures were grown aerobically in LB medium at 37°C under constant shaking at 200 rpm. Purity of the strain was controlled either by microscopic examination or by testing the genetic markers (plasmids and antibiotic-resistance). An effective long term preservation of *E. coli* strains were made by mixing the culture in glycerine buffer (65% Glycerine, 0.1M $MgSO_4$, 25mM Tris-HCl, pH 8.0) 1:1 and then storing it at –80°C in glass vials.

3.2.5 Cultures for *Azoarcus* sp.

Unless stated otherwise, *Azoarcus* cultures were grown aerobically at 37°C in VM-Ethanol medium with constant shaking. Media were usually supplemented with appropriate antibiotic while growing mutant strains. Purity of strains was checked either by microscopic examination or genetic markers. The strains could be successfully stored at 4.C in VM –Ethanol-Agar plates for short term. For long term, the cultures were stored in 10% Dimethylsulfoxide solution (DMSO) in liquid nitrogen.

3.2.6 Set up of N_2 fixing cultures of *Azoarcus* sp. BH72

3.2.6.1 Cultures in semisolid medium

In order to observe the conditions for N_2 fixation, the cells were usually grown in SM medium with 0.2% Agar in glass vials. Under these conditions, the cells can make a thin layer under the agar surface, following the O_2 gradient in the semisolid medium.

3.2.6.2 Batch cultures for N_2 fixation in liquid medium.

Unless stated otherwise, N_2 fixing cells were grown on N-free SM medium under microaerobic conditions in 1l Erlenmeyer flasks. Cells were precultured on SM medium with combined nitrogen (0.05% NH_4Cl, 0.01% yeast extract), washed two times in N-free medium and then inoculated in the same at a final $O.D_{578}$ 0.05. To simulate a N_2 and microaerobic environment, the flasks were sealed with rubber stoppers (suba seals) and the air inside was replaced by N_2 gas. After that, 1.6%

headspace O_2 concentration was set up by injecting sterile air into the flasks and the cultures were usually incubated at 37°C by constant shaking at 100 rpm. In order to measure for subsequent acetylene reduction by gas chromatography, acetylene gas was injected in each flask to final 1% headspace concentration.

3.2.6.3 Cultures in Laboratory fermenter

For setting up N_2 fixing cultures under definitive condition (constant dissolved O_2 concentration of 0.1% at pH 6.8), cultures were grown in a 2 liter lab fermenter (Biostat B, Braun Biotech, Melsungen). By giving air pressure and N_2 injection a stable O_2 concentration could be maintained throughout the culture growth period.

3.3 Gas chromatography

3.3.1 Estimation of oxygen concentration

The oxygen concentrations of N_2-fixing batch cultures were measured by gas chromatograph, model HRGC-4000A (Konik, Barcelona, Spain). The separation of gases was done in a molecular filter column (0.5μm, 80/100 mesh, 2 meter long and internal diameter 0.2 cm) with helium as a carrier gas (1.5 bar column pressure). The injection chamber had a temperature of 112°C and the column temperature was 80°C. The gases were measured by a heat-conducting detector. Room air was injected to calibrate in the beginning.

3.3.2 Estimation of ethylene concentration

Acetylene Reduction Assay (ARA) was used to measure nitrogenase activity. The detection method utilises the reduction of acetylene (substrate analogue of N_2) to ethylene by side products of nitrogenase reaction (H^+). For the separation of acetylene and ethylene, the following gas chromatograph equipped with Porapak-N-column (80/100mesh, 2 m long, 0.2 cm internal diameter) was used. Nitrogen acted as a carrier gas with a column pressure of 1.2 bar. During measurement the chamber temperature was maintained at 112°C and column temperature remained 50°C. The detection used a flame ionisation detector at 220°C fed with H_2 and air pressure. Ethylene (51ppm ± 2%, in N_2) was used to calibrate the detector initially.

3.4 Standard methods for working with nucleic acids

Unless stated otherwise, most techniques used for analysis of nucleic acids (DNA and RNA) was carried out according to standard procedures from Ausbel *et al*., 1987.

3.4.1 Sterilisation

All solutions, reagents and materials needed for working with nucleic acids were autoclaved (30 min at 121°C and 1,3 bar). Glass pipettes and other glassware's were heat sterilised in oven at 180°C, overnight). Special care was taken while working with RNA. All glassware's used for RNA were baked overnight at 180°C. Distilled water used for RNA work was first treated with 0.1% DEPC (diethylpyrocarbonate) and stirred overnight at 37°C. All buffers and solutions used for RNA work were made in DEPC treated water and autoclaved for 1 hour at 121°C. The working place was made RNase free by wiping with 96% ethanol and subsequent flaming the area. Hand gloves were always used while working with RNA for prevention against degradation by stable RNase.

3.4.2 Nucleic acid precipitation

DNA was isolated from the solutions by precipitation. Unless otherwise stated, the precipitation was usually done by adding 1/9 solution volume of 3 M sodium acetate solution pH 5.2. This was followed either by adding 2.5 times the volume ice cold ethanol and 15 min incubation at –80°C or by adding equal volume room temperature isopropanol and incubating for 5 min at room temperature. Finally, the nucleic acid pellet was obtained by centrifugation for 10 min at 13,000 rpm at 4°C. The nucleic acid pellets were usually washed twice with 70% ethanol at room temperature. The pellets were air-dried and finally dissolved in 1X TE buffer or distilled autoclaved water. For RNA, pellets were not strongly dried and finally dissolved in DEPC treated water.

3.4.3 Estimation of nucleic acids (DNA and RNA)

The Nucleic acid concentration of a solution was estimated by measuring its absorption at 260nm with the help of a spectrophotometer (Ultrospec 2000, Pharmacia, Freiburg). The DNA concentration was then calculated using the empirical formula for double stranded DNA: μg DNA/ml = $\Delta E_{260} \times 50$ μg/ml × dilution factor (Sambrook *et al*., 1989). The RNA concentration was measured by using the empirical formula: μg RNA /ml = $\Delta E_{260} \times 40$ μg/ml × dilution factor.

3.4.4 Restriction digestion

Restriction digestion of DNA was done routine wise with 1μg plasmid DNA and 5 units of restriction endonuclease for 2 hours or with 3 μg chromosomal DNA and 20 units of restriction endonuclease for 8 – 12 hours. The incubation temperature and buffer conditions of each enzyme were chosen according to manufacturers instruction.

3.4.5 Agarose gel electrophoreses

Unless otherwise stated, the separation of DNA was routinely done in 0.8% - 1.8% agarose gels in TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0) with 3 – 5 V/cm. Electrophoreses of chromosomal DNA were usually done in gels with TBE buffer (45 mM Tris borate, 1 mM EDTA, pH 8.0). DNA samples were usually treated with 1/10 volume loading buffer {20% Ficoll 400, 0.1% bromophenol blue, 0.1% xylenecyanol 0.1% orange G and 1 mg/ml ribonuclease A (optional)}. Samples with low copy plasmids were incubated with loading dye, containing RNase at 37°C for 20 mins prior to gel loading. Bacteriophage lamda DNA (Amersham Biosciences) digested with *Pst*I restriction endonuclease was used as a standard molecular weight marker (size marker) for DNA. For detection of DNA fragments after electrophoreses, the agarose gel was incubated for 20 min in ethidium bromide solution (0.5 μg/ml) and viewed under UV light (302 nm) with a video camera (Image Master VDS,

amersham pharmacia biotech). Photographs were taken on special thermopapers (K65HM, Mitsuibishi, Ratingen).

3.5 Isolation of nucleic acids

3.5.1 Isolation of chromosomal DNA from *Azoarcus* sp. BH72

For isolation of chromosomal DNA, the method from Dhaese *et al*., (1979), and modified by Hurek *et al*., (1993), was routinely used. By this method, the cells were lysed by detergent, proteins removed by enzymatic digest followed by phenol chloroform extraction and DNA precipitation by ethanol. 1.5 ml overnight culture was pelleted by 1 min centrifugation at 13000 rpm. The cells were washed in TES buffer and suspended in 100 µl TE buffer. Then this was treated with 300 µl 5% N-laurylsarcosin solution (Na salt in TE) so that Pronase E at the end concentration of 2.5 mg/ml could be added. This set up was mixed by vortexing and incubated for 1 hour at 37°C. This was followed by two times extraction with TE buffered phenol and with TE buffered chloroform. Subsequently the genomic DNA was precipitated as described before and finally dissolved in TE buffer.

3.5.2 Plasmid DNA isolation

Methods for plasmid DNA isolation from *E. coli* or *Azoarcus* sp. (usually low copy plasmids) varied according to the purpose for its further utilization, although the basic principle remained the same. However, for standard analytical purpose like cloning and hybridisation, the standard plasmid preparation protocol from Birnboim and Doly (1979), and later modified by Ausbel *et al*., (1987) was routinely used. 1.5 ml of overnight culture (3 ml starting cultures when working with low copy plasmids) was centrifuged down (13000 rpm, 1min) and the pellet suspended in 100 µl TE. The cells were then lysed by adding 200µl (200 mM NaOH / 0.1%SDS) and incubated for 5 min at room temperature. This was followed by adding 150 µl ice-cold 3M sodium acetate pH 5.2 for neutralisation. The precipitated protein and lysed cells were centrifuged down and the supernatant containing the DNA was concentrated by further precipitation and washed by 70% ethanol according to the standard methods as had been discussed previously.

When the plasmid DNA was to be used for sequencing, the isolation was made by using QIAGEN tip 100 columns (catalogue no. 10014, Qiagen, Hilden). This method depends on reversible binding of DNA in the columns. The isolation process using these columns was strictly according to manufacturer's instruction.

3.5.3 Isolation of DNA from agarose gels and solutions

The isolation of DNA fragments from agarose gels and solutions were made using QIAquick Gel Extraction Kit or QIAquick PCR Purification Kit respectively (Nr. 28704 / 28106, Qiagen, Hilden). The general principle of these kits depends on ion dependent reversible binding of DNA fragments in anion exchange columns. The process of using these kits was strictly according to manufacture's instruction.

3.5.4 Isolation of RNA

While handling with bacterial RNA, special care was taken to prevent degradation by RNases. All reagent and solutions were DEPC treated, autoclaved for 1 hour: glasswares were baked and hand gloves were always used while working.

3.5.4.1 Hot phenol method for RNA isolation

This method follows from the protocol from Aiba (Aiba *et al*., 1981). 100 ml culture (O.D. 0.5) was centrifuged down (4000 rpm, 10 min) and suspended in 3 ml "hot phenol buffer" (20 mM sodium acetate, 0.5% SDS, 1mM EDTA, pH 5.5). This was followed by three times extraction by hot (60°C) phenol (equilibrated with 20 mM sodium acetate, pH 5.5. The RNA was finally precipitated from the upper phase by adding 3 volumes of ethanol (-20°C) and finally dissolved in DEPC treated water. The RNA concentration was estimated by using the standard formula, μg RNA / ml = E_{260} $\times$ 40 μg / ml $\times$ dilution factor.

3.5.4.2 Isolation of RNA using kit (peqGOLD Trifast)

For a fast and clean isolation of bacterial RNA, peqGOLD Trifast kit from peQLab (catalogue no. 30-2010) was routinely used. Usually, 1 ml of peqGOLD solution was

added to frozen cells, collected from 50 ml of culture. The cells were suspended in this solution and allowed to stand for 5 min. To this, 200 μl of chloroform was added, mixed and allowed to stand in room temperature for 10 min, during which phase separation could be seen. After 5 min centrifugation, the upper phase (containing RNA) was collected in new cup and precipitated by adding equal volume isopropanol. The RNA pellet obtained after centrifugation was washed 2 times with 75% ethanol and finally dissolved in DEPC treated water.

3.5.4.3 DNaseI treatment of RNA

The RNA isolated by routine procedures could not be used directly for RT PCR (synthesis of cDNA and its subsequent amplification) as it often contained contaminations from genomic DNA. To circumvent this, the isolated RNA was treated with DNaseI. DNaseI is an endonuclease that degrades double stranded DNA (nicks or breaks) to produce 3'-hydroxyl oligonucleotides. Divalent cations like Mg^{++} or Mn^{++} are absolutely crucial for its activity. About 2 -3 μg RNA was mixed with DNase reaction buffer (1X) and about 30 units of RNase free DNaseI (Roche) in a total 50 μl set up and incubated for 20 min at 37°C. This was followed by an immediate inactivation of DNaseI by phenol chloroform isoamylalcohol (pH 4.0) treatment. RNA was precipitated from upper phase using standard precipitation technique by chilled ethanol washed 2 times with 75% ethanol, dissolved in DEPC treated water, and estimated spectrophotometrically.

3.6 Cloning

3.6.1 The cloning vector

Unless stated otherwise, pBluescript SK / KS (Stratagene) and pLAFR3 (Staskawicz *et al*., 1987) were usually used as cloning vectors. Mostly these vectors were used so that there is a possibility to screen recombinant clones (containing the insert fragment) by blue white selection. The plasmids pBSKII and pBKSII differed from each other in context of orientation of their multiple cloning sites. Both used ColE1 Replications origin and could maintain high copy number (of about 200) per *E. coli*

cell The bluescript vectors contained a copy of the *bla* gene so that when transformed by these plasmids the cells were resistant to ampicillin.

For transformation in *Azoarcus*, broad host range plasmid like pLAFR3A was used. This vector provided tetracycline resistance to its host and could be maintained in a low copy number inside the cells.

3.6.2 Construction of recombinant plasmid

3.6.2.1 Preparation of vector and insert (along with its modification if necessary)

For normal cloning, the vector and the cloned DNA fragment were so digested with restriction endonucleases so that they might form compatible ends to be ligated. When this option did not work, the cohesive ends produced by restriction endonucleases were treated with modifying enzymes (T_4 DNA polymerase or DNA polymerase I (*klenow* fragment)) to produce blunt ends To prevent religation of vector by its compatible ends, the digested vector was treated with alkaline phosphatase (Roche) which removed the 5' phosphate from the digested vector prior to ligation with the insert fragment.

The cloning fragments (vector and insert) were restriction digested and run in agarose gel for separation. The respective fragments were isolated and cleaned from the gel slices as had been discussed earlier.

3.6.2.2 Set up of ligation

Normally for a good ligation, 3 –4 fold more of insert fragment was used compared to that of the digested vector. A reduced reaction volume (10 µl) of the ligation set up, usually gave much better result.

For cohesive end ligation, 0.1 unit of T_4 DNA Ligase (GIBCO BRL) was used. For best result, the ligation was carried out for 1 hour at 12°C helping the sticky ends to anneal to each other followed by 2-3 hour incubation at room temperature (22°C) at which the ligase is highly active.

For setting up blunt end DNA ligation, a higher (insert: vector) ratio and more units of T_4 DNA ligase (1-2 unit) was used per set up. The ligation was carried out by incubating the ligation mix at room temperature for 2 – 3 hours. Following ligation, *E.*

coli DH5α were transformed by the ligation mix as will be discussed in the following section

3.7 Transfer of foreign DNA into bacterial cells.

3.7.1 Transfer of DNA in *E. coli* cells.

The transfer of DNA in *E. coli* cells was done usually by employing the method of transformation. For this purpose the cells had to be made competent and were stored in –80°C prior to transformation. Normally the cells could be made $CaCl_2$ competent which then could be transformed by "heat shock method" (Kushner, 1978) or they were made electro competent and subsequently transformed by electroporation.

3.7.1.1 Transformation by $CaCl_2$ and heat shock

According to this method as described by (Kushner, 1978), the cells were washed in a solution containing $MnCl_2$, $CaCl_2$ and $RbCl_2$ so that the cells are competent to take DNA. The exact mechanism of DNA uptake is still not known. From a fresh overnight incubated LB plate, 5 ml preculture of LB+ medium was inoculated and allowed to grow under constant shaking at 37°C until OD_{578} of 0.5. From this preculture, 80 ml of main culture in LB+ medium was inoculated and allowed to grow under the same conditions until OD_{578} of 0.4 was attained. The cells were then cooled down in ice, centrifuged down (5 min, 5000rpm) and suspended in 20 ml TF1 (10 mM MES, 50 mM $MnCl_2$, 10 mM $CaCl_2$, 100 mM $RbCl_2$). After another round of centrifugation, the cells were resuspended in 2 ml ice cold TF2 (10 mM PIPES, 75 mM $CaCl_2$, 75% glycerol) and distributed in eppendorf cups in 100 µl aliquots and immediately frozen in dry ice and stored at –80°C, until transformation.

To 100 µl thawed competent cells, 10 µl of DNA to be transformed was added. It was incubated in ice for 10 min, heat shocked at 42°C for 2 min and immediately mixed with 1ml LB .The cells were incubated at 37°C for 1hour (shaking) and then plated in LB containing antibiotic.

3.7.1.2 Transformation by electroporation

By this method the cells were subjected to a strong electric field so that the membrane structure is disturbed and the foreign DNA is transferred inside the cells (Neumann *et al.*, 1982; Zimmermann *et al.*, 1981). The method followed from Dower (1988) and developed by Ausbel *et al.*, (1987). From a preculture in exponential phase, 500 ml of main culture was inoculated in Erlenmeyer flasks and cultured for 2 – 3 hours till OD_{578} of 0.5 was attained. The cells were then washed many times in 10% ice cold glycerol, suspended in 400 μl of the same, distributed in 100 μl aliquots and immediately frozen. For electroporation, 1 – 10 μg of DNA to be transformed was dialysed against water in special filter plates (VSWPo 2500, Millipore, Eschborn). The electroporation of *E. coli* was done in 0.2 cm cuvettes (Eurogenetic, Seraing, Belgium) with a Gene Pulser (biorad, Munich). Following parameters were installed before starting: 25 μF, 200Ω, 2.5 kV and voltage 12.5 kV / cm. The time constant after electroporation should be 0.48 – 0.5. After 1 hour of outgrowth in LB medium , the cells were plated on antibiotic plates.

3.7.1 Transfer of DNA in *Azoarcus*

In this work, foreign DNA in form of recombinant plasmids were transferred in *Azoarcus* either exploiting transformation by electroporation or by triparental conjugation. The former method was exploited when the gene of interest was intended to integrate in the genome by stable recombination events. In this case the insert was borne in low copy plasmids (eg pBluescript), which is usually not stable in *Azoarcus*. On the other hand, when the gene of interest was intended to be expressed in moderate level in trans, triparental mating by conjugation was the usual choice. In this case, the insert was borne in low copy broad range host vectors like pLAFR.

3.7.1.1 Electroporation of *Azoarcus*

The process of electroporation of *Azoarcus* is similar to *E. coli* in several aspects as had been described. However there are certain differences. Once the cells were made competent, they were electroporated immediately (cannot be stored in –80°C). The cells were grown in VM malate for preculture and also for the main culture. After

electroporation, they were cultured in VM ethanol medium for 90 min and plated in VM ethanol plates with antibiotics. Cuvette length of 0.1 cm was used and time constant after electroporation usually exhibited lower value (0.45), in contrast to *E. coli* electroporation events. Also the efficiency of transformation in *Azoarcus* is drastically low as compared to that in *E. coli*.

3.7.1.2 Conjugation of *Azoarcus* by Triparental mating

Mobilized plasmids from a donor strain, taking the help of transfer gene *tra* from a helper strain can be transferred to a recipient strain by the method of conjugation. The donor (*E. coli* DH5α bearing the transferable plasmid -pLAFR), the helper (*E. coli* DH5α bearing the natural plasmid pRK2013) and the recipient (*Azoarcus* sp. BH72) were grown in separate cultures until an OD_{578} of 1.0 was attained. Each culture was centrifuged down, resuspended to a final OD_{578} 1.0, mixed in the ratio (1:1:100) and plated on KON agar plates. After 5–7 hours of incubation at 37°C, the cells were scraped from the surface of KON plates, washed in selective medium (SEL), and finally plated on SEL plates with antibiotics with different serial dilution. This process could select the transconjugants.

3.8 DNA hybridisation techniques

DNA fragments of interest specifically hybridised to a complementary and labelled DNA fragment (probe) could be detected for analysis. In the following work, Digoxygenin labelled DNA probes had been used. The hybridised probe could be detected secondarily by a specific antibody, which could recognize a steroid haptene and itself is linked to conjugated enzyme at its conserved Fc portion. This could then catalyse a reaction transforming the substrate into a chemeluminogenic or chromogenic product, which could be detected as signal. For hybridisation, the DNA of interest must already bind single stranded to a membrane.

3.8.1 DNA transfer to the membrane (Southern blot)

By this method of Southern transfers (Southern, 1975), the DNA fragments electrophoretically separated in agarose gel, were transferred into a membrane by

the help of capillary action and finally covalently attached to the membrane by UV crosslinking. For the transfer of chromosomal DNA fragments, the agarose gel was incubated for 10 mins in 0.25 M HCl for depurination. This was followed by alkaline denaturation of the gel (2 × 20 min in 1.5 M NaCl, 0.5 M NaOH) for strand separation so that the transfer is facilitated. Alkaline denaturation was followed by neutralization (2 ×20 min in 0.5 M Tris-HCl, pH 7.2, and 1 mM EDTA) before the treated gel was placed below a nylon membrane (Hybond N, Amersham, Braunschweig) for transfer, along with high salt buffer (20X SSC). Following overnight transfer, the DNA was permanently fixed with the membrane by UV crosslinking using 0.4 J/cm^2 energy (Transilluminator Fluonik, Biometra, Göttingen).

3.8.2 Labelling DNA probes for hybridisation

DNA fragments used as probes were labelled with Digoxygenin, using “the DIG – DNA Labelling & Detection Kit “ from Roche. DNA fragment to be used, as probe was first obtained by resriction digest or PCR amplification. The fragment was denatured by boiling for 10 min in a 16μl reaction volume followed by quickly chilling in ice. Then 4 μl of DIG–High Prime mix (containing random primer, polymerase, dNTP and Digoxygenin labelled UTP) was added to the denatured DNA and incubated for 1 hour or overnight at 37°C. The reaction was stopped by heating at 65°C for 10 min. Finally the labelled probe was purified from free dNTPs using sieve chromatography by passing it through Bio gel 250 and eluting it in 1X TE buffer pH: 8. A portion of this purified probe was used in the hybridisation solution.

3.8.3 Hybridisation

The membrane with crosslinked DNA fragment was incubated for 1 hour at 65°C in hybridisation solution (6X SSC, 5X Denhardt’s solution, 0.5% SDS) for prehybridisation. The labelled probe mixed with the hybridisation solution was boiled for 10 min before it was added to the membrane. Hybridisation was carried out at 65°C with constant shaking for minimum 8 hours; overnight incubation was however the best option. Following hybridisation the membrane was washed at 65°C with (2X SSC, for 15 min; 2X SSC / 0.1% SDS for 30 min; and finally 0.1X SSC / 0.1% SDS for 10 min).

3.8.4 Detection of the probe

The hybridised probe was detected by specific antibody against Digoxygenin epitopes. The Fc portion of the antibody was conjugated to enzyme alkaline phosphatase. Depending on the nature of the substrate added at the end, a coloured end product (colorimetric detection) or a precipitate of luminescent product (chemeluminescent detection) occurred. The colorimetric detection was done using the follow up of the "DNA-Labelling and Detection Kit" (Roche Applied Sciences) using manufacturer's instruction. Chemeluminescent detection was performed using TROPIX- system (Serva, Heidelberg), following strictly the manufacturer's instruction. The light emission signal was permanently documented by exposing the membrane to X ray films (X-OMAT, Kodak, Rochester, New York, USA) and subsequent developing and fixing of the film.

3.9 Amplification of DNA by PCR

The polymerase chain reaction (PCR) was routinely used for amplification of DNA fragments. The set up of PCR reactions varied according to purpose needed.

3.9.1 Standard method of amplification of plasmid or genomic DNA

For general purpose, DNA fragments were amplified following the standard protocol from Innis and Gelfand (1990), using 1.25 Units Taq polymerase (Beckman, Munich), standard buffer with 1.5 mM $MgCl_2$, 20 μM of each dNTP and 50 pmole of each primer (forward and reverse) in 50 ul of reaction volume. Usually a standard PCR programme consisted of a denaturation step at 95°C for 5 min linked to a loop of 35 to 45 cycles each having a denaturation step at 95°C, a primer annealing step ranging between 50°C to 65°C (2 – 4°C below the primer melting temperature) and an elongation step by the polymerase at 72°C. The programme usually ended with a final extension step at 72°C for 10 min. The annealing temperature of the primers with the template was determined according to the melting temperatures (Tm) for each primers using the standard formula: Tm (°C) = 81.5 + 0.41×(% GC content)– 600/N +16.6×$\log_{10}$ M where N refers to the number of bases in the primer and logM

refers to the logarithm of the molarity of Na^+ and K^+ concentration present in the PCR reaction.

3.9.2 PCR amplification using ProofStart polymerase

The proof reading activity of normal Taq polymerase is quite low, resulting in incorporation of wrong bases in the PCR product. To circumvent this problem, ProofStart DNA polymerase (Pfu) from Qiagen was used which provided robust PCR performance in high-fidelity PCR. Some unique features adjoined to this method were (an initial 5 min activation step at 95°C, a final concentration of 300 μM of each dNTP, longer extension times at 72°C and about 45 rounds of cycling). When a PCR system did not work well under standard conditions, a special buffer (Q solution provided with the kit) was added. This enabled amplification of a previously failed reaction simply by changing the melting behaviour of DNA.

3.9.3 PCR amplification using RT-PCR beads

Ready-to-go-beads (Amersham Pharmacia Biotech) are basically RT-PCR beads designed as pre-mixed, pre-dispensed reactions for performing RT-PCR. They are provided as dried beads that are stable at room temperature and contain all of the necessary reagents (including M-MuLV reverse transcriptase and Taq DNA polymerase), except primer and template, for performing a one-tube one-step RT-PCR reaction in a volume of 50 µl. The reactions were usually incubated for 30 min at 42°C (formation of reverse transcribed cDNA), a denaturation at 95°C for 5 min and eventual linking to the loop of denaturation, annealing and extension.

3.9.3.1 Semi quantitative RT-PCR

In order to have an idea about the amount of a particular transcript under a given culture condition and to compare this with another condition, a semi quantitative approach was employed. After a reverse transcription of the RNA, when the amplification of cDNA was ongoing, equal amount of samples were pooled out from different tubes (to be compared) at different time points (18-28 cycle number). When electrophored in an agarose gel, the RT-PCR products exhibited differential ethidium bromide staining intensities, which was a linear function (or clear reflection) of their

relative abundance. The primers used in this work (PCR / RT-PCR) are listed below in form of Table 3.

TABLE 3. Description of various primers used for PCR / RT-PCR

Application	name	sequence
Cotranscription of *nifHDK* & *fdxN*	nifKfw	5' CGCCTTCGCGGCACCCGT 3'
	Fdxrev	5' CAGTCACCACAGGACGTGCAT 3'
Cotranscription of *nifL* & *nifA*	RTnifLAfor	5' GAGAACGGCCAGGTCGACGTGGA 3'
	RTnifArev	5' GTTGAAGCCGCACTCCTCGTCGAGCA 3'
RTPCR (*nifA*)	nifArev3RT	5' TCGTCCAGGTGCTCGCGGCTG 3'
	nifAfor1RT	5' ATGAGCGCGGCCGGTCCGATG 3'
	nifArev2RT	5' CACGGTTTCGTGCCCGGCGCG 3'
RTPCR(16SrRNA)	1401rev	5' CGGTGTGTACAAGACCC 3'
	104f	5' GGCGAACGGGTGMGTAAYGCACTGG 3'
	1346rev	5' TAGCGATTCCGACTTCA 3'
Cotranscription of *ntrB* and *ntrC*	RTntrBbridgeF	5' GGATGAGATTCGCGACAAGAT 3'
	RTntrCbridgeR	5' CGGTCATGATGATGACCGGAA 3'
Cotranscription of *ugk* and *glnK*	RTugk-F	5' GCGGCATATCCGGTTACCGAAGA 3'
	RTglnK-R	5' GATCTTCACCTTGGGCAGGAAGTCGA 3'
ntrB amplification	ntrBfor	5' AAGCTTGCTCGATCCTCCTCCTCCCAG 3'
	ntrBrev	5' ATTAATTCAGGTCCGATCGGTAATCGGC 3'
ntrC amplification	ntrCfor	5'AAGCTTAATACCGTCTGGATCGTGGATGACG 3'
	ntrCrev	5' ATTAATTCAGTCCTCGTGCTCGCCTTCG 3'
glnB amplification	glnBfor	5' GCATGAAGAAGATCGAAGCGATCATC 3'
	glnBrev	5' CGGGATCCGATTGCGGCTTCGTTGGTTTC 3'
glnK amplification	glnKfor	5' GCATGAAGTTCATCACAGCGATCATC 3'
	glnKrev	5' CGGGATCCCAGGGCGTCGGCCCCGGTTTC 3'
glnY amplification	glnYfor	5' GCATGAAACTGATCACCGCAATCATC 3'
	glnYrev	5' CGGGATCCGAGGGCGTCGGCACCGGACTC 3'
GS3 amplification	GS3forRT	5'GCTACGTCCTGGCCCAGTTCGTCGATA 3'

	GS3revRT	5'GCAGTAGCGAGAACTCCGGCTCCA 3'
GOGAT amplification	GOGAT RTfor GOGAT RTrev	5'GAGTGTTCATCGAGCACGTCGGCATC3' 5'GTTGTGCAGATGGGACTTGCCGCAT3'
Assimilatory nitrate reductase amplification	nir forRT NO3revRT	5'GCTGTCCTACGGCGTGCTCGAAGC3' 5'GCTGGCTGCTTGGACACCGG3'
Sequencing primers for pBluescript & pBK-CMV	T3 forward T7 reverse	5' AATTAACCCTCACTAAAGGG 3' 5' GTAATACGACTCACTATAGGG 3'

3.10 Primer extension

To map the transcription start point of genes, "Primer Extension Method" (Boorstein and Craig, 1989) was performed. The method involved isolation of RNA followed by annealing of the Cy5 labelled primer with the transcript of query and its subsequent extension by AMV reverse transcriptase till the 5' end of the transcript was reached. Finally, by running a sequencing gel with the labelled single stranded cDNA product along with a sequencing reaction of that particular gene, the precise base of the gene, with which the 5' base of the RNA matched (transcription start point of the gene) could be located.

About 30 μg of RNA was incubated with 2.5 pmole of Cy5 labelled primer and 1X AMV reverse transcriptase buffer (Roche Applied Sciences) at 70°C for 2 min in a 20 μl reaction volume followed by "switching off" the thermo block and allowing it to gradually cool to 42°C. The reverse transcription of RNA was performed subsequently at 42°C for 30 min by addition of 1.25 mM each of dCTP, dGTP, dATP, dTTP and 10 U AMV reverse transcriptase to the reaction. After a phenol chloroform isoamylalcohol extraction and ethanol precipitation, the product was dissolved in 1X TE. This was loaded in ALF sequencing gel along with sequencing reaction of the gene, with a prior heating at 65°C for 2 min. The primers used for primer extension are listed in Table 4.

TABLE 4. Description of Cy5 labelled primers used for primer extension.

Application	name	primer sequence
Primer extension *nifH*	TH25rCy	5'TGCCGATACCGCCCTTGCC3'
Primer extension *nifLA*	Cy5PEnifLA rev	5'GATCGCCGACTGCTCCACGG3'
Primer extension *ntrB*	Cy5PEntrB rev	5'CTCTCTGCCGCGATCGGC3'
Primer extension *ntrB*	Cy5PEntrB revUS	5'GCGCTCGTGCAGGTCGAC3'
Primer extension *ugk*	Cy5PEugk rev	5'GTGGGCGGTACCGATCAG3'
Primer extension *glnK*	Cy5PEglnK rev	5'GAACGGCTTGATGATCGCTG3'

3.11 Sequencing of DNA

DNA sequencing was routinely performed in the laboratory following dideoxymethod (Sanger *et al*., 1977). The sequencing reaction was set up using "Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7- deaza- dGTP" (Amersham, Braunschweig) following the manufacturer's instruction with Cy5 labelled primers (Thermo-Hybaid) and template DNA (clean plasmid preparations, minimum 5 μg for sequencing each clone). For each clone, 4 reactions were set up each having ddATP, ddCTP, ddGTP or ddTTP as terminators of these particular bases respectively. The reactions were carried out in a thermo cycler (Cyclogene, Thermo-Dux, Wertheim), under the following set up parameters: 1 min 97°C, followed by 25 cycles of 30 s at 95°C (denaturation) and 30 s at 60°C (primer annealing and DNA synthesis). The sequencing samples were later loaded in a 21% polyacrylamide gel " ReproGel™ Long Read Solutions" (Amarsham Pharmacia Biotech, Uppsala, Sweden), containing 6.2 M urea and separated with 0.6 X ALF TBE buffer in the "ALF Express DNA Sequencer". The fluorescent labelled fragments could be detected by laser and photosensors. The evaluation of the sequencing reactions was done with "ALFwin Software".

3.12 Protein chemistry methods

3.12.1 SDS–PAGE

Preparation of total cell extracts

Cells grown in liquid culture were centrifuged down and suspended in 1 ml extraction buffer (62 mM Tris-HCl pH 6.8, 5% β-mercaptoethanol, 10% glycerol) per 150 mg fresh weight along with 2% SDS and incubated at 95°C for 15 min. The preparation was then centrifuged for 2 min at 13000 rpm and the clear supernatant was collected in a new eppendorf cup and stored at –70°C.

Estimation of protein concentration

The protein concentration when required was estimated following the principle from Bradford (1976) using the BioRad Protein Assay kit (BioRad, Munich), following manufacturer's instruction.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS PAGE was performed following the principle from Laemmli (1970) using discontinuous or disc electrophoresis (Ornstein, 1964) and (Davis, 1964) where a non-restrictive large pore gel, referred to as stacking gel is layered on top of a separating gel. Each gel layer is made with a different buffer and the running buffer is different from the gel buffers. Set up: separation gel: 10-18% polyacrylamide, (30:1 acrylamide: basacrylamide), 0.375 M Tris-HCl, pH 8.8, 0.1% SDS; stacking gel: 5% polyacrylamide, 0.125 M Tris-HCl, pH 6.8, and 0.1% SDS. The polymerisation took place only after addition of 10% ammoniumpersulphate (3 µl / ml) and TEMED (2 µl / ml). For the separation of the two bands of nitrogenase, "a low cross linker gel" with a different polyacrylamide mix (acrylamide: bisacrylamide :: 172 : 1) was used.

Unless stated otherwise, the protein samples to be loaded were treated with 4X loading buffer (62.5 mM Tris-HCl pH 6.8, 15% glycerol, 2.3% SDS, 1.25% β-mercaptoethanol and 0.03% bromophenol blue) and denatured by heating at 95°C for 3 min, before loading in the gel. As molecular weight marker, SDS-PAGE standards, Broad Range (BioRad, catalogue: 161-031) was used when the gels were prepared only for Coomassie staining. Gels, which were transferred to membranes for Western blot, were usually loaded with "Colour Markers For SDS-PAGE and Protein Transfer, Wide Range (MW 6.5KD – 205KD)" (Sigma, C-3437). 1X TGS

(0.025 M Tris-HCl pH 8.4, 0.192 M glycine, and 0.1% SDS) was used as standard running buffer for SDS-PAGE. The gel electrophoresis was done in a mini gel chamber (Biometra, Göttingen) or Mini PROTEAN gel chamber (BioRad). The gels were usually run initially at 10 – 15 mA constant current; the current being increased to 20 – 25 mA when the bromophenol blue entered the separating gel.

3.12.2 Gel staining

The staining of gels was done by Coomassie method. The gels were stained in staining solution (0.25% Coomassie Brilliant Blue R-250 in 45% v/v methanol and 10% v/v acetic acid) for one hour followed by subsequent destaining in (45% methanol and 10% acetic acid) until the protein bands became clear.

3.12.3 Western blot and immunodetection

For the purpose of identification of proteins with specific antibodies, the proteins after being separated by SDS-PAGE were electrotransfered (30 min, 8V) with the help of a Semi-Dry-Electroblotters (Trans-Blot Sd, BioRad, Munich) on a nitrocellulose membrane (BB 85, Schleicher and Schüll, Dassel) placed below the gel. The semidry condition was maintained over the period of transfer by soaking pieces of Whatmann filter papers with transfer buffer (25 mM Tris, 0.2 M glycine, 0.0075% SDS in 20% methanol) and placing the gel & membrane in between them. After the transfer, the membrane was incubated with 5% fat free milk powder in 1X TBS (150 mM NaCl, 10 mM Tris-HCl pH7.5) for 1 hour to prevent non-specific binding of antibodies to the membrane (blocking). The primary antibody (polyclonal) was added in 1:5000 dilution in TBS along with 0.5% BSA and incubated with the membrane overnight at 4°C with constant shaking. Following several washes in TBS (5 –6 times, 5 mins each), the membrane was treated with secondary antibody (pork anti rabbit) for 2 hours in (1:10000) dilution in TBS with 0.5% BSA. After repeated wash in TBS there after, the membrane was ready for chemiluminiscent detection by “ECL Western blotting detection reagents” (Amersham, Braunschweig) following manufacturer’s instruction. The signals were detected by exposing the membrane on X Ray films (Kodak) and subsequent development in developer and fixation. The heterologous antibodies for dinitrogenase reductase (NifH) from *R. rubrum* (presented by P.Ludden) was used to detect the NifH from *Azoarcus* sp.

3.12.4 2D – gel electrophoresis

This process involves separation of proteins first according to its charge (isoelectric point, pI) and then according to its mass. Therefore the cells were treated with a non-ionising detergent (Nonidet P 40) and finally via phenol extraction and precipitation, the protein pellet was isolated.

3.12.4.1 Protein extraction

Cells grown in liquid cultures were centrifuged down and pellet washed in ice cold PBS (150 mM NaCl, 10 mM phosphate pH 7.0). Cells were suspended in 1 ml extraction buffer (0.7 M Saccharine, 0.5 M Tris, 30 mM HCl, 100 mM KCl, 2%β-mercaptoethanol) per 50 mg fresh weight and lysed by ultrasonication (Branson Sonifer, Modell 250). After 30 min incubation in ice, the cell lysate was centrifuged down for 5 min at 1000g. The supernatant was collected, mixed with 700 µl phenol (buffered with 100 mM Tris-HCl, pH 8) and incubated in ice for 30 min. After centrifugation (1000g, 4°C, 10 min), the upper phase was collected and precipitated overnight at –20°C with 5-volume precipitation buffer (0.1 M ammonium acetate in methanol). After centrifugation (5000g, 4°C, 10 min), the protein precipitate was dissolved in loading buffer (9,5 M urea, 2% nonidet P40, 2% ampholine pH 3.5 – 10 (Amersham Pharmacia), 100 mM dithiothreitol) and stored at –70°C for future use.

3.12.4.2 Isoelectric focussing (I dimension)

For isoelectric focussing, IPG – strips (Pharmacia) were used; they contain different immobilised ampholyte (immobiline) which build up a linear pH gradient. Once placed within a strong voltage, in this gradient, the proteins position themselves according to their characteristic isoelectric points, and therefore separate themselves from each other. It is possible to use IPG-strips having pH gradient ranging from 3.5 to 10 or 4 to 7. Once the strips are rehydrated, it is necessary that the strips are soaked in protein solution (300 – 800 ug protein, 8 M urea, 0.5% nonidet P40, 100 mM DTT and 0.5% ampholyte (pH 3.5 – 10)) minimum for 6 hours. The first dimension electrophoresis was carried out in Multiphor system (Pharmacia) according to manufacture's instruction. The IPG strips after isoelctric focussing could be stored at –70°C for future use.

3.12.4.3 SDS PAGE (II dimension)

After the isoelectric focussing, the IPG stripes were treated with equilibration solutions (A & B), each for 15 min, before proceeding towards the II dimension (SDS PAGE); (equilibration solutions: 50 mM Tris HCl pH 6.8, 8 M urea, 30% glycerol, 4% SDS and 3.5 mg/ml DTT (solution A) and 45 mg/ml idoacetamide (solution B)). By this treatment the disulphide bridges are reduced by DTT and iodoacetamide helped in covalent modification. At the end, the IPG strips were placed on 12–18% discontinuous gels (with stacking and resolving zones) and embedded on top of the same with a embedding solution (SDS PAGE buffer with 1.5% agarose) so that the strips are held firmly and the loss of proteins from the strips to the SDS PAGE are minimised. The SDS PAGE was usually run at 55 volts, overnight and then the gel was usually processed for a Coomassie staining or western blot.

3.13 Estimation of β-glucuronidase activity (GUS assay)

The β-Glucuronidase encoded by the *gusA* gene from *E. coli* was used as reporter gene to study the expression of different promoters in *Azoarcus* sp. (transcriptional fusions). The plasmid constructs, having the gene of interest fused with the reporter gene, were either integrated with the *Azoarcus* genome or expressed from a low copy stable plasmid (pLAFR3A), extra chromosomally. Examples of few genes used as transcription fusion with *gusA*, reported in this work are the *nifH*, *nifLA*, *glnB*, *glnK*, *glnY* etc.

Initially, the successful incorporation of *gusA* in the transformed strains was detected by the enzymatic conversion of Xglu to a blue chromogenic product; however the detection method is qualitative in nature. For quantitative estimation of the enzyme activity, para-nitrophenyl-β-D-glucuronide (pNPGlu) was used as a chromogenic substrate for the enzyme whereby the yellow end product could be spectrophotometrically estimated. For this, cells were grown in liquid medium till the optical density of 0.4 was attained. 2 ml of these cells were centrifuged down, washed and suspended in 2 ml of GusA buffer (60 mM Na_2HPO4, 40 mM NaH_2PO4 pH 7.0, 1 mM EDTA and 14 mM β-mercaptoethanol). From this, 1ml of cell suspension was used for OD estimation at 600nm and remaining 2 × 0.5 ml for setting up of the enzyme test. For partial perforation, 0.5 ml of each cell suspension was treated with 25 µl TE buffer and 25 µl 1% SDS, mixed completely by vortexing

and incubated at 37°C for 10 min. Thereafter followed the addition of the substrate (100 µl from 4mg/ml stock solution) and the reaction was stopped by adding 200 µl of stop buffer (2.5 M 2-amino-2-methylpropandiol), when the assay developed yellow colour. The exact time between the addition of substrate and addition of stop buffer was noted down which was used for calculating the enzyme activity. Following centrifugation, the extinction of the yellow supernatant from the enzymatic reaction was measured at 420 nm against a blank (without the cells) under the same conditions. The β-glucuronidase activity was calculated using the standard formula:
Miller Units (U) = $\Delta E_{420} \times 1000 / (t \text{ min} \times OD_{600})$.

3.14 Microscopy

For general examination of colonies, the compound microscope (Axioplan 2, Zeiss) was routinely used. On the other hand, to investigate detailed feature of colonies and examine them under high resolution, the binocular microscope from (Olympus, model: SZX – ILLB200, Olympus optical co. LTD, Japan) was used. Phase contrast images both at high and low resolutions were obtained using the same instrument, both under dark and bright fields.

3.15 Computer programmes used for data evaluation

For the generation of nucleic acid sequences and determination of restriction maps, as well as for the translation of amino acid sequences along with the determination of open reading frames, DNASTAR-programme was used routinely (DNASTAR, London, Great Britain). Translation of protein sequences was also performed using "Translate tool" (www. Expasy.ch/tools/dna.html). Determination of functional domains within protein sequences was carried out using "Smart-Suchmaschine" (http://smart.embl-heidelberg.de) or NPS@ (Network Protein Sequence @nalysis, Lyon, France). Search for homologous proteins was carried out using the data banks of BLAST-programme (Altschul *et al*., 1989). Alignment of homologous proteins with protein of interest was performed using clustal X programme.

4 RESULTS

The aim of the presented work is to study the regulation of genes related to N_2 fixation and N-assimilation in strain BH72 and the involvement of NtrBC in such regulation. Under section 4.1, promoter characterization of the *nifH* and the *nifLA* of strain BH72 by primer extension is carried out along with the study of cotranscription of these genes with their linked partners. Emphasis has also been given to study the transcription regulation of *nifLA* by RT-PCR. Both these candidates are profusely expressed during N_2 fixation and are active members of the N-regulatory cascade. Section 4.2 deals with the identification and genetic organization of the *ntrBC* genes in strain BH72. In section 4.3 the generation of a marker exchange deletion mutant of the *ntrBC* of strain BH72 is in focus. Section 4.4 deals with the study of typical phenotypes manifested by the *ntrBC* mutant. Several aspects of regulation of the *ntrBC* operon in strain BH72 have been studied in section 4.5. Section 4.6 is a broad section dealing with the putative targets under the NtrBC control. This aspect has been dealt under three broad subheadings: transcriptional regulation of genes for N_2 fixation by NtrBC (4.6.1), transcriptional regulation of *gln* genes of strain BH72 and role of NtrBC (4.6.2) and study of genes for N-assimilation and the role of NtrBC in this respect (4.6.3).

4.1 Transcript analyses of genes (*nifH* and *nifLA*) related to N_2 fixation in strain BH72

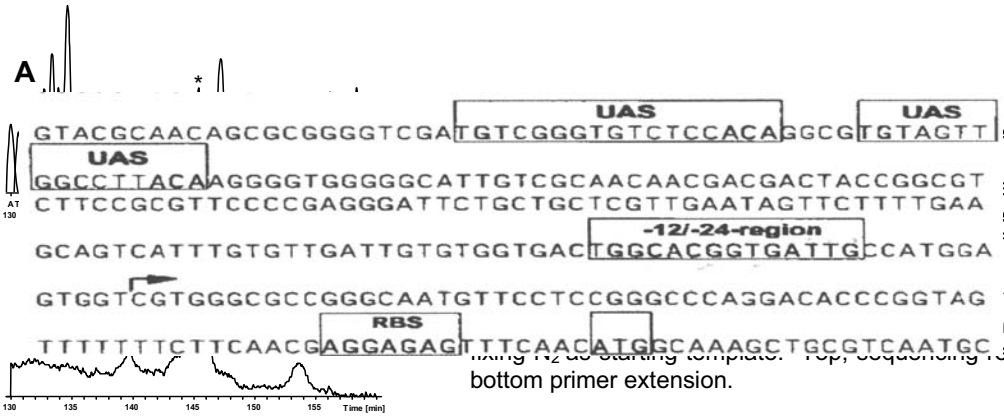
The aim of this section is to analyse the transcripts of important genes required in strain BH72 for N_2 fixation. *nifH* encoding the dinitrogenase reductase and *nifA* encoding its essential transcription activator are major players of the N-regulatory cascade and highly expressed during N_2 fixation. That is why for the initial standardization of transcript analyses experiments in strain BH72; they are the ideal candidates of choice. The transcription analysis carried out in this section basically involved mapping the 5' end of the *nifHDK* and the *nifLA* transcripts by primer extension and proving the cotranscription status of genes linked to *nifHDK* or *nifA,* respectively. Differential expression analysis of the *nifA* transcript according to N-availability is also carried out.

4.1.1 *nifHDK* is cotranscribed with *fdxN* from its upstream σ^{54} promoter

The upstream-untranslated region of *nifHDK* in strain BH72 has a high sequence identity with the sigma(54) promoter consensus (Fig 4.1 A) (Egener *et al*., 2001). To verify that this promoter is utilized for the *nifHDK* transcription, the transcription start site of this gene was mapped by primer extension. The result shown in Fig 4.1B indicates the transcription start at minute 158.6 corresponding to nucleotides 541/542 (159 and 158.2 min). The –12/-24 region of the promoter is precisely located 12 base upstream of this start point base. So it is quite likely that this promoter is utilized in transcribing the *nifHDK.* It was thus possible that primer extension was done non-radioactively, with automated sequencing.

To proof that the *nifHDK* operon in BH72 is transcriptionally linked with its downstream gene *fdxN*, a PCR involving an reverse transcription step was carried out with RNA isolated from strain BH72 fixing N_2. The first primer for the RT reaction and the PCR was designed to anneal to the 5' end of the ferredoxin gene, while the second primer was targeted to *nifK.* An RT PCR product of expected *size* (Fig 4.1C) was detected only in presence of RNA and active reverse transcription and not after heat inactivation RT (control for DNA contamination) or without addition of RNA (Egener *et al*., 2001).

Fig 4.1 Promoter region of *nifHDK* and its transcript analysis to determine the transcriptional start point along with the detection of its cotranscription with *fdxN*. In (**A**) the promoter region of *nifHDK* with the putative sigma(54) region along with upstream elements is shown. An arrow shows the transcriptional start point. In (**B**), the transcription start of *nifH* determined by primer extension is shown. The transcription start point is localized at 158.6 min corresponding to nucleotides 541/542 (159 and 158.2 min) using the primer TH25rCy and plasmid pEN322 bearing the *nifH* region and it's upstream as template. Top, sequencing reaction using pEN322 as template and TH25rCy as reverse primer; bottom, primer extension with RNA from N_2 fixing cultures of BH72 as template and the same primer as the sequencing reaction. In (**C**), analysis of the *nifHDK* and *fdxN* cotranscription by RT-PCR using 1 μg RNA from N_2 fixing cells of strain BH72 and primers annealing to *nifK* (nifKfw) and *fdxN* (Fdxrev) spanning 384 bp. Size marker (lane 1), negative control (no RNA added) (lane 2), RT inactivated prior to RNA addition (lane 3), RNA added without heat inactivation (lane 4) and PCR amplification from genomic DNA (lane 5).



nifLA 145.16 ed with *nifLA* or the primer for the BH72 action; bottom primer extension.

g 4.2B Analysis of *nifLA* cotranscription by RT :R. Primers RT were used to plify a 422 bp i *nifL* and *nifA* er reverse trans ript from 1 µg IA isolated from n BH72. Lane & 6, size marke); Lane 2, no IA added; L ated reverse nscriptase; Lan ith RNA from N_2 fixing cells; and Lane 5, PCR amplification from genomic DNA of strain BH72.

4.1.2 *nifA* is cotranscribed with *nifL* utilizing the σ^{54} promoter

The next candidate for transcript analysis was *nifLA,* NifA being the essential transcription activator of the *nifHDK* (Egener et al., 2002). The ORFs of the *nifL* and the *nifA* of strain BH72 are closely adjacent (intergenic region 78 bp). Only upstream of *nifL,* are motifs characteristic of σ^{54}-dependent promoters corresponding well to the consensus (Merrick, 1992). Primer extension with an RNA preparation from N_2 fixing culture of strain BH72 and Cy5-labelled reverse primer (Cy5PenifLArev) corroborated the transcription start site. Using plasmid pSO8 as template for parallel sequencing reaction and the same primer as above, the transcription start was localized at minute 145.16, corresponding to the nucleotide marked by an asterisk in (Fig 4.2A) (minute 145.11) which was at position +1 with respect to the –12 / -24 motif of σ^{54} promoter.

Cotranscription of the *nifL* and *nifA* was confirmed by RT PCR using a forward primer annealing to the 3' end of the *nifL* and a reverse primer annealing to the 5' end of the *nifA*: RT PCR using DNaseI treated RNA of strain BH72 revealed an amplification product of the expected *size* (422 bp) which was also obtained by PCR using chromosomal DNA as template but not in an RNA free control or after heat inactivation of reverse transcriptase(Fig 4.2B).

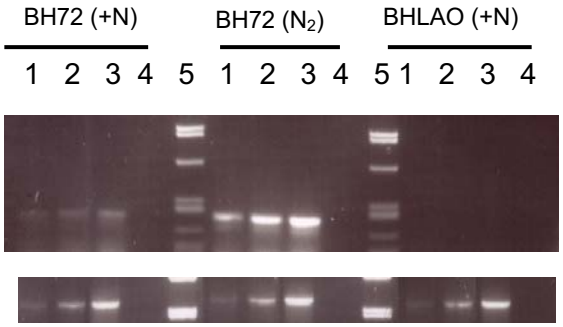


Fig 4.3 Analysis of *nifA* expression by RT-PCR. 1 μg RNA isolated from strain BH72 cells grown under ammonium excess (+N) or on N_2, and from the *nifL::Ω* mutant BHLAO grown on ammonium, was used for RT-PCR with primers specific for *nifA* (nifArev3RT, nifA for1RT and nifArev2RT) (**A**). Samples were taken after 18 (lane 1), 23 (lane 2), and 28 (lane 3) cycles; lane 4, reverse transcriptase heat inactivated; lane 5, DNA size marker. Product of expected size 421 bp for *nifA* obtained. (**B**). As a quality control of the RNA, the same RNA preparations were used to get a 1242 bp product obtained from 16S rRNA-directed RT-PCR using primers 1401rev, 104f and 1346rev with 10 ng RNA as template; lanes are numbered as in **A**

4.1.3 *nifA* in strain BH72 is expressed differentially according to N-availability.

It has already been reported by Northern blot experiments that the *nifLA* transcript was detectable in aerobically grown cells on combined N_2 but was more abundant during N_2 fixation (Egener *et al*., 2002). The differential expression of *nifA* was confirmed by RT PCR: One microgram RNA isolated from strain BH72 cells growing under ammonium excess (+N) or on N_2, and from the *nifLA*::Ω mutant (BHLAO) growing on ammonium was used in semi quantitative RT-PCR for amplification of the *nifA* gene (Fig 4.3A). Samples were removed after 18 (lane1), 23 (lane2), or 28 (lane3) PCR cycles, respectively (Egener *et al*., 2002). RT PCR amplification products of the *nifA* (0.42 kb band) were more abundant in RNA extracts from N_2 fixing cells than in extracts from ammonium grown cells and was completely absent in extracts from ammonium grown cells of BHLAO (*nifLA* insertion mutant) (Fig 4.3A). The use of equal amounts of RNA in all the three extracts was confirmed in a similar way by directing an RT PCR using primers for 16S rRNA (1.24 kb band) (Fig 4.3B).

4.2 Identification and genetic organization of the *ntrBC* genes

Identification of putative *ntrC* clones of *Azoarcus* was carried out by triparental mating and subsequent complementation of *Azotobacter vinelandii* transconjugants (Tanja Egener, 1999). Partial chromosomal DNA digests of *Azoarcus* sp. BH72 had been cloned in low copy vector pLAFR3A in order to generate a cosmid gene bank. After subsequent transfection of the gene bank in *E. coli*, it was used as a donor strain (bearing the mobilizable plasmid pLAFR3A) for triparental conjugation, the recipient being an *ntrC* mutant of *Azotobacter vinelandii* (unable to use nitrate as sole nitrogen source), the helper being *E. coli* strain bearing plasmid pRK2013. Finally the transconjugants which could complement growth in selection medium (BS-medium with 10 mM nitrate as only nitrogen source) were chosen. The *ntrBC* coding regions were present on a big plasmid (pTECE) having six *Pst*I sites. A 6.5 kb *Sau*3A-*Bam*HI fragment from pTECE was further subcloned into pBK-CMV vector and named as pNTRC2 (Egener, 1999). This clone was used to generate further subclones for sequencing of the *ntrBC* region. Further attempts have been made in this section to align the translated NtrB and NtrC with known protein sequences from the data base, characterize important motifs within these putative proteins, analyse the *ntrB* and *ntrC* transcripts and elucidate their cotranscription.

4.2.1 Cloning and sequencing of the *ntrBC* region

For the purpose of sequencing the *ntrBC* region and to get an idea about the possible restriction sites within it, a phage bank clone bearing the *ntrBC* along with its upstream and downstream regions was chosen. This clone (pNTRC2) had a 6.5 kb *Sau*3A - *Bam*HI fragment in the pBK-CMV vector. Initially pNTRC2 was digested individually by *Hin*dIII, *Pst*I and *Xho*I and in each case the largest fragment *i.e.,* the

portion of the clone still attached to the vector (9.1 kb, 8.3 kb and 5,7 kb respectively) were separated from other fragments after running in a gel and then religated. In this way, using general Cy5 labelled T_3 and T_7 primers annealing to the vector, sequencing could be more easily accomplished. By this approach *ntrBC* and its flanking regions could be sequenced. The *Hin*dIII, *Pst*I and *Xho*I religated subclones were named as pSNTR2.1, pSNTR2.2 and pSNTR2.3 respectively. In order to facilitate sequencing of the *ntrBC* region, pSNTR2.1 was further digested by *Sma*I and *Pst*I. The 1.2 kb and 1.8 kb fragments coming from *Sma*I digest were further subcloned into pBluescript-KS and named as pSNTR2.7 and pSNTR2.8, respectively. Similarly, *Pst*I digested fragments (0.54, 0.76, 0.50, and 0.30 kb) from pSNTR2.1 were subcloned to pBluescript-KS and named as pSNTR2.9, pSNTR2.6, pSNTR2.5 and pSNTR2.10, respectively. The subclones along with their putative restriction sites are shown in Fig 4.4.

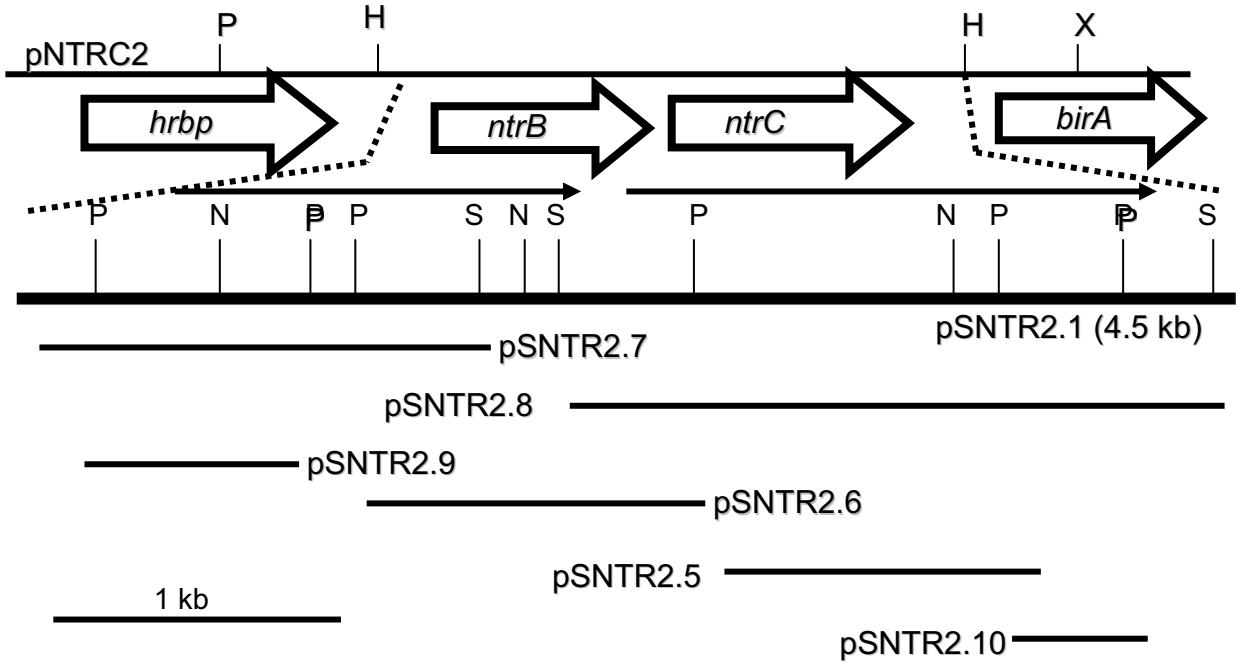


Fig 4.4, Chromosomal organization of the *ntrB* and the *ntrC* genes along with its flanking genes, restriction map and subclones generated for sequencing. The description of each of the subclones is provided in Table 2 (Material and Method). The restriction map exhibits the restriction sites of the following enzymes: H, *Hin*dIII, P, *Pst*I, X, *Xho*I, S, *Sma*I, and N, *Nru*I. *ntrBC* is flanked by genes homologous to *hrbp* (human RNA binding protein) and *birA* (biotin CoA bifunctional protein).

Overlapping sequences from these fragments were arranged in the form of contigs using seqman (DNASTAR programme). Finally several contigs were arranged to give consensus sequences. The final consensus was blasted against the NCBI data bases. The putative NtrB protein from *Azoarcus* sp. BH72 showed 48% identity and

60% similarity to NtrB from *Ralstonia solanacearum* or 45% identity and 57% similarity to *Herbaspirillum seropedicae* histidine kinase or 39% identity to NtrB protein from *Pseudomonas aeruginosa*. In all cases these function as sensor kinases. The putative NtrC protein from *Azoarcus* shared its highest homology with NtrC proteins from *Ralstonia solanacearum* (63% identity and 73% similarity) and *Herbaspirillum seropedicae* (62% identity and 74% similarity).These proteins function as N-response regulators and transcriptional activators. Thus both these putative proteins from *Azoarcus* showed high homology to β proteobacterial relatives.

Sequence analysis of the genomic region upstream of the *ntrBC* showed homology to the RNA binding proteins from *Homo sapiens* (38% identity). These are predicted to be proline rich proteins. Similar proline rich proteins have also been reported from plants such as putative proline rich proteins from *Arabidopsis thaliana* (Lin *et al*., 1999). Sequence analysis of the downstream region of the *ntrBC* showed 32% identity to the *birA* gene from *E. coli* and 31% identity to the *birA* gene from *Salmonella typhimurium*. This gene is known to code for a bifunctional protein acting as biotin operon repressor and biotin synthetase holoenzyme. The sequence, reading frames, and putative restriction sites of the *ntrB* and the *ntrC* are presented as attachment 4.5

4.2.2 Alignment of the NtrB and NtrC amino acid sequences of *Azoarcus* sp. BH72 with known sequences from databases

For this purpose, the translated protein sequence from the *ntrBC* open reading frames was aligned with known protein sequence from the databank using the Clustal X method. The protein sequence generated from the first ORF aligned well with other known NtrB proteins (Fig 4.6). The protein sequence from *Azoarcus* was compared not only with members of other β-but also with α -and γ- *Proteobacteria*. In general, *Azoarcus* NtrB was found to be more or less similar in length compared to other members. However amino acid similarities were higher with those of *Herbaspirillum*, *E. coli* or *Klebsiella* compared to that with *Rhodobacter sphaeroides* of the α subgroup. From the alignment, it appears that the NtrB proteins are much less conserved in their N terminal ends whereas the central and C terminal domains show short and discrete patches of highly conserved regions. This is quite in correlation with the function of NtrB like sensor kinases where a relatively non-conserved N-terminal domain (input domain) helps to integrate discrete and different signals, which can then be processed by a more general histidine kinase activity of

the transmitter module. The imprints of a typical transmitter module of a sensor kinase like conserved histidine residue (AHEIKNPL) and glycine rich motifs for ATP binding (GSGLGL) are also present in the putative NtrB protein sequence from *Azoarcus* sp. strain BH72.

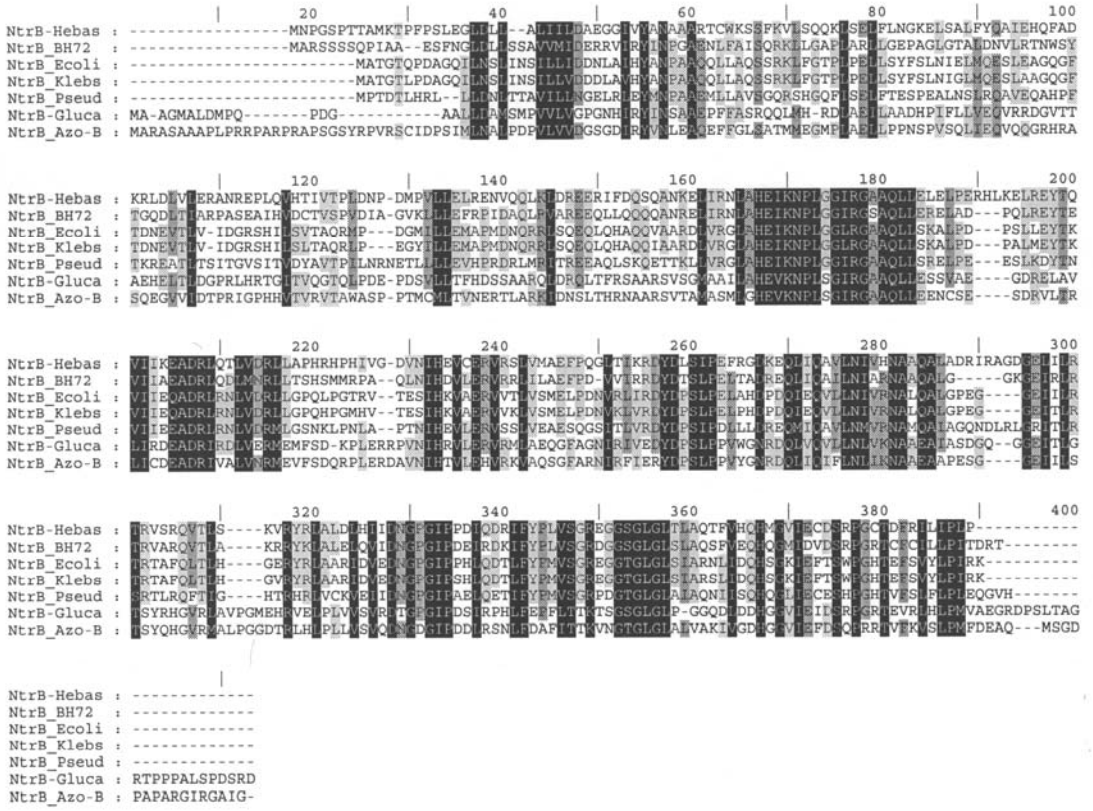


Fig 4. 6 Alignment of amino acid sequences of different NtrB-proteins. Protein sequences from *Herbaspirillum seropedicae*, *Azoarcus* sp. strain BH72, *Escherichia coli*, *Klebsiella pneunomiae*, *Pseudomonas syringae*, *Gluconoacetobacter diazotrophicus*, and *Azotobacter vinelandii*. Percent sequence identities are as follows: black: 100%, dark grey : 80%, light grey : 60%, and white : under 60%.

The NtrC proteins act as global transcription activators for many *ntr* regulons. Alignment of *Azoarcus* NtrC with its close relatives revealed the following features (Fig 4.7). In contrast to NtrB, it shared more conserved regions. The N terminal receiver domain of *Azoarcus* NtrC possesses conserved aspartate residues (DDD): a typical site for phosphotransfer. This is linked to the central domain by a Q linker. A common feature of the central domain of most response regulators is a stretch of conserved residues GESGTGKE. This is also present in *Azoarcus* NtrC (between residues 170 to 180) and is probably responsible for ATP binding and subsequent transcription activation.

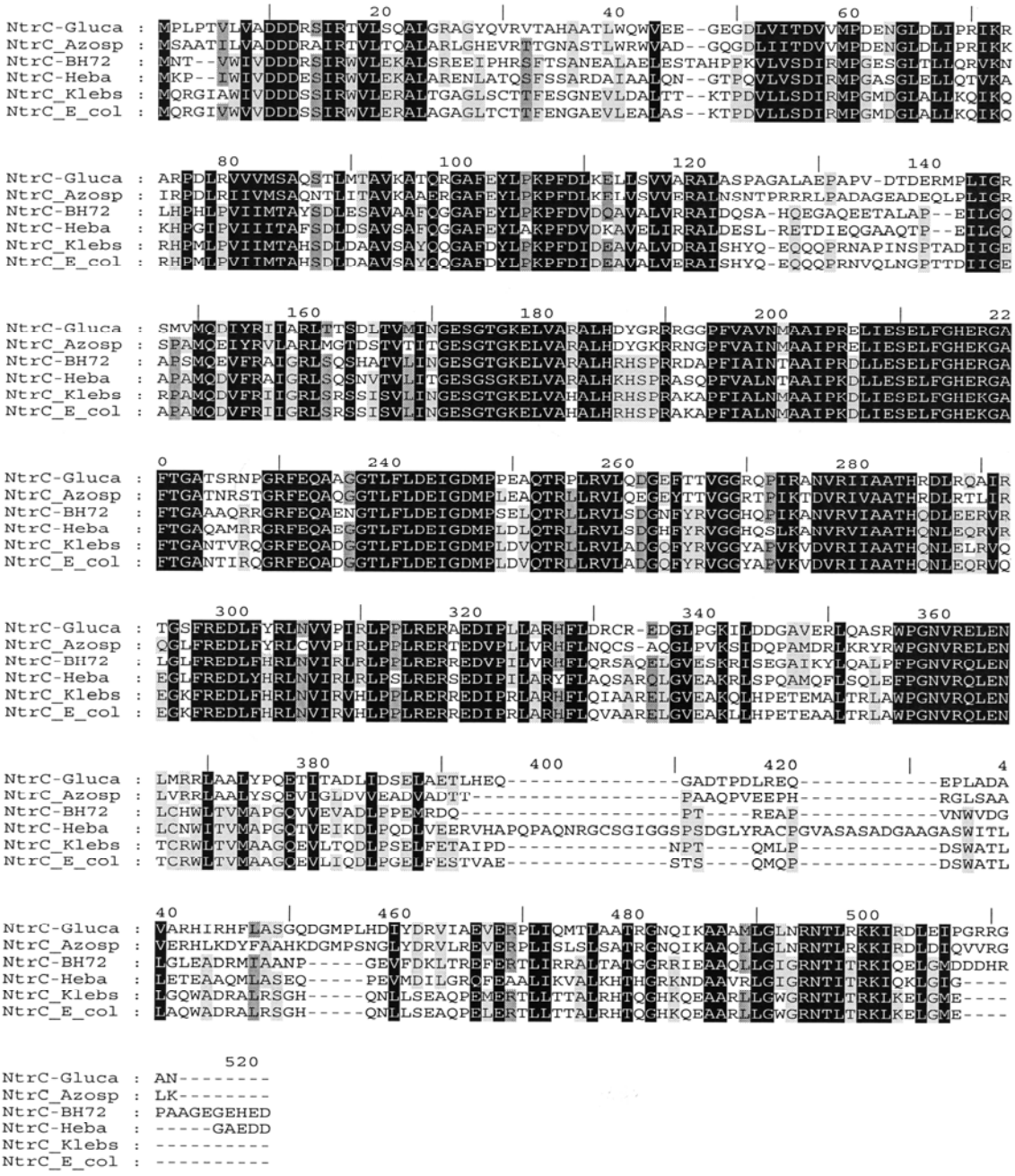


Fig 4. 7 Alignment of amino acid sequences of different NtrC proteins. Protein sequences from *G. diazotrophicus*, *A. brasilense*, *Azoarcus* sp. strain BH72, *H. seropedicae*, *K. pneumonia*e, and *E. coli*. Percent sequence identities are as follows: black : 100%, dark grey : 80%, light grey : 60% and white : under 60%.

4.2.3 Predicted functional motifs of the NtrB and NtrC from *Azoarcus* sp. BH72

The nucleotide sequence of *ntrB* and *ntrC* was translated by ExPASy (www.expasy.ch/tools/dna.html) and generated predicted proteins for both the genes under query. For NtrB, this predicted protein had 357 amino acids (molecular weight 39.69 kD, pI 6.56) and for NtrC 476 amino acids (Molecular weight 53.15 kD, pI 5.87).

In order to further investigate the functional motifs present in these predicted proteins the translated sequences were analysed using the programme SMART (Simple Modular Architecture Research Tool). Website address:
(www.smart.embl-heidelberg.de)

For NtrB from strain BH72, SMART predicted the following motifs: 12-79 residues PAS domain, 137-202 residues histidine kinase domain and 240-355 residues histidine kinase like ATPases. PAS motifs appear in *Archaea*, *Bacteria* and *Eucarya* and are involved as signal sensor domains. Several PAS domain proteins are known to detect their signal by way of an associated cofactor. The histidine kinase A N-terminal domain is a dimerisation and phosphoacceptor domain of histidine kinases and is found in many bacterial sensor proteins. The histidine kinase-like ATPases include several ATP binding proteins like histidine kinase, DNA gyrase B, topoisomerases, phytochrome-like ATPases and DNA mismatch repair proteins.

With NtrC as query, SMART prediction was as follows: 2-115 residues REC, 158-301 AAA and 303-314 low complexity. The REC (cheY-homologous receiver domain) is present in CheY and involved in clockwise rotation of *E. coli* flagellar motors. This domain contains a phosphoacceptor site that is phosphorylated by histidine kinase homologues and forms a part of bacterial two component-systems. It is usually found N-terminal to a DNA binding effecter domain. AAA (ATPases associated with a variety of cellular activities) profiles detected a fraction of this vast family. The key feature of this motif is that it shares a conserved region of 220 amino acids that contain one ATP binding site. The AAA domains in these proteins act as ATP dependent protein clamps and function in nucleotide binding. Surprisingly, no DNA binding helix-turn-helix (HTH) motif, a characteristic feature for DNA binding response regulators was predicted by this programme. However it is known that in SMART diagrams domains with low scores are not exhibited. Therefore the putative NtrC protein sequence from strain BH72 was submitted to another programme: NPS@ (Network Protein Sequence @nalysis) to find out whether such a motif really exists in the protein. The result produced a score of 6.15 for sequence

GRRIEAAQLLGIGRNTITRKIQ at position 436. This score suggests a very high probability that the protein of interest contains a helix-turn-helix motif.

4.2.4 *ntrB* and *ntr*C in strain BH72 are transcriptionally linked

From sequencing and reading frame analyses it is quite clear that both the sensor histidine kinase NtrB and its response regulator NtrC are oriented in the *Azoarcus* genome in same direction one next to the other. However a known promoter consensus could not be detected before the start codon of the *ntrB*. It has been shown previously that members of two component systems are physically and functionally linked in the form of an operon, transcribed together and are often controlled for cooperative translation (Govantes *et al*, 1998). Therefore in order to check the *size* of the *ntrBC* transcript and to know whether both components are transcriptionally linked (as would be the case if they share a common operon architecture), total RNA was isolated from N_2 fixing microaerobic batch cultures of strain BH72. After a DNaseI treatment, the transcripts of *ntrB* and *ntrC* were reverse transcribed separately using Amersham pharmacia beads with ntrBrev and ntrCrev primers for 30 min at 42°C followed by subsequent amplification of the cDNA with ntrBfor and ntrCfor primers respectively; the cycling conditions were 95°C 1min, 60°C 1min and 72°C 1,5 min for 35 cycles. The RT PCR amplification products from this experiment showed bands of expected size (1.07 kb *ntrB* fragment and 1.43 kb *ntrC* fragment) along with a negative control with heat inactivated beads (Fig 4.8 A). To check the quality of RNA, RT-PCR of 16S rRNA was performed from the same RNA preparation using 10 ng and using 1401 rev, 104f, and 1346rev primers.

To further verify whether *ntrB* and *ntrC* of strain BH72 are transcriptionally linked (cotranscribed), the RNA from N_2 fixing cultures of strain BH72 was reverse transcribed by RTntrCbridgeR and PCR amplified with primers annealing to the 5' end of the *ntrC* (RtntrCbridge R) and 3' end of the *ntrB* (RtntrBbridge F). The reverse transcription was carried out at 42°C. Following 35 PCR cycles of 95°C for 1 min, 55.5°C for 1 min, and 72°C for 1 min an amplification product of expected *size* (460 base pairs) which was also obtained from chromosomal DNA as template but not obtained in an RNA-free control or after heat inactivation of reverse transcriptase (Fig 4.8B). Therefore, *ntrB* and *ntrC* are clearly cotranscribed.

Fig 4.8 RT-PCR analyses of the *ntrBC* operon of *Azoarcus* sp. strain BH72. **(A)** Analysis of *ntrB* and *ntrC* transcripts by RT-PCR. Lane 1, *ntrB* primers without RNA; Lane 2, *ntrC* primers without RNA; Lane 3, heat inactivated reverse transcriptase prior to RNA addition; Lane 4, *ntrB*- and Lane 5, *ntrC*-amplification product from 1 μg RNA of N_2 fixing cells of strain BH72, respectively; Lane 6, size marker; Lane 7, 16S rRNA directed RT PCR as control. **(B)** Analysis of the *ntrBC* cotranscription by RT-PCR. Lane 1, no RNA added; Lane 2, RT heat inactivated prior to RNA addition; Lane 3, RT PCR product from RNA without heat inactivation; Lane 4, amplification from genomic DNA; and Lane 5, size marker.

4.3 Generation of a marker exchange *ntrBC* mutant of *Azoarcus* sp. strain BH72

These experiments were carried out to investigate, how *ntrBC* in strain BH72 regulates different cellular processes and whether it is absolutely crucial for its survival.

4.3.1 Construction of a marker exchange deletion mutant of the *ntrBC*

The strategy was to delete a major portion of the genetic region of *ntrBC* and replacing it by a resistance cassette. The cassette used in this case was a spectinomycin / streptomycin resistance *c*artridge (2kb), derived from plasmid pHP45Ω (4.3Kb). The Sp/Sm *c*artridge is flanked both sides by short inverted repeats carrying a transcriptional and translational stop signals in all the three reading frames along with a short polylinker stretch flanking symmetrically on both sides. The Ω resistance *c*artridge generates a polar effect on the transcription of the operons in which it is inserted. For this purpose, the *Sma*I digested Ω fragment from pHP45Ω

was cloned in the *Nru*I digested pSNTR2.1. Three *Nru*I sites are present within the *ntrBC* region: two within the *ntrB* region and the other in the middle of *ntrC* region. Clone (pSNTR2.lΩ) was chosen by checking its restriction pattern with *Hin*dlll digest to ensure that the cartridge is inserted into the two outermost *Nru*I sites, replacing the portion in between. Finally pSNTR2.1Ω was transformed in *Azoarcus* sp. BH72 by electroporation. Double recombinants were then chosen (rare clones where two point homologous recombination took place) by plating the transformation mix on VM ethanol plates with spectinomycin and streptomycin and choosing the resistant.

4.3.2 Construction of a nonpolar *ntrB* mutant of strain BH72

These experiments were carried out to check whether the response regulator is still functional when the sensor is knocked out. For this purpose the kanamycin resistance *c*artridge (1.2 kb) in the pUC4K vector was digested by *Eco*RI and the fragment was treated with *Klenow* fragment to generate blunt ends as described in material and methods. To generate the vector, *Asp*718 and *Sst*I fragments from pSNTR2.1 (having the entire the *ntrBC* region) were cloned in pBluescript-KS using the same enzymes. This strategy imposed ampicillin resistance to the vector (pBS-KS NTR2.1) to distinguish it from the *c*artridge resistance (kanamycin). A partial digest of this vector was carried out using *Nru*I and the bands were separated in an agarose gel. After cloning of the kanamycin cassette in this partially digested vector, the positive clone (pBS-KS NTR2.1 kan) was selected where the *c*artridge is cloned into the first two *Nru*I sites by replacing part of *ntrB* while the third *Nru*I site in the *ntrC* was still intact. Following an electroporation of this plasmid in *Azoarcus* sp. strain BH72, transformed strains were selected on plates with kanamycin where both the single and double recombinants grew. Finally the double recombinants were negatively selected by transferring the transformants in ampicillin plates where only the single recombinants grew. Thus only kanamycin resistant double recombinants are the putative nonpolar mutants where the kanamycin promoter can still drive the transcription of its downstream gene.

4.3.3 Validation of constructs by Southern hybridisation and genomic PCR amplification.

To confirm the *ntrBC* mutant genetically, a Southern blot was performed using genomic DNA from both the wild type and the mutant strain. The genomic DNA from both the strains (wild type and the *ntrBC* mutant) (3 μg) were digested with *Hin*dIII and *Pst*I respectively. A 540 base pair *Pst*I digested fragment within the *ntrB* region was used as a probe for southern hybridisation at 65°C and washings were done under conditions of high stringency. The result of the Southern hybridisation is presented in Fig. 4.9. The probe hybridised with a large 6 kb *Hin*dIII fragment in the wild type strain. The *ntrBC* mutant strain under similar condition hybridised to a 750 bp fragment: the internal *Hin*dIII site being generated from the insertion of the Ω *c*artridge. Similarly the *Pst*I digested wild type genomic DNA hybridised with a short 540 bp fragment corresponding to the *size* of the probe. Since the internal *Pst*I sites of the mutant are replaced by the *c*artridge, the probe hybridised to a larger 2.5 kb fragment instead.

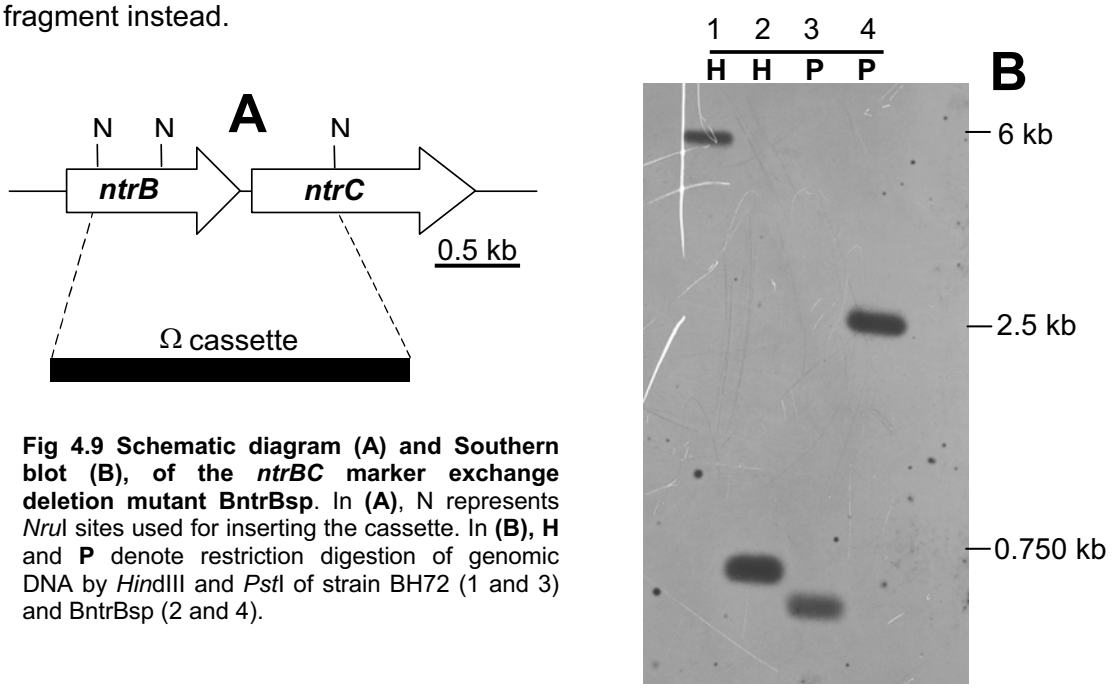


Fig 4.9 Schematic diagram (A) and Southern blot (B), of the *ntrBC* marker exchange deletion mutant BntrBsp. In **(A)**, N represents *Nru*I sites used for inserting the cassette. In **(B), H** and **P** denote restriction digestion of genomic DNA by *Hin*dIII and *Pst*I of strain BH72 (1 and 3) and BntrBsp (2 and 4).

The genomic arrangement of the *ntrB* non polar mutant was verified by PCR amplification of *ntrB* and *ntrC* from its genomic DNA using the standard forward and reverse primers for the respective genes; the cycling condition were 30 cycles of 95°C for 1 min, 63°C for 1 min and 72°C for 3 min. As expected, a 1.43 kb fragment was generated from the *ntrC* amplification, suggesting intactness of this gene. Using

the *ntrB* primers a 1.58 kb fragment was amplified instead of 1.07 kb; the correct *size* of the intact *ntrB* (Fig 4.10). This can only be explained if 0.771 kb internal *Nru*I fragment is replaced by 1.28 kb Kanamycin *c*artridge (1.07 – 0.771 + 1.282 = 1.584). The presence of the intact *Nru*I site within the amplified *ntrC* product was further confirmed when the 1.43 kb *ntrC* amplification product fragmented into 0.858 kb and 0.573 kb when digested by *Nru*I.

Fig 4.10 Schematic diagram (A) and PCR amplification (B) of the *ntrB* marker exchange nonpolar mutant BntrBKan. In **(A)**, N represents the *NruI* sites within the *ntrBC* region for the insertion of the kanamycin cassette. The *ntrB* nonpolar mutant is generated by replacing the *ntrB* region flanked by two *Nru*I sites with the Kan cassette, the entire *ntrC* being intact. **(B)** PCR confirmation of the *ntrB* nonpolar mutant. Primers used to amplify *ntrB* are ntrBfor and ntrBrev respectively while those used to amplify *ntrC* are ntrCfor and ntrCrev. Lane 1 (size marker), lane2 (PCR amplification of entire *ntrB* from BH72), lane 4 and lane 5 (amplification product of the *ntrB* and the *ntrC* respectively from BntrBkan) and lane 3 (restriction digestion of the *ntrC* amplification product from lane 5 by *Nru*I)

4.4 Phenotypes of the *ntrBC* mutants

The *ntrBC* mutant (BntrBsp) and the nonpolar *ntrB* mutant BntrBkan generated by marker exchange mutagenesis were viable and able to fix N_2 under microaerobic conditions. The growth characteristics, morphology and physiology of these mutants were compared with wild type strain BH72 in order to provide deeper insights about NtrBC function and hints about its putative targets.

4.4.1 Growth characteristics of wild type and the *ntrBC* mutants

One of the ways to study growth characteristics of an organism is to follow up its growth kinetics. The growth is usually carried out either on complete or synthetic media with different N sources or N-free medium (N_2 fixing). Samples were collected at defined time intervals (both from the wild type and the mutant strains) to measure OD at 578 nm. The plot of the OD against time intervals produced growth curves of a

particular strain from which growth rate and generation times could be calculated using the standard formula: growth rate (μ) = $lnOD_2 – lnOD_1 / (t_2 – t_1)$ where OD_2 and OD_1 are the respective OD's at time t_2 and t_1, respectively. The generation time calculated as ($ln2 / \mu$) from this formula is a measure of its growth characteristics. The results from this experiment are presented in TABLE 1. The generation times of all the three strains were comparable on complex medium, as well as on mineral medium with ammonium as N-source (1.3 hrs in average for all the three strains, compared). So ammonium as a N-source did not have a pronounced effect on the growth rate of the mutants. One of the interesting observations is the prolonged generation time of BntrBsp (9.56 hr) while growing aerobically with nitrate as sole N source compared to strain BH72 (2.1 hr) or BntrBkan (3.3 hr) under similar condition. A similar effect of nitrate has been observed in *glnB*$^-$*K*$^-$ double mutant of strain BH72 (Martin *et al*., 2000). The N_2-dependent growth was made in a fermenter with 0.8% oxygen where the growth of BntrBsp was found to be affected (generation time: 3.62 hr) in comparison to the wild type strain BH72 (generation time: 1.8 hr).

TABLE 1. Growth of *Azoarcus* sp. strain BH72 (wild type), the *ntrBC* and the *ntrB* knockout mutants on different nitrogen sources. The sign (-) means not carried out. All data are mean values of at least three independent experiments.

		Generation time in (h) during growth on			
strain	Relevant genotype	N_2	10mM NH_4Cl	10mM NO_3^-	VM ethanol
BH72	wild type	1.8	1.32	2.1	1.3
BntrBsp	*ntrBC*$^-$	3.62	1.3	9.55	1.39
BntrBkan	*ntrB*$^-$	-	1.38	3.3	1.53

4.4.2 Comparison of colony/cell morphologies of the wild type with the *ntrBC* mutant growing on nitrate as sole N-source: phenotypes of impairment exhibited by BntrBsp

The colonies of the strain BH72 and strain BntBsp looked identical while growing on complex medium agar plates. However when streaked on plates with nitrate as sole N source and incubated at 37°C, the mutant colonies appeared drastically different. BntrBsp colonies were more rounded and centrally opaque in contrast to whitish BH72 colonies. Microscopic observation of the same under bright field illumination revealed the colony profile in much more detail (Fig 4.11). The cells of BntrBsp colonies appeared rounded and smooth surfaced with deep central invagination. In

contrast, strain BH72 cells appeared rough surfaced with no such concavities. Under dark field illumination the *ntrBC* mutant colonies appeared more refractive. This was even clearer under phase contrast with bright field illumination where the *ntrBC* mutant cells appeared elongated with a central shining region much in contrast with wild type cells. It seems as if the *ntrBC* mutant was severely N-limited during growth on nitrate and accumulated polyhydroxy butrate under these conditions. The unreleased data from *Azoarcus* genome project have revealed the existence of putative genes encoding enzymes for PHB biosynthesis. So it is quite reasonable to speculate that PHB biosynthesis is upregulated in the *ntrBC* mutant due to N-limitation in presence of nitrate.

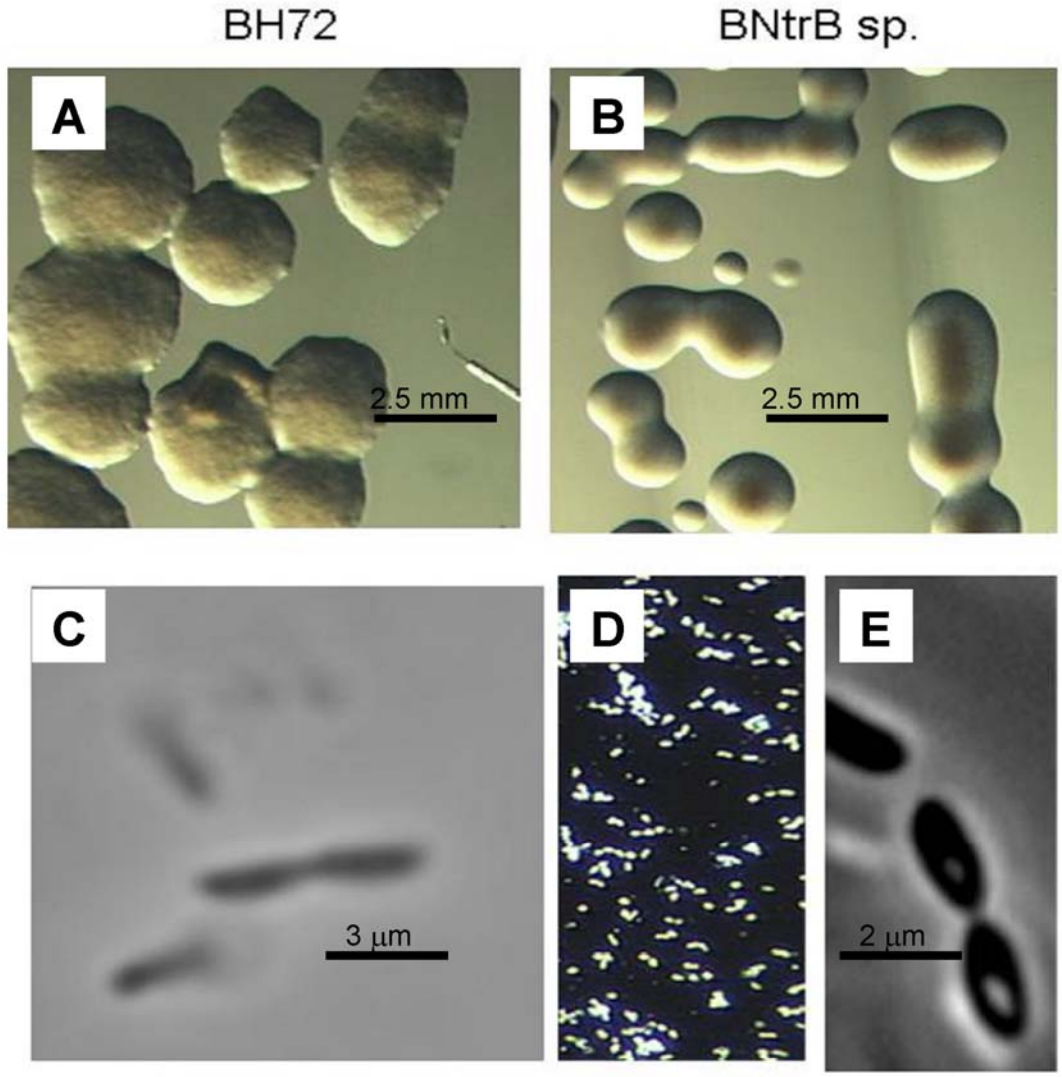


Fig 4.11 Comparision of cell morphologies of the wild type strain with the *ntrBC* mutant growing on plates with nitrate as sole N-source. The cell morphologies from wild type colonies (**A**) or from the *ntrBC* mutant colonies (**B**) as viewed under bright field illumination on a stereo microscope (Binocular). The view of wild type cells under phase contrast in a bright field illumination (**C**). The BntrBsp cells viewed under dark field with lower resolution (**D**) or under bright field with a higher resolution (**E**).

4.4.3 Twitching motility is upregulated in BntrBsp

Twitching motility is a form of bacterial translocation over firm surfaces that require retractile type IV pili (Liu *et al*., 2001). It is already known that *Azoarcus* sp. strain BH72 is capable of exhibiting twitching on solid complex medium when incubated at 37°C for 3 days and then left at room temperature. It has already been reported that heterologous expression of type IV pilin gene from *Pseudomonas syringae* pv. *tomato* DC3000 in strain *Azoarcus* sp. BH1599, an isogeneic mutant having transposon insertion in *pilA*, could functionally complement the defect of this mutant in "twitching motility" (Plessel, 2001). So it is evident that type IV pili are involved in twitching motility in strain BH72. Interestingly it was observed that BntrBsp twitched more under similar conditions as compared to the wild type. For quantification of twitching motility overnight precultures of strain BH72 and BntrBsp in complex medium were washed and suspended in physical saline. A dilution was made in order to have 50 cells per 100 μl per VM ethanol plates. They were incubated at 37°C for 3 days when the colonies started to show twitching. Plates were scanned and the colony areas from scanned images were calculated using the software "image quant" of the fluoro phosphoimager (Typhoon). About 47 BH72 colonies from total 5 plates and 121 BntrBsp colonies from 3 plates were used for calculating the area (square units) occupied by individual colonies by outlining individual colonies manually. The results are shown in Fig 4.12. It appeared that BntrBsp exhibited 2.2 times more larger colony areas as compared to the wild type under similar conditions. Since it has been already shown that both the wild type and the *ntrBC* mutant have similar growth rates in complex medium (generation time: 1.3 hr) it can concluded that the larger colony areas of BntrBsp was not due to faster growth but as a result of better twitching compared to strain BH72.

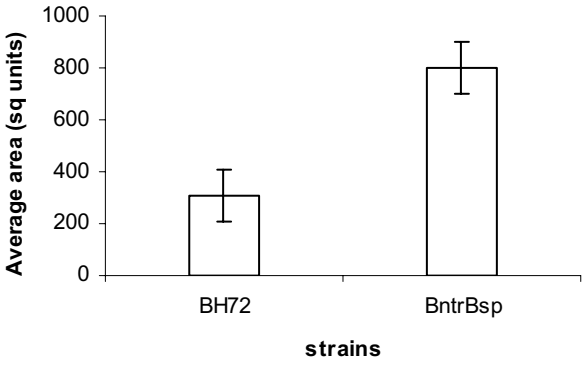


Fig 4.12 Comparison of twitching motility of strain BH72 and strain BntrBsp. The area of 47 independent colonies of strain BH72 from 5 independent plates were compared with 121 colony areas of BntrBsp from 3 independent plates. The bar in each case represents standard deviations of calculated areas.

4.5 Transcriptional regulation of the *ntrBC* operon

In the following section the main question addressed is the transcription regulation of *ntrBC* in strain BH72. Generally the enteric *ntrBC* 's are linked to an upstream *glnA* gene and transcribed from –12 / -24 type N-regulated promoters (MacFarlane *et al*., 1985). On the other hand, *ntrBC* of the α *Proteobacteria* including rhizobia share a –10 /- 35 promoter consensus and are transcriptionally linked to an upstream ORF1 of unknown function. To get a clear scenario about the organization of the *ntrBC* promoter of *Azoarcus* sp. strain BH72 an attempt was made to map the 5' end of the *ntrBC* transcript. The other questions addressed in this section deal with the expression profile of *ntrB* and ask whether any autoregulation mechanism exists to control its expression.

4.5.1 Mapping the 5' end of the *ntrBC* transcript (primer extension)

The promoters of the principal *nif* genes of strain BH72 (*nifH* and *nifLA*), studied in section 4.1 share a -12 / -24 consensus, typical for N-regulated promoters. The transcription start point of these genes mapped precisely with respect to the location of the upstream consensus. However no putative –12 /-24 consensus was detected upstream of *ntrB*; neither a bacterial –10 / -35 sigma (70) promoter consensus could be found. Interestingly no proper Shine-Dalgarno sequences which are usually located 3 – 8 base pair upstream of the start codon and are necessary for ribosomal binding and translation initiation, could be detected upstream of the *ntrB* and the *ntrC* start codons of *Azoarcus* sp. BH72.

In order to investigate the transcription start point of *ntrB,* two Cy5 labelled reverse primers (Cy5PntrBrev and Cy5PEntrbrevUS) were designed, one annealing at about 30 – 35 bp down stream from the start codon of *ntrB* and the other annealing at about 30 – 35 bp upstream (US) of *ntrB* start codon. The rationale for designing primers annealing at such sites is to cover a greater probability of transcripts originating at undefined origins, upstream of *ntrB.* Primer extension was carried out according to the method described earlier with RNA isolated from N_2 fixing cultures of strain BH72. A peak was detected using the upstream primer very close to the primer peak. The sequencing reaction at such an early time point is

often erratic, and so the starting base could not be detected precisely with such an approach (data not shown). However the downstream primer under similar conditions

produced a single extension peak at 213 min, corresponding to the base C (Fig 4.13). Thus the start point was mapped about 150 bp upstream from the *ntrB* start codon. However attempts failed to detect a known bacterial promoter consensus upstream of this putative transcription start site.

A

CGAAGCCCGCAAGAGCCTGGGCGAGCAGGAAGCGGTCCGGCTCGGCGACGAGCGCAACTACCA

GCGCGTGCTCGACCGCCTGCAGCCGTTCAAGGACAAGGTCGACCTGCACGAGCGCAATATCGAA

GCACTGAAGCGCGAGATCGCCAACCTGCGCTGACGCGAAGCCGGGGAGCACGCGCCCCGGTCC

GACTCACCTCCGTTCCGGTCGCCCGTTCCCCATGGCTCGATCCTCCTCCTCCCAGCCGATCGCGG

start codon

CAGAGA

B

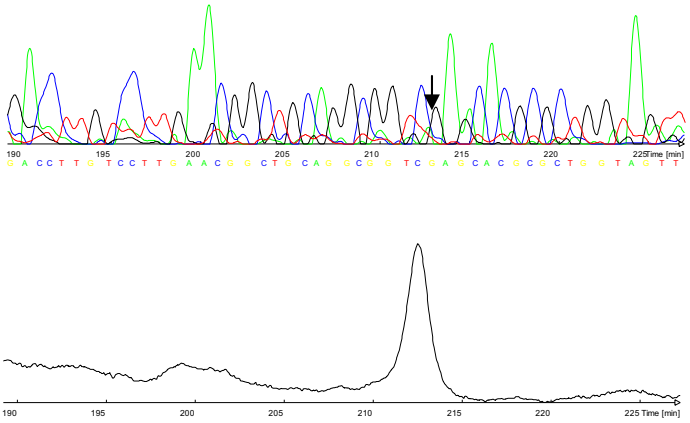


Fig 4.13 Analysis of transcription start point of *ntrBC* of strain BH72 by primer extension. (**A**), sequence of the upstream region of *ntrB* beyond its start codon. No standard bacterial promoter consensus could be detected in this upstream region. (**B**), Primer extension analysis localizing the transcription start at 213.07 min, corresponding to the nucleotide "C" marked with an arrow. Top, sequencing reaction and bottom, primer extension.

4.5.2 Undetectable expression of the *ntrB::gusA* in strain BH72 grown on different nitrogen sources.

One of the ways to quantify gene expression is to construct a transcriptional fusion of the upstream region of the gene of interest with a promoter less *gusA* reporter gene. While screening the putative pSNTR2.1Ω, some clones were obtained where the Ω *c*artridge was inserted into an *Nru*I site deleting a portion of the *ntrC* region while the *ntrB* with its upstream region was still intact (refer to section 4.). By this cloning, an extra *Hin*dIII site is generated downstream of *ntrB* coming from the catridge in addition to the original *Hin*dIII site present upstream of *ntrB*; This clone was digested by *Hin*dIII and ligated to *Hin*dIII digested pBluescript-GUS-SK. By this strategy, the transcription termination loops present within the catridge could be avoided from being incorporated within the reporter construct. The correct orientation of the reporter construct was checked by *Bam*HI digest; the resulting plasmid (pSNTRGUS) was electroporated in *Azoarcus* and ampicillin resistant single recombinants were isolated representing the desired reporter strain. GUS assays were performed with this strain (BHNTRGUS) under N excess (ammonium or nitrate as N-source) as well as under N limitation (N_2 fixing). The results are shown in Fig 4.14. Very low and close to undetectable levels of GUS units were measured in all cases even after incubation for 8 hrs. A positive control assay under similar conditions with *glnK::gusA*, could be done in 2 mins, as expected. So no conclusions could be drawn on the effect of different N-sources on *ntrB* expression in strain BH72.

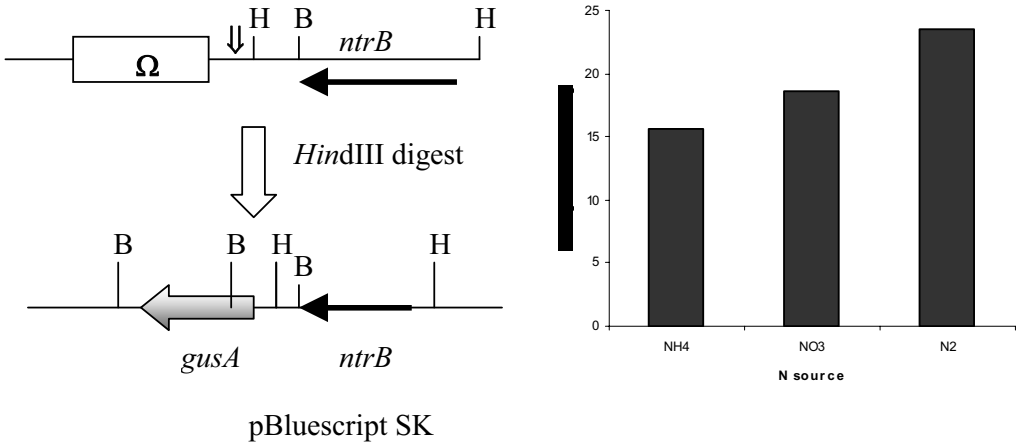


Fig 4.14 Expression of the *ntrB* measured by GUS activity.(**A**)Cloning strategy of the *ntrB::gusA* for chromosomal integration. H – *Hin*dIII, B – *Bam*HI, **Ω** represents resistance catridge and ⇓ represents the location of transcription termination loop. (**B**)Very low or near to undetectable levels of GUS activity was estimated in each case by culturing BHNTRGUS in ammonium (NH4), nitrate (NO3) or N_2 fixation (N2).

4.5.3 Effect of nitrogen sources on the *ntrB* transcription: RT PCR approach.

In order to analyse about the transcriptional regulation of the *ntrBC*, an RT-PCR approach was used, since reporter gene studies failed. RNA was isolated both from ammonium grown and N_2 fixing cells of strain BH72. DNaseI treated RNA from both were reverse transcribed by ntrBrev primers using the RT PCR beads at 42°C for 30 min followed by subsequent cDNA amplification of the *ntrB* transcript as described in section 4.1. At defined time points of amplification cycles, samples were removed and separated in an agarose gel (Fig 4.15 A). To ensure that an equal amount of RNA was taken as starting template in both the cases, 16S rRNA was reverse transcribed at 42°C for 30 min by 1401 rev fro 10 ng of same RNA preparations and PCR amplified with 104f and 1346rev primers at 95°C 1 min, 65°C 2 min, and 72°C 2 min (Fig 4.15 B). From the figure it is clear that the abundance of the *ntrB* transcript is much higher during N_2 fixation as compared to ammonium grown cultures. This points to the differential expression of *ntrB* depending on the N availability. 16S rDNA bands of equal intensities in both the cases confirmed that equal amounts of RNA were used under both the conditions for amplification.

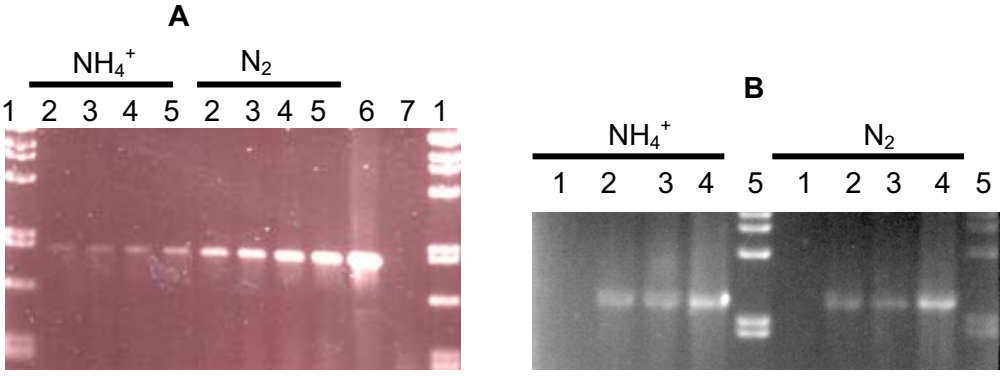


Fig 4.15 Analysis of the *ntrB* expression by RT-PCR. **(A)** 1 μg RNA isolated from strain BH72 under ammonium excess (NH_4^+) or N-limiting (N_2) was used for RT-PCR with primers specific for *ntrB*. Samples were taken after 18, 20, 23, and 26 PCR cycles respectively, corresponding to lanes 2, 3, 4, and 5 respectively; lane 6, PCR amplification from genomic DNA; lane 7, RNA with heat inactivated beads; and lane 1, size marker. **(B).** As a RNA control, the same RNA preparations were used for 16S rRNA directed RT PCR. Samples were removed after 18, 22, and 26 PCR cycles (lanes 2, 3, 4 respectively); RNA with heat-inactivated beads in lane 1 and size marker in lane 5, respectively.

4.5.4 *ntrBC* of strain BH72 is not likely to be autoregulated

NtrC as a transcription activator is known to regulate its own expression apart from regulating its targets. Typical NtrC binding motifs (GCTGGCGCAGGTGC) have been detected in the upstream region of many *ntrC* regulated promoters. In *Rhizobium leguminosarum* it has been reported that NtrC negatively regulates its own operon (Patriarca *et al*., 1993). An attempt to detect NtrC binding motifs upstream of *ntrBC* from strain BH72 failed. On the other hand to get a clear picture about the existence of autoregulation, a 150 bp fragment near the *ntrB* start which is still intact in the *ntrBC* insertion mutant within the *ntrB* (before the *Nru*I site where the Ω *c*artridge is inserted) was reverse transcribed by the primer RTntrBrev at 42°C from DNaseI treated RNA (500 ng) isolated from the *ntrBC* mutant growing in ammonium or N_2 fixing respectively. The cDNA was amplified using RTntrBfor primer in cycles of 95°C 1min, 59°C 1min and 72°C 1 min. Samples were removed after 18, 20, 23, 25 and 28 cycles and run in a gel along with a negative control for DNA contamination (heat inactivated beads) as had been mentioned earlier (Fig 4.16). The result shows the expression of the 150 bp N-terminal fragment in the *ntrBC* mutant under both conditions tested. Interestingly, there is still a differential expression (N_2 / NH_4^+) of the N-terminal *ntrB* fragment in the *ntrBC* mutant, which is also an argument for no autoregulation. A similar experiment with strain BH72 produced a comparable expression profile as that of BntrBsp (data not shown). A control for equal amounts of RNA for both the cases was carried out by RT PCR of 16S rRNA as before. The results of the experiment indicate that the *ntrB* from *Azoarcus* sp. BH72 is not involved in regulating its own expression (autoregulation).

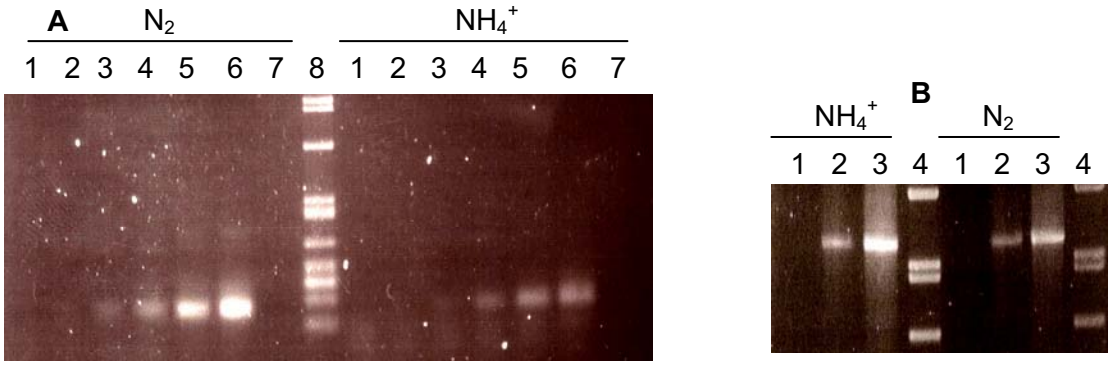


Fig 4.16 Analysis of autoregulation of *ntrBC* by RT- PCR. **(A)** 0.5 μg RNA from BntrBsp growing under N_2 fixation (N_2) or combined nitrogen (NH_4^+) was used to reverse transcribe and amplify 150 bp fragment from the N-terminal of *ntrBC* still intact in BntrBsp. Samples were collected after 16, 18, 20, 23, 25, and 28 cycles (lanes 1, 2, 3, 4, 5, 6, respectively) ; RNA with heat inactivated beads (lane 7) and size marker (lane 8). **(B).** Control for quality and equal amount of RNA as starting template was done by 16S rRNA directed RT-PCR by taking 0.5 μg RNA each time and removing the samples after 18, 23, and 26 cycles (lanes 1, 2, & 3 respectively); the size marker being in lane 4.

4.6 Putative targets of NtrBC of strain BH72

In this section, the goal is to analyse how the NtrBC of *Azoarcus* sp. strain BH72 plays a role in regulating the expression of putative downstream target genes. The *ntr* regulon is generally known to control various genes involved in N_2 fixation and N-assimilation. These two aspects are studied under two different broad subheadings.

4.6.1 Transcriptional regulation of N_2 fixation genes by NtrBC

In this section, the regulation exerted by NtrBC of strain BH72 on the *nif* genes coding for structural genes for nitrogenase (*nifHDK*) and its transcription activator (*nifLA*) has been studied. At the same time, the regulatory function of NtrBC on *gln* genes acting as sensors for the N status of the cell and in many ways related to N_2 fixation have been investigated.

4.6.1.1 The *nifA* expression in strain BH72 is NtrBC regulated in a nitrogen dependent manner

In *Azoarcus* sp. BH72, N_2 fixation and *nifHDK* transcription occur only under microaerobic and N-limiting conditions (Egener et al., 1999; Hurek *et al*., 1987). It is also known that *nifA* is an essential transcription activator for *nifHDK*, the structural genes for nitrogenase. In contrast to other members of β *Proteobacteria*, strain BH72 harbours a *nifL* like gene similar to the members of γ *Proteobacteria* and helps in inactivating NifA in response to O_2 and combined nitrogen (Egener *et al*., 2002). In order to study the effects of the *ntrBC* mutation on *nifHDK* and *nifLA* expression, upstream regions of *nifH* and *nifLA* were transcriptionally fused with a *gusA* reporter gene and cloned in a low copy vector pLAFR3A (pNHGUS and pLGUS) respectively (Egener, 1999). These plasmids were conjugated into strains BH72 and BntrBsp. The transconjugant strains arising from the wild type - BH72(pNHGUS) and BH72(pLGUS) and from the *ntrBC* mutant: BntrBsp(pNHGUS) and BntrB(pLGUS) were assayed for GUS activity by growing all the transconjugant strains on ammonium or N_2, respectively. The results are presented in Fig 4.17.

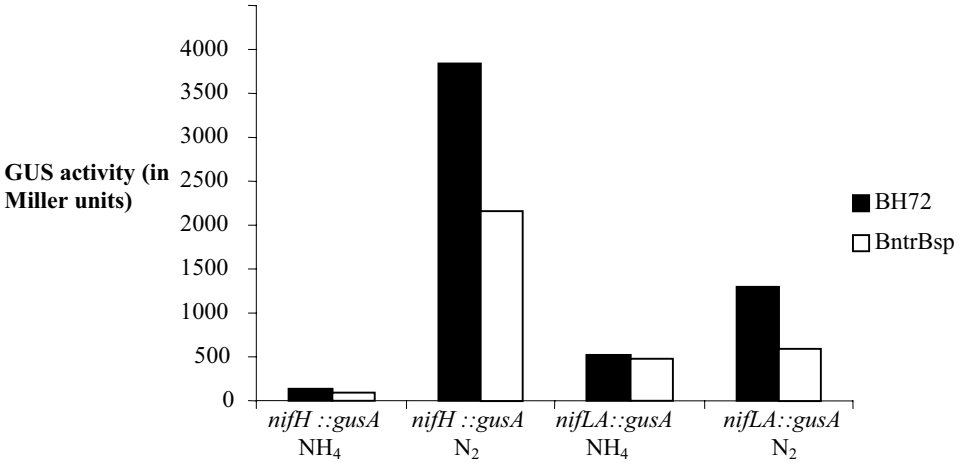


Fig 4.17 Expression of *nifH* and *nifLA* in strain BH72 and BntrBsp. Cultures were grown either in presence of combined nitrogen (10 mM ammonium) aerobically or microaerobically (0.8% O_2 in head space) with N_2 as substrate. The GUS activity was measured after 10-12 hours of growth (exponential growth phase). The data represent results from three parallel measurements of two independent experiments.

From the GUS measurements it is clear that the *nifH* promoter activity in strain BH72 is strongly induced under N_2 fixation. In strain BntrBsp the *nifH* promoter is also induced under N_2 fixation and suppressed under N excess. However the *nifH* GUS activity in the *ntrBC* mutant fixing N_2 is relatively lower as compared to the wild type under similar condition. On the other hand, the *nifLA* GUS activity in strain BH72 is not completely repressed under N excess and induced about three fold during N_2 fixation. This is quite consistent with previous *nifLA* expression patterns studied in northern blot experiments (Egener *et al*., 2002). Interestingly in the *ntrBC* mutant, the *nifLA* expression was not enhanced on N_2 compared to its expression on ammonium, along with reduced *nifH* expression on N_2 suggesting that NtrBC might play a role in regulating the *nifLA* transcription in response to N-deficiency. This in turn may have a direct or indirect influence on *nifH::gusA* expression

4.6.1.2 Effect of nitrate on the derepression of nitrogenase genes in BntrBsp

One of the interesting features of the *ntrBC* mutant is its inability to completely repress N_2 fixation when growing microaerobically on nitrate as sole N-source. The nitrogenase activity measured as (nmol of C_2H_4 / mg of protein) by acetylene reduction assay (ARA) showed that BntrBsp exhibited acetylene reduction activity of 6,230 ± 440, on nitrate which is much higher as compared to its fixation on N_2 (3,980 ± 230). However the nitrogenase activity detected for the wild type strain BH72 on N_2 was estimated to be (9,238 ± 440). The expression of *nifH* and *nifLA* in wild type and

ntrBC mutant while growing microaerobically on nitrate has been compared in Fig 4.18. The high GUS activity as a consequence of *nifH* expression in BntrBsp suggests derepression of the nitrogenase. Consistent with the nitrogenase activity, the *nifH::gusA* expression in BntrBsp was found to be higher on nitrate as compared to that on N_2. It is interesting to point out that the nitrogenase is derepressed in a similar way in *gln* B^-K^- double mutant while growing on nitrate with *nifH::gusA* expression of 2,860 miller units and nitrogenase activity of 4,010 ± 360 units (Martin *et al*., 2002). So it is clear from these data that the extent of derepression of nitrogenase on nitrate in *ntrBC* mutant is comparable to *gln* B^-K^- double mutant of strain BH72. Another interesting observation here is the relative high (compared to the *nifLA* expression on N_2) and more or less comparable *nifLA* expression in both the wild type and the *ntrBC* mutant. Thus in the same *ntrBC* mutant background, the expression of *nifLA* is differential according to different nitrogen source (nitrate or N_2). So it is clear from these results that source of nitrogen (ammonium or nitrate) also have an effect on *nifLA* expression in strain BH72.

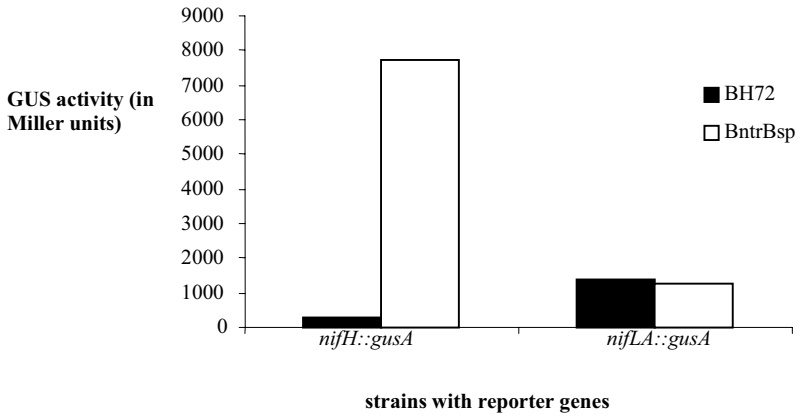


Fig 4.18 . Effect of nitrate on the *nifH::gusA* and *nifLA::gusA* expression in strain BH72 and BntrBsp, respectively. Cultures were grown microaerobically on 10 mM nitrate (0.8% O_2 in head space) and GUS activity was measured after 10 hours. The data represent values from three parallel measurements from two independent experiments.

4.6.2 Transcriptional regulation of the *gln* genes of *Azoarcus* sp. BH72: role of NtrBC

P_{II} like signal transmitter proteins, found in *Bacteria*, *Archea* and plants mediate control of nitrogen / carbon assimilation. They indirectly regulate the activity of key metabolic enzymes and transcription factors by protein – protein interactions. Many proteobacteria harbor two paralogous P_{II} like proteins, GlnB and GlnK, whereas a novel third P_{II} paralogue (GlnY) has recently been identified in strain BH72 (Martin *et al*., 2000). It is already known that P_{II} like proteins in strain BH72 play distinct roles in mediating nitrogen and oxygen control of the *nif* gene transcription and nitrogenase activity (Martin *et al*., 2002). However the regulation of these genes in strain BH72, is still not clear, especially at the level of transcription. At the same time, sequences upstream of the *gln* genes lack conserved bacterial promoter sequences, which make it even difficult to get an idea about the nature of regulation. So attempt has been made in this section to study the transcriptional regulation of these *gln* genes by RT-PCR and GUS assays and find the role of NtrBC in this context.

4.6.2.1 *glnK* regulation and effect of N_2 on its expression: role of NtrBC

In *Azoarcus* sp BH72, the *glnK* gene is downstream genetically linked to *amtB* (a putative ammonium transporter). This physical linkage of *glnK* with *amtB* has been reported for many bacteria. Northern blot analysis to study *glnK* transcription in strain BH72 revealed detectable expression of a large 1.8 kb transcript only during N_2 fixation, which was repressed in presence of ammonium (Martin *et al*., 2000). This speaks in favour of an N-regulated expression of the *glnK* operon. However a typical sigma 54 promoter (-24 / -12) or general sigma 70 (-10 / -35) type promoter consensus was not apparent in the *glnK* upstream region; instead a 750 bp ORF is present. This ORF termed *ugk* (upstream *glnK*) was found to have 43% identity to a putative protein harbouring signal peptide from *Ralstonia solanacearum* and 35% identity to a similar protein from *Caulobacter crescentus*. Interestingly, a typical sigma 54 promoter consensus sequence characteristic for many N-regulated promoters is present upstream of *ugk*. Therefore, it is interesting to explore whether a functional linkage exists between the *glnK* and the *ugk* of strain BH72 and whether the σ^{54}- promoter consensus upstream of the *ugk* can still drive transcription of *glnK* gene located downstream. Thus it can be speculated that Ugk harbouring a signal peptide may be secreted into the periplasm, which may aid the sensor AmtB to sense ammonium and propagate a signal transduction within the cell with the help of GlnK.

Nitrogen-dependent transcriptional regulation of the *glnK* gene was further verified by RT PCR and also by constructing a reporter gene fusion with its upstream sequence. Western blot was also carried out from protein extracts of strain BH72 to substantiate the differential expression of GlnK according to N availability. The *glnK* expressions were parallely studied in the *ntrBC* mutant to check whether its expression is altered under similar conditions.

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4.6.2.1.1 Cotranscription of the *glnK* and *ugk*.

To establish this hypothesis, total RNA isolated from BH72 fixing N_2 was reverse transcribed by a reverse primer annealing near the 5' end of *glnK* (RTglnK-R) at 42°C for 30 min followed by amplification of cDNA with a forward primer annealing to the 3' end of *ugk* (RTugk-F). 35 cycles of 95°C for 1 min, 59°C for 1 min, and 72°C for 1 min was carried out and the products were separated on a gel (Fig 4.19). The results show a 450 bp band spanning the *ugk* – *glnK* region obtained from DNaseI treated RNA and also from the positive control with genomic DNA, but not from the heat inactivated RT PCR bead representing a negative control for genomic DNA contamination. This speaks in favour of cotranscription of *glnK* with *ugk*.

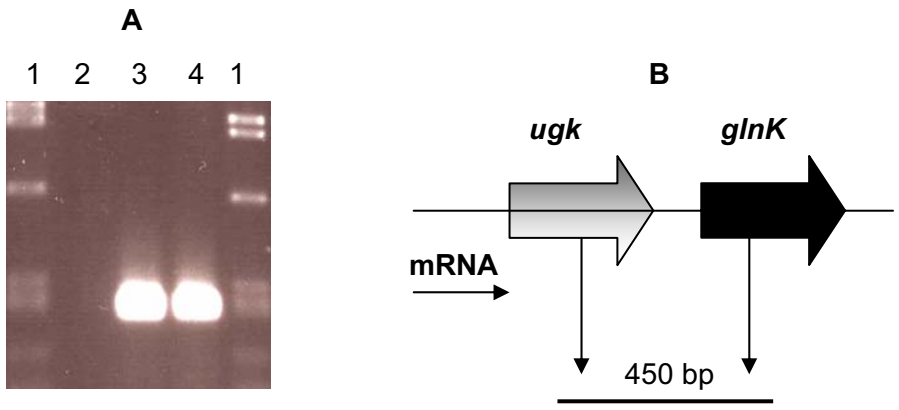

Fig 4.19 Analysis of cotranscription of *ugk* and *glnK*. (A). RT RCR analysis of cotranscription of *ugk* and *glnK* using primers RtugK-F and RTglnK-R. Lane1, size marker; lane 2, RNA from N_2 fixing cultures of strain BH72 with heat inactivated beads; lane3, RNA added without heat inactivation; and lane 4, amplification from genomic DNA. **(B).** Diagrammatic representation of the *ugk- glnK* region.

4.6.2.1.2 Primer extension studies to map the 5' end of the *ugk* and *glnK* transcript.

In order to locate the transcriptional start point of the *glnK* transcript, a primer extension analysis was carried out from RNA isolated from strain BH72 fixing N_2.The Cy5 labelled reverse primer (Cy5PeglnK rev) annealed about 20 bp downstream from the *glnK* start codon. The result of the primer extension is shown in Fig 4.20 C. The primer extension peak at 163.8 min matched with the base A (163.56 min) of the sequencing reaction (Fig 4.20 B). An attempt to find a known promoter consensus upstream of this start point failed. It is also quite likely that the predicted position for a transcriptional start may represent a site for active transcript processing. However the primer extension result substantiated not only the transcriptional linkage *glnK* with *ugk* (Fig 4.20 B) but also for the processing of a large transcript. On the other hand, Northern blot experiments, detected a single 1.8 kb transcript in *glnB*$^-$ with a *glnK* probe under nitrogen fixation, indicating cotranscription of *glnK* with *amtB* (Martin et al., 2000). Thus it is reasonable to think for a large transcript bearing *ugk*, *glnK* and *amtB* and its subsequent processing under N_2 fixation. A similar approach was made to map the transcriptional start point of *ugk* with a Cy5 labelled reverse primer (Cy5PEugk rev) annealing about 50 bp downstream from the putative start codon of the *ugk*. The primer extension peak at 120.87 min mapped with base T (121.03 min) of the sequencing reaction (Fig 4.20 C). In fact it maps within the putative Shine-Dalgarno sequence, present immediately upstream of the *ugK* start codon and so base spacing required between the transcription start site and the upstream sigma (54) consensus is not strictly maintained (Fig 4. 20 A). This can also be interpreted in terms processing of the major transcript. However a northern blot should be performed to get a better overview about the nature of transcript processing.

Fig 4.20 Analysis of transcription start point of the *ugk* and the *glnK* by primer extension analysis in strain BH72. (A) Sequence of the upstream region of *ugk* showing its start codon, and the putative RBS. Asterisks mark the bases that are conserved in the sigma (54) promoter region present at its upstream. The Arrow marks the putative transcription start point of *ugk*. **(B)** Sequence of the genomic region upstream of *glnK*, showing the *glnK* start codon and spanning the intergenic region between *ugk* and *glnK*. The thin arrow indicates the start point of *glnK* transcription as mapped by primer extension analysis; the thick arrow representing the stop codon of *ugk*. **(C)** Primer extension analyses of *ugk* showing the sequencing reaction (top) and the primer extension reaction (second). The primer extension peak at 120.87 min match with base" A" of sequencing reaction indicated by an arrow. Primer extension of *glnK* transcript with sequencing reaction (third) and extension reaction (fourth) of Fig 4.20 C. The primer extension peak at 163.8 min match with base "A" indicated by an arrow in the sequencing reaction.

A

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CGTTTGGTGCTTTGCGACAAAAGGGGCGCACCAATCCGGTGCAGACGCGCCCG

CACCGCAGGGGGCGCTGCCCGAAAAGGCCTGCGACATGCCCCTCGCTTTCGT

CAAAACCCCAGTCGTTGCGGGGTTTGCGACAAGCTGGCACGGACCCTGCATTA

GCTGCCCTGCGATAAACACATCAACGCTGGAGAAAGACATGCGCAAGAACATTC

ATATGAAGGATAAGAGCGATTATGACGCTGACGCACGCTATATCCTGTCCGTCA

CCAAATCGTTTTAATTGTCATAGGCTTCCGGCCGCGGGTGGCGACACCCTGGC

CGGGTAACAGGAGAACCCGATGAAGTTCATCACAGCGATCATCAAGCCGTTCA

4.6.2.1.3 Western blots to study the effect of nitrogen on GlnK expression in strain BH72.

Equal amounts of total protein extracts of strain BH72 isolated from ammonium grown and N_2 fixing cultures, respectively, were separated by SDS PAGE, electrotransfered to a membrane and used for Western blot analysis with a GlnK antiserum. The result (Fig 4.21) shows weak expression of GlnK in presence of combined nitrogen and strong induction during N_2 fixation, speaking in favour of its N-regulated expression in the wild type strain. The lower band in both cases represents an unspecific cross-reaction of the GlnK antiserum to a protein of slightly lower molecular weight that does not belong to the P_{II} protein family (Martin *et al*., 2000).

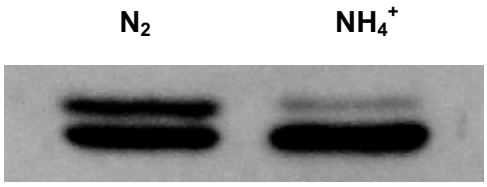

Fig 4.21 Western blot analysis of GlnK from strain BH72 growing with N_2 or ammonium. The upper band represents GlnK.

4.6.2.1.4 Nitrogen-dependent differential *glnK* expression in strain BH72 and its down- regulation in the *ntrBC* mutant: RT-PCR approach

RNA isolated from strain BH72 as well as BntrBsp growing in combined nitrogen or N_2 fixing conditions, respectively, were used for reverse transcription and subsequent amplification of the *glnK* transcript using glnKfor and glnKrev primers. RT PCR was carried out with glnKrev primer at 42°C for 30 min followed by PCR amplification with glnKfor and glnKrev primers at 95°C 1 min, 57°C 1 min, and 72°C 2 min. Samples were removed at defined time intervals of the PCR cycles. A similar approach was made to amplify the 16S rRNA in each case to check the quality of RNA, taking 10 ng of the same RNA preparation in each case, reverse transcribing at 42°C 30 min by 1401 rev primer and PCR amplifying with 104f and 1346 rev at 95°C 1 min, 65°C 2 min and 72°C 2 min. The results are shown in Fig 4.22. Consistent with the results obtained from Western blot analysis, a differential expression of the *glnK* transcript according to the N-availability was detected by RT-PCR analysis in strain BH72. The abundance of the *glnK* transcript was much higher when cells were grown on N_2 as compared to that on combined nitrogen. A more quantitative evaluation of the

differential expression of the *glnK* transcript in strain BH72 emerged when the same gel was evaluated after scanning with the fluoro phosphoimager, Typhoon (Fig 4.22 C). The transcript abundance of the *glnK* in strain BH72 quantified by the fluoro phosphoimager was found to be three times higher, when cells were grown on N_2 as compared to its abundance on combined nitrogen. This is in good agreement with the GUS measurements of the *glnK* expression under similar conditions, which is discussed in the following section. However attempts to amplify *glnK* transcripts from total RNA of the *ntrBC* mutant failed under both conditions tested (Fig 4.22 A). However 16S rRNA directed RT PCR produced an amplification product of correct *size* (1.2 kb) in all cases tested, confirming that an equal amount of RNA was used as starting template (Fig 4.22 B). Therefore it can be concluded that *glnK* exhibits an N-regulated differential expression profile in strain BH72 and is strongly downregulated in BntrBsp, suggesting a possible function of NtrBC in regulating *glnK* expression.

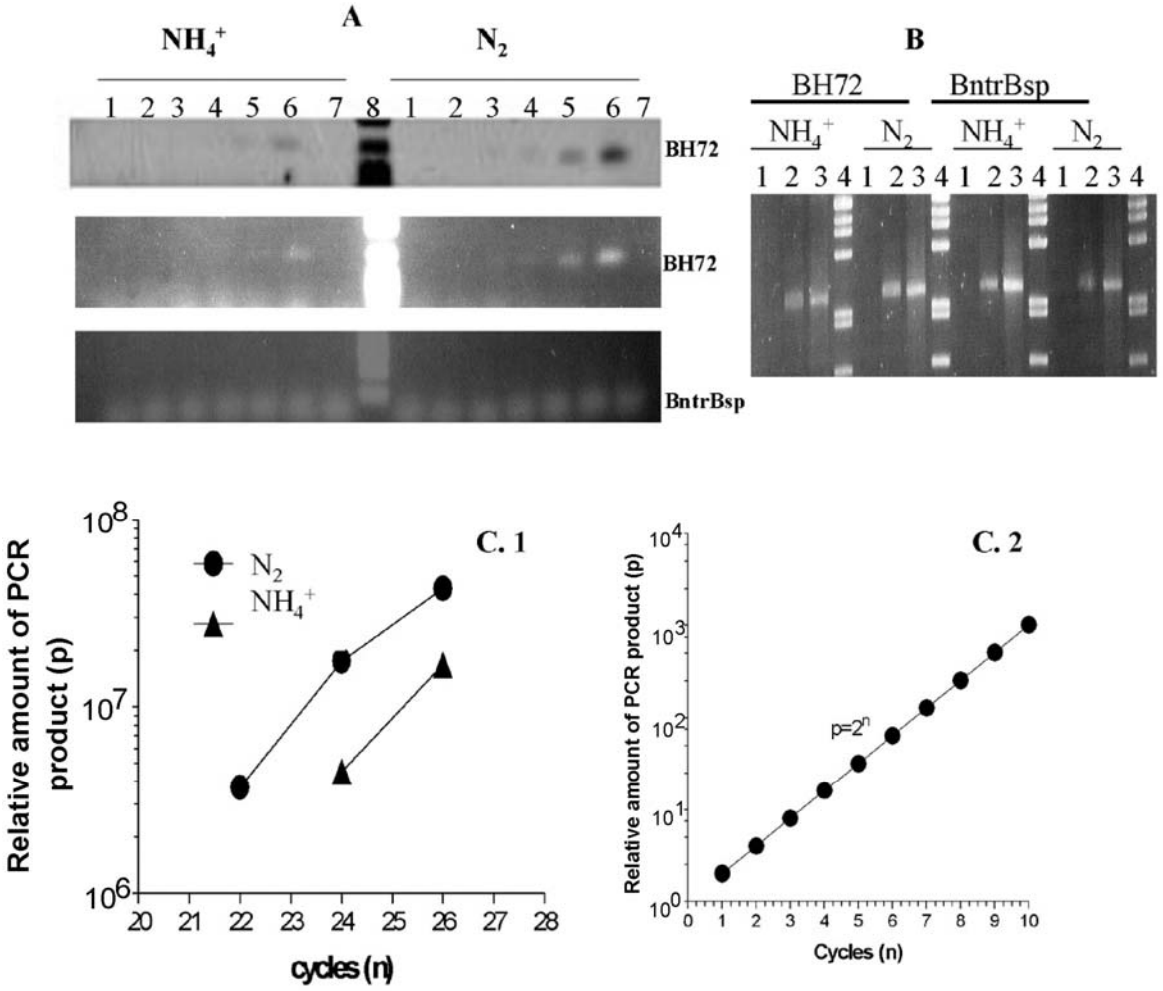

Fig 4.22 Analysis of the *glnK* expression by RT PCR in strains BH72 and BnrBsp. (A). Differential expression of *glnK* (strongly induced under N_2 fixation). The *glnK* transcript was reverse transcribed and samples were taken after 16, 18, 20, 23, 25, and 28 PCR cycles (lane 1, 2, 3, 4, 5, and 6) from wild type and BntrBsp (both grown on combined nitrogen or nitrogen, respectively) middle and the lower panel; top panel, image of the middle panel scanned with fluoro phosphoimager. Lane 7, heat inactivated reverse transcriptase prior to RNA addition, and lane 8, size marker. **(B)** RT-PCR directed amplification of 16S rRNA gene from all of the four combinations, BH72 and BntrBsp each, growing in presence of NH_4^+ or N_2, respectively; samples being taken at 16, 23, and 28 PCR cycles (lanes 1, 2, and 3). Lane 4, size marker. **(C)** Quantification of the bands from the scanned gel of **(A)** showing that the *glnK* transcript is relatively more abundant during N_2 fixation than in the presence of combined nitrogen in strain BH72 (**C.1**) **C.2**, standard PCR kinetics of any PCR reaction, showing the exponential increase of products with time.

4.6.2.1.5 Confirmation of nitrogen dependent *glnK* expression by GUS reporter gene and its down regulation in the *ntrBC* mutant

The aim of this section is to construct transcription fusion of *glnK::gusA* and analyse the effect of nitrogen on *glnK* expression in wild type and *ntrBC* mutant. A 2.4 kb Asp718 – *Sal*I fragment from pDZD17 was ligated into pBluescriptII SK. The *gusA* gene had been already cloned in this vector into the *Eco*RI site against the *lac* promoter. Both wild type BH72 and BntrBsp strains were transformed with this plasmid (pSGLK-GUS) by electroporation, as described in material and method.

Ampicillin resistant single recombinants from both the strains were chosen; the genetic recombination status was confirmed by Southern blot hybridisation (data not shown). The recombinant strains BGLK-GUS and BNtrBGLK-GUS generated from the wild type or *ntrBC* mutant, respectively were checked for GUS activity by growing them on N excess (ammonium) and N_2-dependently. The results are shown in Fig 4. 23. It shows that the *glnK::gusA* expression in BGLK-GUS is twice as high under N_2 fixation as compared to ammonium. At the same time there is a drastic reduction of GUS activity in the *ntrBC* mutant at both conditions tested. Thus the GUS measurement profile corroborated the interpretation made from RT-PCR experiments, suggesting a possible role of the NtrBC in regulating the *glnK* transcription in strain BH72.

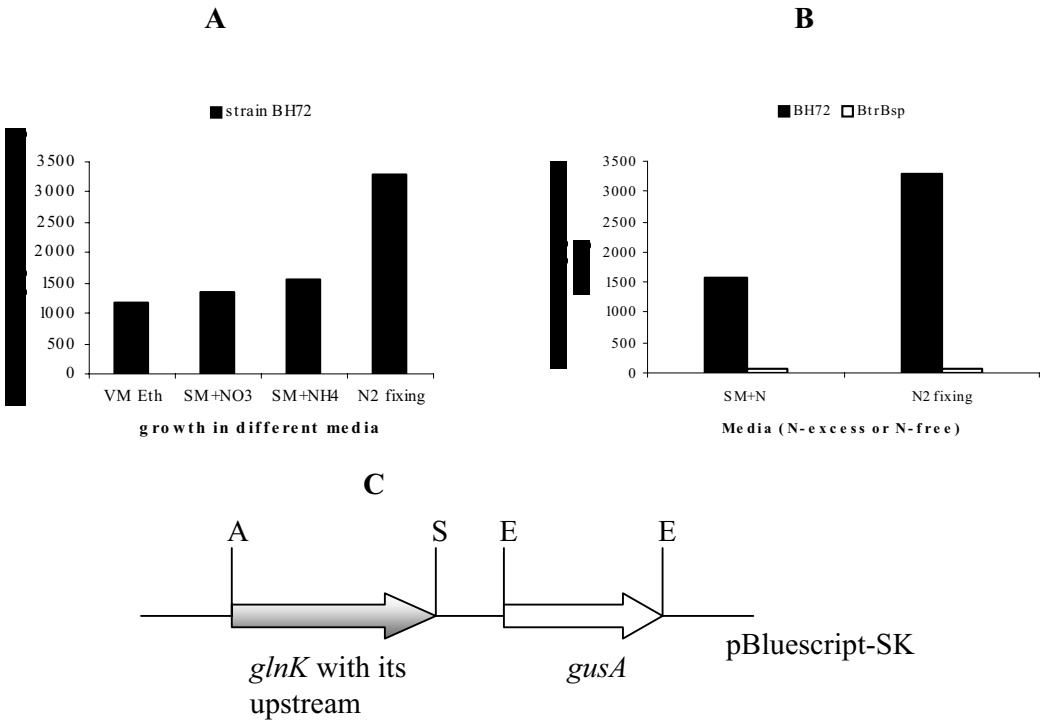

Fig 4. 23 Expression of the *glnK::gusA* fusion in BH72 grown on different N-sources (A), and comparison of the *glnK* expression in strain BH72 and BntrBsp (B) along with restriction map (C). Cultures were grown in VM Ethanol (complex medium), SM+N (with 10 mM nitrate), SM+N/NH4 (10 mM ammonium) or on N_2 in strain BH72 (**A**) or strains BH72 and BntrBsp (**B**). Results are from 3 parallel measurements from two independent experiments. pBluescript with *glnK::gusA* used for electroporation (**C**). A, *Asp*718; S, *Sal*I; and E, *Eco*RI.

4.6.2.1.6 Retention of ammonium "switch off" response and nitrogenase modification in BntrBsp under low *glnK* expression levels

Nitrogenase inhibition in response to ammonium (switch off) is observed in wild type BH72 and it has been shown that GlnK is essential for this process (Martin *et al*., 2002). For nitrogenase switch off (repression analyses), N_2 fixing cells both from strain BH72 and BntrBsp, were incubated on N free medium followed by addition of 2 mM NH_4Cl, and nitrogenase activity was subsequently estimated for both the strains before and after ammonium addition by an acetylene reduction assay. Both the wild type and mutant strain exhibited ammonium switch off response despite of the fact that the mutant had highly reduced *glnK* expression (Fig 4. 24 A). It has been shown that the NifH protein of *Azoarcus* sp. strain BH72 was covalently modified after addition of ammonium: a NifH protein of lower electrophoretic mobility (difference of approximately 1.5 kDa) accumulated. Western blot analysis of protein extracts from the ammonium induced switch-off experiments indicated that the BntrBsp mutant still responded to a switch off similar to the wild type (Fig 4.24B). It has been already established for strain BH72 that presence of both P_{II} (GlnB and GlnK) as well as AmtB is required for this response to ammonium. Therefore it seems to be likely that either a novel candidate or low levels of GlnK in the *ntrBC* mutant are sufficient to restore an ammonium induced fast response and a covalent modification of nitrogenase.

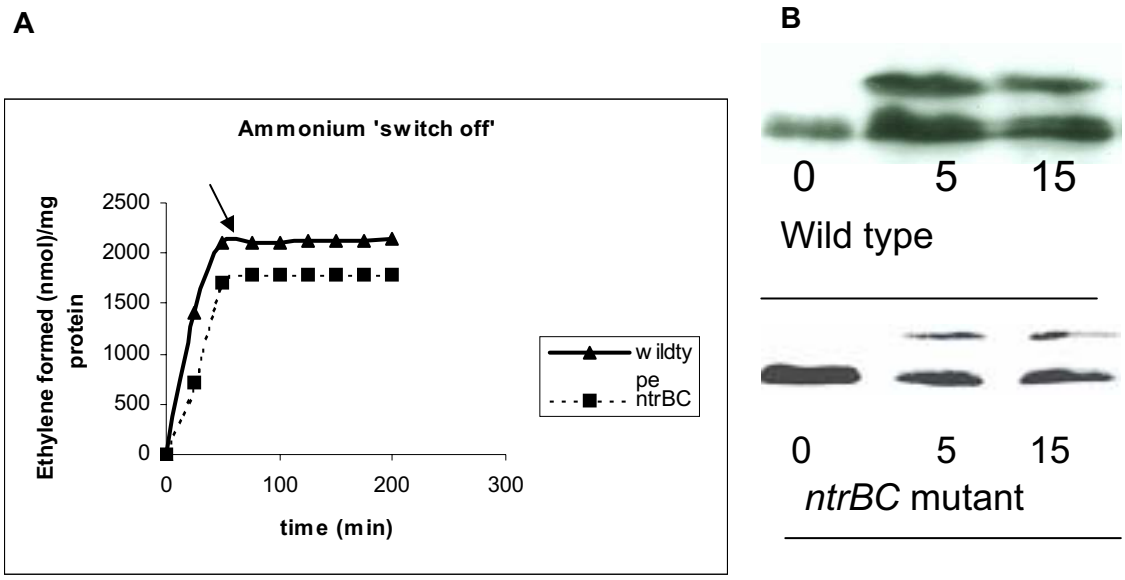

Fig 4. 24 (A) Analysis of an **ammonium induced switch off** response in the wild type and in BntrBsp by the acetylene reduction assay. The arrow indicates the time of ammonia addition (2 mM). **(B)**. Western blot analysis of **NifH modification after ammonium addition** to N_2 fixing cultures of the wild type and BntrBsp. The numbers indicate the time in minutes after ammonium addition.

4.6.2.2 Analyses of the *glnB* expression and role of NtrBC

The *glnB* gene has been reported to encode for small trimeric P_{II} proteins and plays an important role in regulating nitrogen fixation and N-assimilation in many prokaryotes (reviewed by Merrick and Edwards, 1995). One of the functions of GlnB is to stimulate NtrB dependent dephosphorylation of NtrC under N-excess, thereby preventing transcription from the *ntr*-dependent operons. Therefore, *glnB* belongs to the *ntr* regulatory cascade. It is the goal of this section is to study *glnB* expression in *Azoarcus* sp. BH72 (by GUS reporter gene analysis and RT PCR) and to find out the role of the NtrBC in this context.

4.6.2.2.1 The *glnB::gusA* expression in strain BH72 is affected by nitrogen

To construct a transcriptional fusion of *glnB::gusA*, plasmid pDZD3 was digested by *Sst*I and *Bgl*II so that part of the *glnB* along with its upstream region falls out. This DNA fragment was then ligated to *Sst*I and *Bam*HI digested pBluescript-KS. To this construct, the *Hin*dIII and *Sma*I digested GUS fragment from pBluescript–SK was modified by *Klenow* fragment and ligated into *Eco*RV digested *glnB*-pBluescript-KS. The orientation of the *gusA* gene with respect to the *glnB* region was confirmed by restriction digestion. This plasmid (pSGLB-GUS) was used to transform strains BH72 and BntrBsp by electroporation. Attempts to transform BntrBsp failed, but single recombinants of strain BH72, which were ampicillin resistant and had the *gusA* reporter gene integrated in the genome upstream of *glnB* could be rescued. This recombinant strain BGLB-GUS was tested for its GUS activity by growing the strain in various N-sources and on N_2. The results of the GUS activity are presented in Fig 4. 25. Surprisingly, the strain exhibited a differential expression pattern; being highly induced on N_2 and suppressed in complex medium or minimal medium with N (ammonium or nitrate). This is quite contradictory to previous reports where the *glnB* expression (studied by Northern analyses) appeared to be constitutive, independent of the N-status of the external medium (Martin *et al*., 2000).

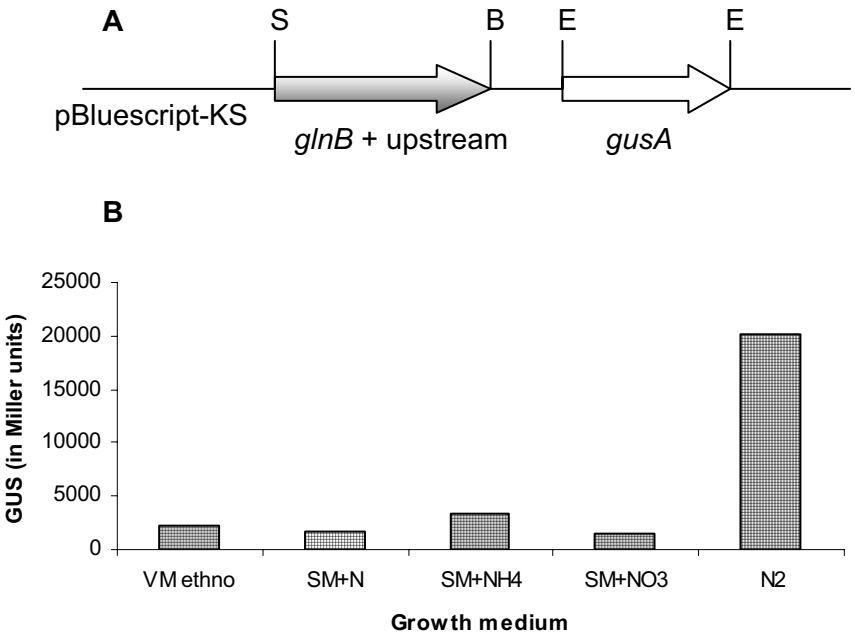

Fig 4. 25 Expression of *glnB::gusA* in strain BH72 along with the restriction map of the construct. (**A**) Restriction map of the *glnB::gusA* construct used for electroporation. S, *Sst*I; B, *Bam*HI / *Bgl*II; E, *Eco*RV. (**B**) Expression of *glnB::gusA* in strain BH72. GUS activity was measured from cultures grown in complex medium (VM ethanol), minimal medium with 10 mM ammonium (SM+N / SM+NH_4), minimal medium with 10 mM nitrate (SM+NO_3), or N_2 fixing (N_2). Each data represent 3 parallel measurements from 2 independent experiments.

4.6.2.2.2 RT-PCR to analyse *glnB* expression in strain BH72 and its down regulation in BntrBsp

To further investigate whether *glnB* expression in strain BH72 is indeed differential in response to the N-status and to have an idea about its expression profile in BntrBsp (since no GUS reporter strain could be obtained for BntrBsp), RT PCR was carried out to amplify the *glnB* gene. Equal amounts of RNA isolated from strain BH72 and BntrBsp each growing in ammonium or under N_2 fixation, respectively, were reverse transcribed at 42°C for 30 min by reverse primer glnB rev and subsequently PCR amplified by glnBfor and glnBrev primers at 95°C 1 min, 57°C 1 min, and 72°C 2 min using RT-PCR beads. Equal aliquots of samples were removed at defined time points of the cycles from both the strains and the PCR products were separated on a gel. To ensure that equal amounts of template were used in all cases, an RT PCR of the 16S rRNA gene was performed with 10 ng RNA from the same preparations at 42°C 30 min with the reverse primer 1401 rev followed by PCR cycles of 1 min 95°C, 2 min 65°C and 2 min 72°C with 104f and 1346rev primers The result of this experiment is shown in Fig 4.26. It appeared that in strain BH72, the *glnB* abundance

levels were more or less comparable under N_2 fixation and ammonium. At this point it is important to note that predictions from the GUS assays do not correspond to these findings. Interestingly in strain BntrBsp, the transcript (expression) appeared to be down-regulated under N_2 fixation as compared to that in ammonium.

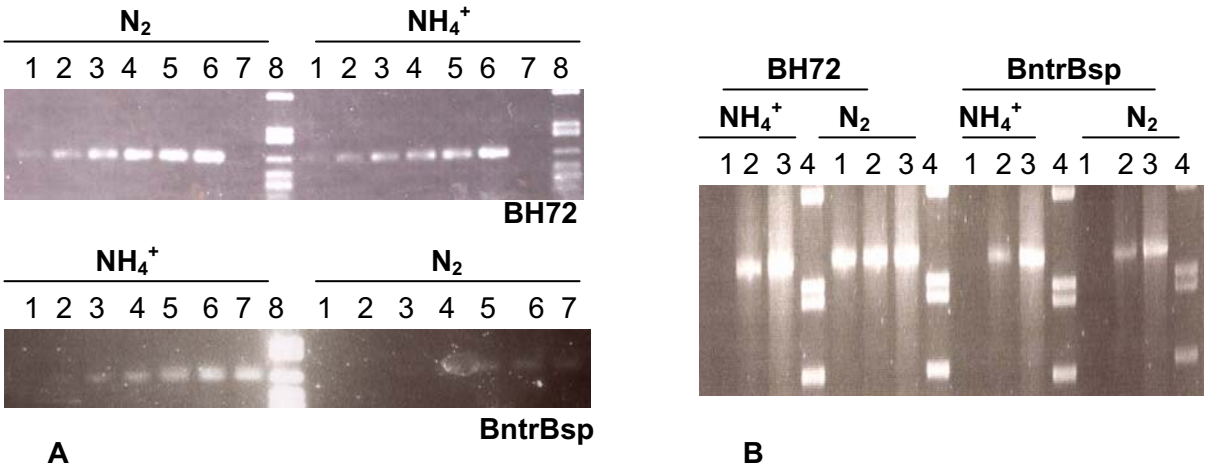

Fig 4.26 Analysis of the *glnB* transcription by RT-PCR. (A) Cultures of strain BH72 and BntrBsp growing either in presence of combined nitrogen or N_2 fixing were used for RNA isolation and subsequent *glnB*-directed RT-PCR. Samples were taken after 16, 18, 20, 23, 26, and 28 PCR cycles (lanes 1, 2, 3, 4, 5, and 6 for strain BH72 and lanes 2, 3, 4, 5, 6, and 7 for strain BntrBsp); RNA with heat inactivated beads (lane 7 for BH72 and lane 1 for BntrBsp) and size marker in lane 8. **(B)**. 16S rRNA directed RT-PCR from equal amounts of all RNA preparations as control. Samples taken at 18, 23 and 28 PCR cycles (lanes 1, 2, and 3 respectively); lane 4, size marker.

4.6.2.2.3 Comparable protein levels of GlnB in strain BH72 independent of nitrogen

Since results coming from reporter gene fusion and RT-PCR analyses of *glnB* transcriptional regulation differed from each other, attempt was made to study the expression pattern at the level of protein. Equal amounts of protein isolated from ammonium grown or nitrogen fixing cultures of strain BH72 were separated by SDS-PAGE electrophoresis and transferred to membranes for Western blot analysis with antiserum against GlnB. The result of the Western blot analysis is presented in Fig 4.27. It appeared that the GlnB was expressed in strain BH72 and that the expression level under N-excess and N_2 fixation was comparable. Thus at least for strain BH72, the Western blot results correspond to the findings of RT PCR analysis of the *glnB* transcript.

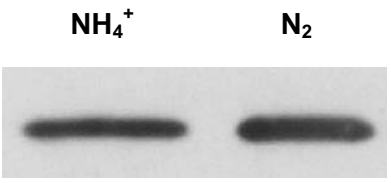

Fig 4.27 Western Blot analysis of GlnB from strain BH72. Equal amounts of protein isolated from cultures in ammonium or N_2 fixing were used for SDS PAGE and Western blot with GlnB antiserum

4.6.2.3 Detection and analyses of the *glnY* in the *ntrBC* mutant

As mentioned earlier, *Azoarcus* sp. BH72 is unique to have a third GlnY like P_{II} protein along with GlnB and GlnK. The GlnY was only detected in the *glnK glnB* double mutant and occurs only in its uridylyted state (Martin *et al*., 2002). An ORF coding for a putative ammonium transporter (*amtY*) is located immediately downstream of *glnY* (Martin *et al.*, 2000). Interestingly GlnY could be detected in strain BntrBsp of *Azoarcus* sp. along with the other P_{II} proteins (verified by RT PCR and 2D gel electrophoresis analysis). Also an attempt has been made to analyse the nature of the *glnY* expression with GUS reporter gene.

4.6.2.3.1 Detection of the *glnY* transcript in BntrBsp by RT PCR

For this purpose, RNA was isolated from strain BntrBsp growing microaerobically in a minimal medium with nitrate as sole N-source. As has been mentioned earlier, nitrogenase is not completely repressed under such conditions and the *ntrBC* mutant still fixes N_2. About 1 μg of RNA isolated from this culture was treated with DNase I and used for reverse transcription by the reverse primer (glnYrev) at 42°C for 30 min with RT PCR beads (amersham pharmacia) as mentioned in the material and methods section. The cDNA was subsequently amplified by PCR (95°C for 1 min, 57°C for 1 min and 72°C for 2 min) with primers specific for *glnY* (glnYfor and glnYrev). The *glnB* and *glnK* genes were similarly reverse transcribed and PCR amplified with their specific primers (glnB for and glnB rev and glnK for and glnK rev), respectively. The results of this experiment are shown in Fig 4.28. The *glnY* transcript was specifically amplified along with *glnB* and *glnK* while no amplification product was obtained with heat-inactivated beads (thus test for DNA contamination in the template) or with water (without any template).

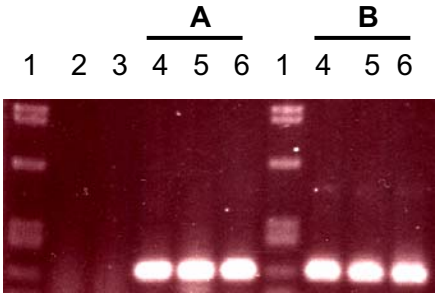

Fig 4.28 Analyses of *glnY* expression by RT PCR along with *glnB* and *glnK* from BntrBsp growing microaerobically on nitrate. Lane 1, size marker; lane 2, negative control without RNA; lane 3, heat inactivated reverse transcriptase prior to RNA addition; lane 4, 5, and 6, (RT PCR product of *glnB, glnK* and *glnY* from DNaseI treated RNA **(A)** or PCR products from genomic DNA of BntrBsp **(B)**

4.6.2.3.2 2D-gel and Western blot analyses confirming GlnY expression along with the P_{II} proteins

For confirmation at the protein level, total protein was isolated from BntrBsp growing microaerobically on nitrate. Two-dimensional gel electrophoresis (2D-PAGE), and Western blot analyses were performed to separate and identify the P_{II} proteins. The antiserum raised against GlnK-MalE, which can detect both GlnK and GlnY, was used to detect the P_{II} proteins. The results of the 2D gel and Western blot are shown in Fig 4.29. A single GlnY like spot appeared at a less acidic pI (pI 6.4) and could clearly be differentiated from the other two more acidic P_{II} – protein spots (pI 5.2). The two distinct spots of P_{II} represent its modified (uridylylated) and unmodified forms with the spot towards the less acidic pI (pI 5.5) and less electrophoretic mobility representing the uridylylated form. It has already been reported that in strain BH72 cells growing on various N_2 sources except ammonium, two P_{II} like protein spots were always detectable in the wild type; both proteins were present as a mixture in a single spot (Martin *et al*., 2000). So in this situation although the GlnK antiserum binds to both, GlnK and GlnY, it is quite likely that the two GlnK spots may be a mixture of two P_{II} proteins (both GlnB and GlnK) in their modified and unmodified form. Although the nitrogenase is not completely repressed in BntrBsp under this condition, the unmodified form of P_{II} is relatively more abundant.

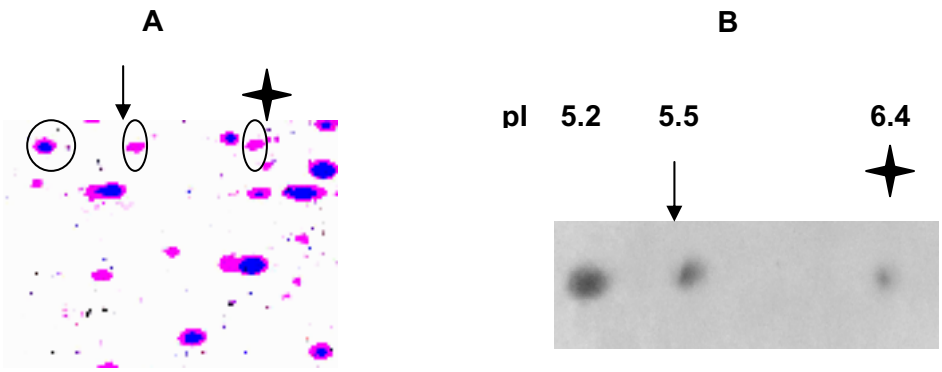

Fig 4.29 Analysis of P_{II} expression pattern along in BntrBsp by two-dimensional gel electrophoresis (A) and Western blot analysis (B) with antisera against GlnK. The *ntrBC* mutant strain was grown microaerobically with 10 mM nitrate as sole N-source. The arrow indicates the position of the modified P_{II} (UMP). The star indicates the GlnY spot at a relative more basic pI (pI 6.8) compared to that of the P_{II} proteins (GlnB and GlnK), both in their modified and unmodified forms.

4.6.2.3.3 Low but detectable *glnY::gus* expression in BntrBsp

In order to construct a transcription fusion of *glnY::gusA*, a 1.5 kb *Sal*I – *Sal*I fragment from pDZD18 was inserted into the *Sal*I site of pBluescript-SK-GUS (pSGLY-GUS). This plasmid was electroporated into strains BH72 and BntrBsp so that the reporter gene fusion construct with *glnY* got integrated within the genome by a single recombination event. The single recombinants were grown on different nitrogen sources and the GUS activity was measured (Fig 4. 30). The GUS activity of BGLY-GUS was nearly undetectable under all conditions tested. On the other hand, the NTRGLY-GUS had relatively low but detectable GUS expression levels. Interestingly the GUS activity was much higher in N-free medium (N_2 fixation) compared to that in SM+N. Also the *glnY::gusA* expression in BntrBsp was found to be relatively higher while growing microaerobically on mineral medium with nitrate as sole N-source as compared aerobically under similar condition.

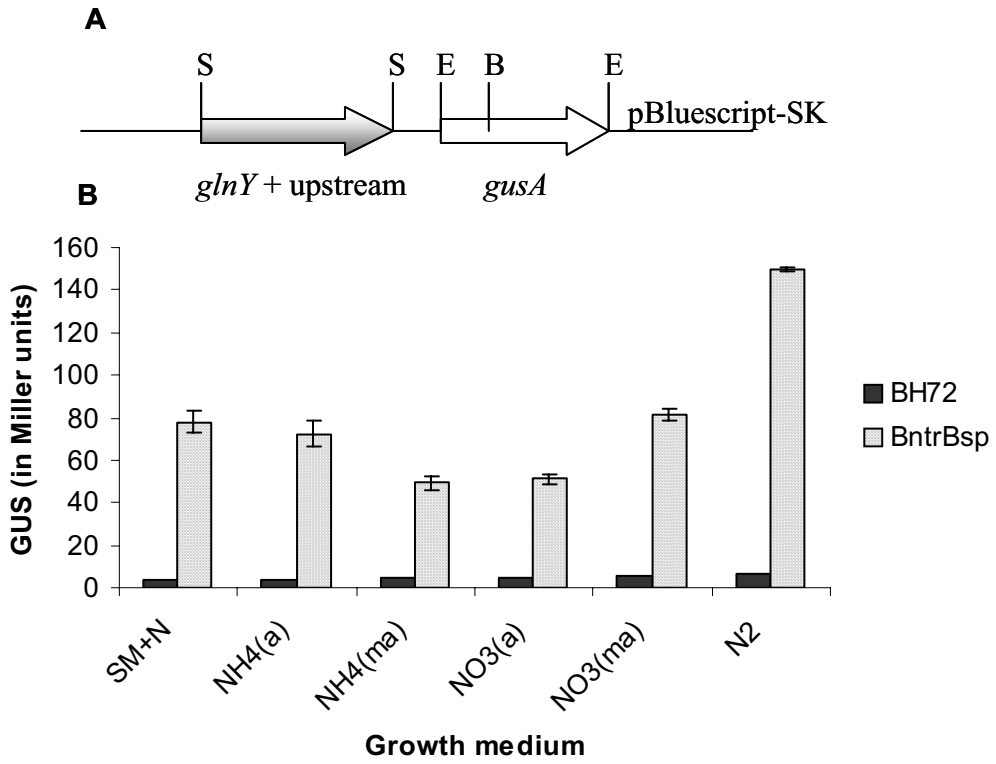

Fig 4. 30. Restriction map of *glnY::gusA* construct and its expression in strain BH72 and BntrBsp . (A) Restriction map of *glnY::gusA* construct in pBluescript-SK. S, *Sal*I; E, *Eco*RI; B, *Bam*HI; **(B)** Expression of *glnY* in strains BH72 and BntrBsp. The GUS activity was below the detection level in the wild type strain BH72 under all conditions tested. SM+N (Minimal medium with 10 mM ammonium and 0.01% yeast extract), NH_4 (in presence of only 10mM ammonium) or NO_3 (with 10mM nitrate) growing aerobically –NH_4 (a) and NO_3 (a) or microaerobically with 0.8% 0_2 – NH_4(ma) & NO_3(ma). "N_2 "represents growth under N_2 fixation. Results were obtained from three parallel GUS measurements of two independent experiments. Bars represent standard deviations

4.6.3 Study of genes for N–assimilation in *Azoarcus* : role of NtrBC

Genes involved in nitrogen assimilation are part of the *ntrBC* regulatory cascade. The enzyme glutamine synthetase (GS) is the main assimilatory enzyme converting ammonium to glutamine and strongly induced under N-limitations. Similarly, glutamate synthetase (GOGAT) also acts as another key enzyme for ammonium assimilation. On the other hand assimilatory nitrate reductase converts nitrate to nitrite and acts as the rate-limiting enzyme when the source of nitrogen is nitrate. The nitrite salts are toxic for the cells. Therefore cells detoxify nitrite rapidly by assimilatory nitrite reductase, which reduces nitrite into ammonium, which can be

easily utilized by the cell. It has been reported that transcription of many of these assimilatory genes are NtrBC controlled (Persuhn *et al*., 2000)

In order to study the transcriptional regulation of a few of these assimilatory genes, several contigs arising from the genome data of *Azoarcus* sp. strain BH72 (confidential and unreleased data from the *Azoarcus* genome project in cooperation with University of Bielefeld, Germany) were blasted against Genbank using BlastX (translated BLAST search tool). Primers were designed to specifically amplify certain regions of these ORFs. After standardizing the conditions for PCR amplification from genomic DNA for each of these putative assimilatory genes, transcripts both from the wild type and the *ntrBC* mutants were reverse transcribed and amplified by PCR. Differential transcription according to the N-source was observed, speaking in favour of an N-regulated expression pattern of these genes.

4.6.3.1 Identification of a putative glutamine synthetase III in *Azoarcus* sp. BH72

In order to identify the gene encoding for glutamine synthetase III, in strain BH72, a contig of 21.251 kb (contig 281) was blasted in parts against Genbank. A 1500 bp region located 13.500 kb downstream from the start of the contig was found to have 58% identity and 72% similarity to glutamine synthetase III from *Mesorhizobium loti*. The corresponding genetic region from *Azoarcus* was translated into protein and a putative protein with high similarity to *glnIII* gene from *Mesorhizobium loti* counter part was chosen. This predicted protein was then aligned with known protein sequences retrieved from related organisms by Clustal X. The result of this alignment is presented in Fig 4.31. This alignment identified a putative glutamine synthetase III in Azoarcus sp. BH72 by sequence comparison with GS proteins from other bacteria.

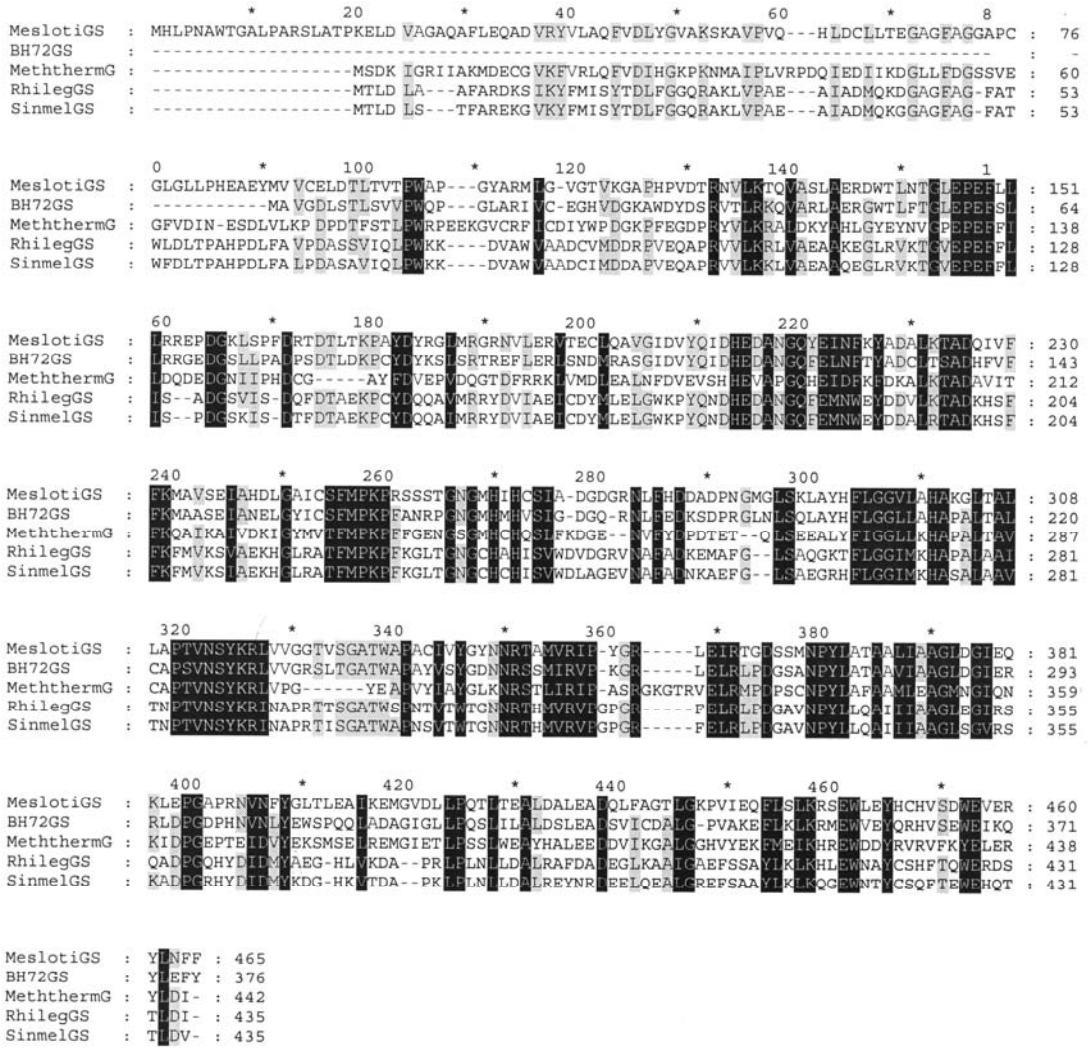

Fig 4. 31 Alignment of amino acid sequences from different glutamine synthetase proteins (GS). Putative GS protein sequence from *Azoarcus* sp. BH72 aligned with sequences from *M. loti, M. thermotrophicus*, *R. leguminosarum* and *S. meliloti*. The degree of similarity in each case is represented as follows: black :100%, dark grey : 80% and white : below 80%.

4.6.3.2 GSIII transcription in strain BH72 is nitrogen dependent but free from NtrBC regulation

Equal amounts of RNA isolated from both strains - BH72 and BntrBsp grown in ammonium and on N_2 was reverse transcribed (using RT beads) using reverse primer GS3 rev followed by PCR amplification (95°C for 1 min, 61°C for 1 min, and 72°C for 2 min.) of a central 350 bp fragment of the putative *glnIII* counterpart from strain BH72 using GS3 rev and GS3 for primers respectively. The results are shown in Fig 4. 32. No expression of *glnIII* was observed under N excess in both the strains, BH72 and BntrBsp. However there was strong expression of the putative GS protein under N- limitation (N_2 fixation) for both strains. 16S rRNA directed RT PCR

confirmed equal abundance of templates in all cases. These results indicated that the putative *glnIII* gene is nitrogen regulated but not under the control of NtrBC.

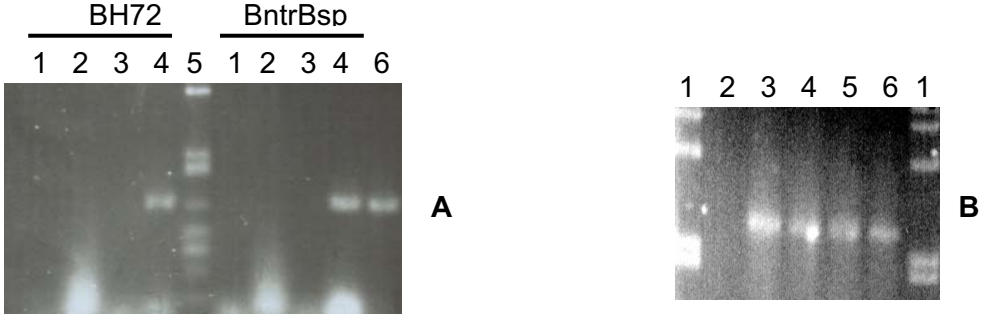

Fig 4.32 Analysis of glutamine synthetase III (*glnIII* gene) expression in strain BH72 and BntrBsp by RT-PCR. **(A)**RT-PCR of *glnIII* gene, using 1 μg RNA from N_2 fixing cultures of strain BH72 or BntrBsp. Reverse transcription carried out at 42°C for 30 min with the reverse primer GS3 rev followed by amplification for 35 cycles (95°C 1min, 61°C 1min and 72°C 2 min) using GS3 for and GS3 rev primers. RT-PCR product of *glnIII* from cells grown either in presence of ammonium with heat inactivation of reverse transcriptase prior to RNA addition (lane 1) or without heat inactivation (lane 2) or during N_2 fixation with heat inactivation of reverse transcriptase (lane 3) or without heat inactivation (lane 4); size marker (lane 5); amplification product from genomic DNA (lane 6). **(B)**, 16S rRNA directed RT-PCR from 10 ng RNA of the same preparation (as in A). Reverse transcription carried out with 1401rev primer (42°C 30 min) followed by amplification with 104f and 1346rev primers for 35 cycles of 1 min 95°C, 2 min 65°C, 2 min 72°C. 16S rRNA RT-PCR product from strains BH72 or BntrBsp growing in presence of ammonium (lane 3 and lane 5 respectively) or N_2 fixing (lane 4 and lane 6 respectively); heat inactivated reverse transcriptase prior to RNA addition (lane 2); size marker (lane 1).

4.6.3.3 Identification of the genetic region in *Azoarcus* BH72 putatively encoding glutamine-2-oxoglutarate amino transferase (GOGAT)

A similar approach was made to identify the *glt* gene encoding for putative GOGAT enzyme as it was done to identify *glnIII* of *Azoarcus* sp. BH72. Within the same contig (281), a 1.44 kb region (10620 bases downstream from the start) showed homology to the Glutamate synthetase large subunit like protein of *Sinorhizobium meliloti* (73% identity and 78% similarity). The translated amino acid sequences generated using ExPASy was aligned with known GOGAT amino acid sequences of other organisms using Clustal X. The alignment is shown in Fig 4.33. It shows that the putative protein sequence from strain BH72 aligned well with others and showed high level of identity with known sequences.

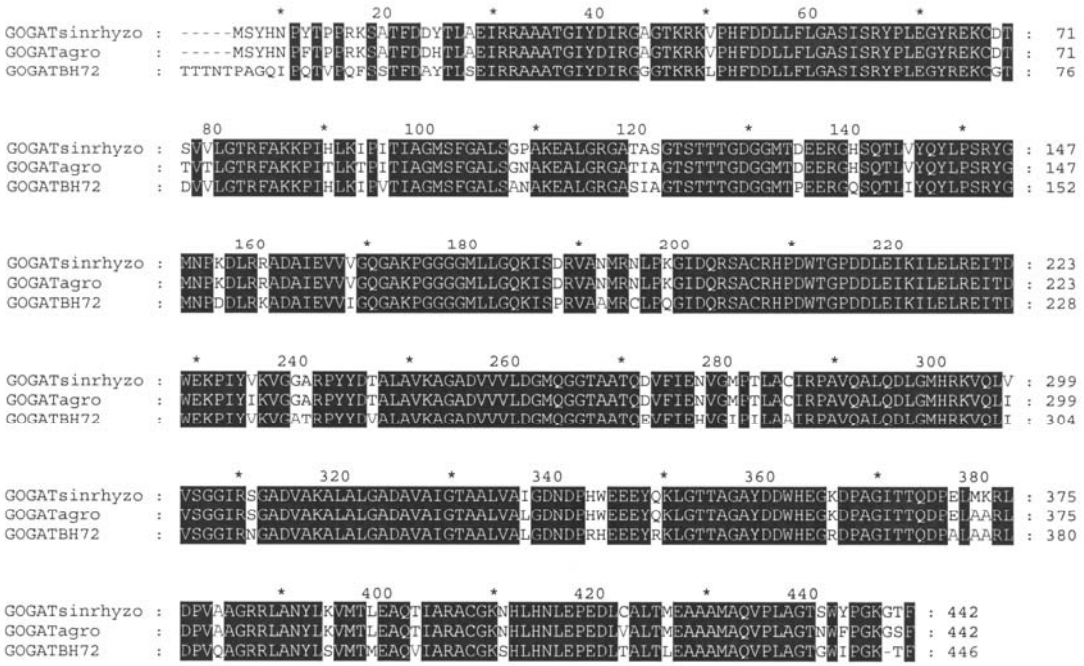

Fig 4.33 Alignment of amino acid sequences from different glutamate synthetase (GOGAT) proteins. Putative GOGAT protein sequence from *Azoarcus* sp. BH72 aligned with protein sequences from *S. meliloti* and *A. tumefaciens*. Only identity (100% homology) represented by black shade is shown.

4.6.3.4 Effect of nitrogen on *glt* expression and its control by NtrBC in strain BH72

Equal amounts of RNA from strain BH72 and BntrBsp were used to reverse transcribe and amplify a 440 bp fragment from the central part of a putative *gogat* counterpart using GOGATfor and GOGATrev primers. The reverse transcription was carried out at 42°C and the annealing temperature for the primers was 61°C. The result is shown in Fig 4.34. It clearly indicates that *glt* gene is differentially expressed in both the strains, the transcript being much more abundant under N_2 fixation, an observation similar to the *glnIII* expression in strain BH72. However the expression of *glt* in BntrBsp under N_2 fixation is relatively lower as compared to that in strain BH72 under similar conditions. Therefore, the expression of *glt* gene in strain BH72 appears to be partially regulated by NtrBC.

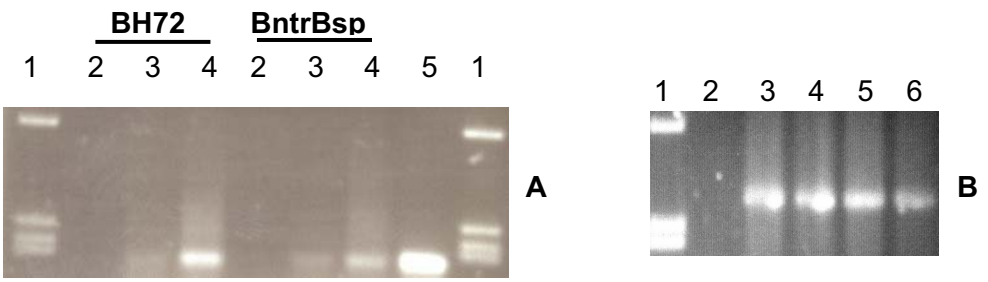

Fig 4.34 Analysis of *glt* gene (GOGAT) expression in strain BH72 and strain BntrBsp by RT-PCR. (**A**). One μg RNA, in each case was reverse transcribed by GOGAT RTrev primer at 42°C for 30 min, followed by PCR amplification by GOGAT RTfor and GOGAT RTrev primers for 35 cycles of 95°C 1 min, 61°C 1 min, and 72°C 2 min. RT-PCR product of *glt* gene from cells grown either in presence of 10 mM ammonium (lane 3) or N_2 fixation (lane 4); negative control with heat inactivated reverse transcriptase prior to the addition of RNA (lane 2); PCR amplification of *glt* from 100 ng genomic DNA using the same primers (lane 5); and size marker (lane 1). (**B**) 16S rRNA directed RT-PCR from equal amount of same RNA preparations (as in A) was carried out at 42°C for 30 min with 1401rev primer followed by amplification cycles of 95°C 1min, 65°C 2 min, 72°C 2 min by 104f and 1346rev primers. RT-PCR product from strains BH72 or BntrBsp growing in presence of ammonium (lane 3 and lane 5 respectively) or N_2 fixation (lane 4 and lane 6 respectively); heat inactivated reverse transcriptase prior to RNA addition (lane 2); size marker (lane 1).

4.6.3.5 Analysis of the nitrate assimilatory enzyme: nitrate reductase

As mentioned earlier, the *ntrBC* mutant (BntrBsp) showed signs of severe starvation while growing on plates of minimal medium with nitrate as sole N-source. The growth of the mutant is also prolonged when growing aerobically on such a medium. Therefore it is quite tempting to speculate that NtrBC of strain BH72 has a regulatory effect on the assimilatory nitrate reductase expression. To establish this fact, the putative assimilatory nitrate reductase of *Azoarcus* was detected by homology with other related genes and an attempt has been made to study its transcriptional regulation.

4.6.3.5.1 Identification of the genetic region in strain BH72 corresponding to the assimilatory nitrate reductase

A similar approach was made to detect the assimilatory nitrate reductase gene from strain BH72 as it was done previously to detect *glnIII* and *glt* using raw data from the *Azoarcus* genome project, Bielefeld. A 2.58 kb region from the beginning of contig 342 was blasted against Genebank. This region was found to have maximum homology to *Pseudomonas aeruginosa* PA01 assimilatory nitrate reductase (58% similarity and 50% identity). This genetic region from contig 342 was further translated into a protein sequence by ExPASy and the amino acid sequence generated by this way was aligned with other assimilatory nitrate reductase protein

sequences downloaded from the database using ClustalX. The result of this alignment is presented in Fig 4.35. It appears that the putative protein sequence from strain BH72 is very similar to other assimilatory nitrate reductases.

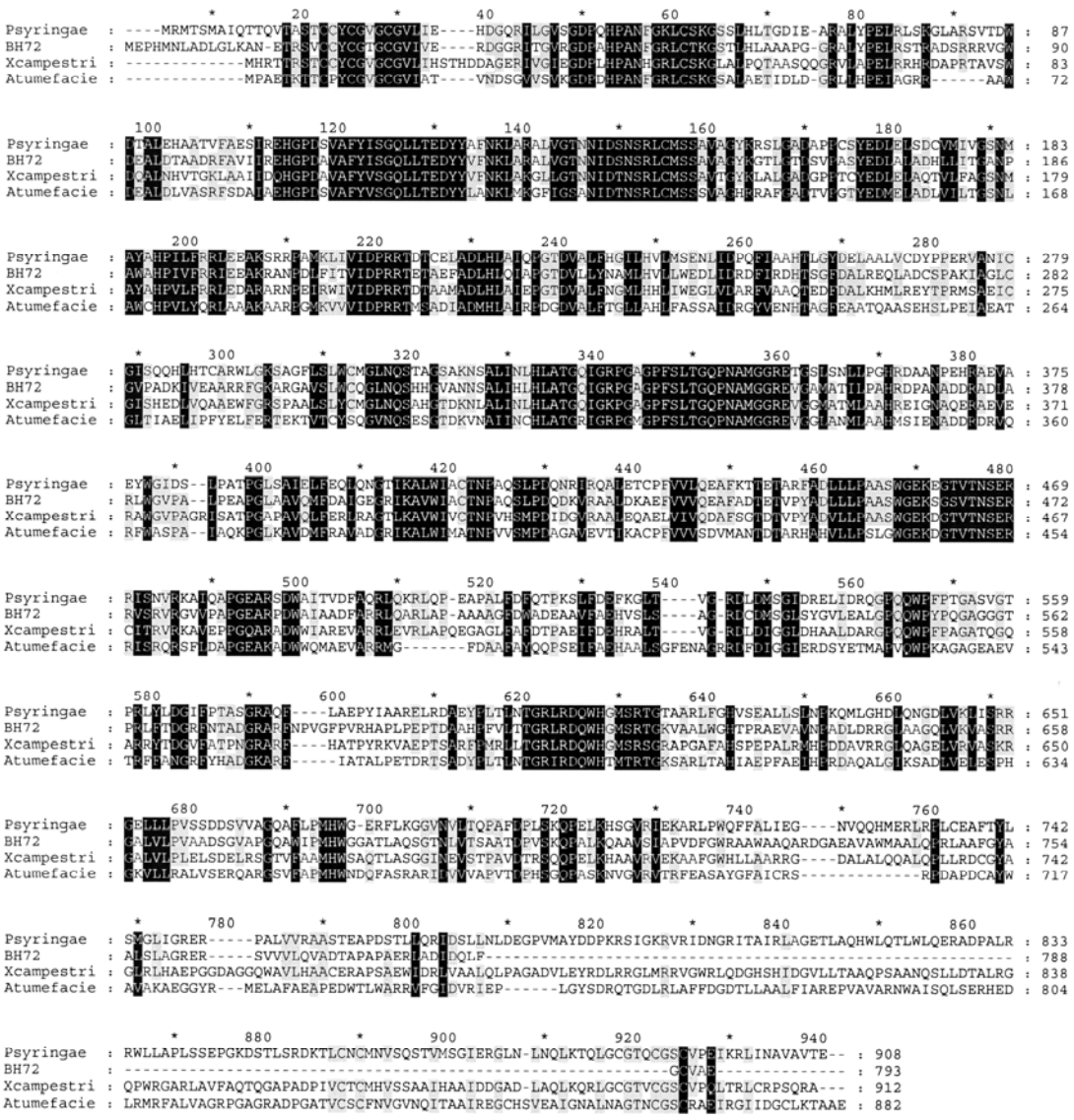

Fig 4.35 Alignment of amino acid sequences from different assimilatory nitrate reductase proteins. The putative protein sequence from *Azoarcus* sp. strain BH72 is aligned with the enzyme sequences derived from *P. syringae*, *X. campestris*, and *A. tumefaciens*. The degree of sequence similarity is represented as follows: black : 100%, dark grey : 80%, and white : less than 80%.

Nitrate reductases can be also of the dissimilatory type (respiratory nitrate reductase). Usually these are membrane associated and involved in reduction of nitrate to molecular N_2. To ensure that the above mentioned gene is of the assimilatory type and not a dissimilatory one, the protein sequence of this putative nitrate reductase from strain BH72 was aligned with other dissimilatory nitrate

reductase sequences from the data base. The result of such an alignment is shown in Fig 4.36.

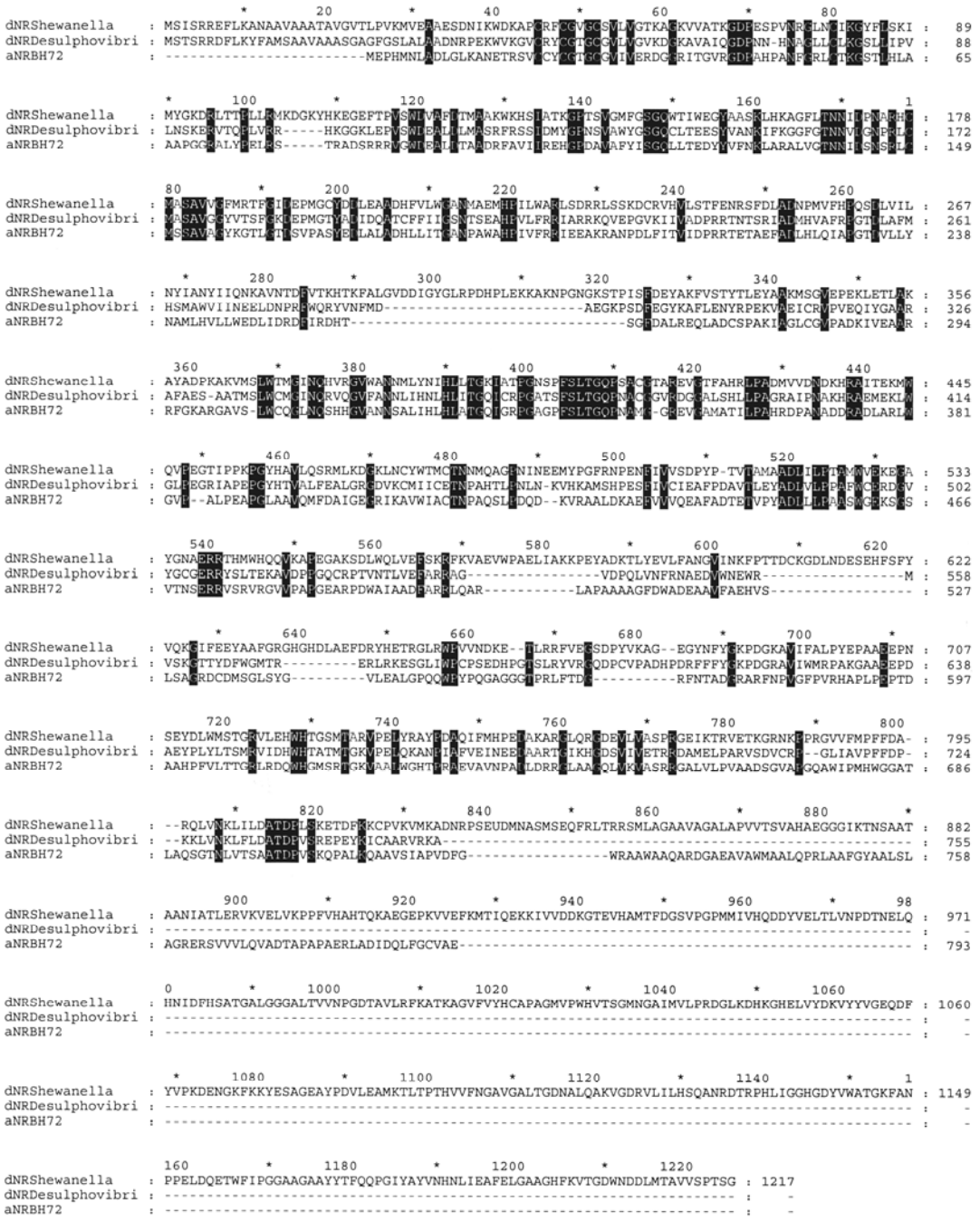

Fig 4.36 Alignment of the putative assimilatory nitrate reductase sequence from strain BH72 with different dissimilatory nitrate reductase sequences. The dissimilatory sequences from *Shewanella violacea* and *Desulphovibrio* are aligned with the putative assimilatory nitrate reductase sequence from strain BH72. Only regions of identical amino acids (100% sequence similarity and shaded in black) have been shown. The putative *Azoarcus* counter part is much less similar to diifferent dissimilatory nitrate reductase sequences than to other assimilatory nitrate reductases from reference organisms (Fig 4.35).

From the alignment in Fig 4. 36, it is quite clear that the putative assimilatory nitrate reductase sequence from strain BH72 shows low similarity as well as identity

to potential dissimilatory nitrate reductase sequences of *S. violacea* and *Desulphovibrio*. Nitrate reductases in general, share similar functional domains within their structure. Usually a Molybdopterin oxidoreductase Fe_4S_4 domain is present near the N-terminus of nitrate reductase. The central part of the enzyme bear typical Molybdopterin dinucleotide cofactor binding domain which may constitute an entire subunit and a Molybdopterin oxidoreductase domain. A [2Fe – 2S] binding domain is present near the C-terminus which function as a redox regulatory component and is often absent in dissimilatory nitrate reductases. Dissimilatory nitrate reductases in addition, bear signal peptide sequences and transmembrane helices at their N-terminals, typical for membrane proteins. It was evident from protein sequence analysis by programme SMART, that dissimilatory nitrate reductases from *Shewanella violacea* or *Desulphovibrio* possessed signal peptide sequences and transmembrane helices, which were absent in the putative assimilatory nitrate reductase from strain BH72, in consistent to other cytoplasmic assimilatory nitrate reductases from different organisms. This line of evidence is also clear from the alignment, where the N-terminal region of the protein sequence from strain BH72, appears shorter in length compared to the dissimilatory nitrate reductase sequences. All these speak in favour of cytosolic and assimilatory nature of the putative nitrate reductase sequence from strain BH72.

4.6.3.5.2 The expression of the assimilatory nitrate reductase gene in strain BH72 is NtrBC dependent.

An attempt was made to amplify the transcript for assimilatory nitrate reductase by RT-PCR, both from strains BH72 and BntrBsp, to analyse its expression. Equal amounts of RNA (1 μg) from strain BH72 and BntrBsp growing aerobically on 10 mM nitrate as sole N-source were DNaseI treated and amplified after reverse transcription using primers specifically designed for assimilatory nitrate reductase. Unfortunately the standard method of reverse transcription with RT beads was not working in this case. Therefore a new method was adopted. Initially the reverse primer (NO3revRT) was allowed to anneal to RNA by allowing the template primer mix to gradually cool down from 70°C to 42°C, followed by reverse transcription with AMV reverse transcriptase for 1 hour at 42°C. About 7.5 μl of this reaction mix was then used as a template for PCR amplification using the Proof start enzyme from Qiagen: the cycling conditions being 94°C 30 sec, 63°C 30 sec and 72°C 25 sec for 45 cycles with nirforRT and NO3revRT primers, respectively. The results are shown in Fig 4.37. Interestingly a PCR product (506 bp band) was obtained which was much

less intense in BntrBsp compared to that of strain BH72, although equal amounts of RNA were used as template for both the cases. A 16S rRNA directed RT-PCR was carried out using equal amount (10 ng) of same RNA preparation from strains BH72 and BntrBsp respectively. Reverse transcription carried out at 42°C for 30 min with 1401rev primer followed by 35 PCR cycles of 95°C 1 min, 65°C 2 min, and 72°C 2 min with 104f and 1346rev primers produced bands of comparable intensities in both the cases, verifying once more, that equal amount of starting template have been taken for both the strains. These results show that the assimilatory nitrate reductase gene in the *ntrBC* mutant (BntrBsp) is down regulated and that NtrBC is directly or indirectly involved in the regulation of the assimilatory nitrate reductase in strain BH72.

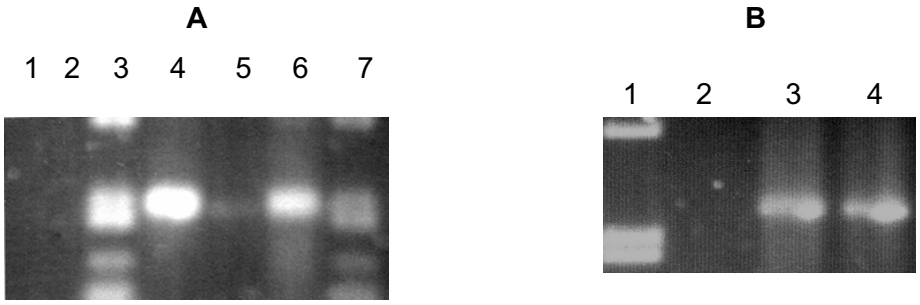

Fig 4.37 Analysis of the expression of assimilatory nitrate reductase by RT-PCR in the wild type and the *ntrBC* mutant. **(A)** RT-PCR analysis of the assimilatory nitrate reductase transcript with RNA as templates which was isolated from BH72 and BntrBsp growing aerobically on 10 mM nitrate as sole N-source. Reverse transcriptase heat inactivated prior to addition of RNA from BntrBsp (lane 1) and BH72 (lane 2); size marker (lane 3), PCR amplification product from genomic DNA (lane 4), RT-PCR amplification of RNA after 45 cycles from BntrBsp growing on nitrate (lane 5), the same from strain BH72 (lane 6), size marker (lane 7). **(B)** 16S rRNA directed RT-PCR taking equal amounts of RNA from strain BH72 or BntrBsp, respectively. Size marker (lane 1), RNA with heat inactivated reverse transcriptase (lane 2), 16S rRNA amplification product from strain BH72 (lane 3) and from strain BntrBsp (lane 4).

5 Discussion

Although biological nitrogen fixation is carried out only by certain *Eubacteria* and *Archea,* the capacity for N_2 fixation is dispersed widely among a phylogenetically diverse group of these organisms. In a broad sense, nitrogenases from phylogenetically diverse diazotrophic organisms show similarity in structure and enzymatic mechanism. However the nitrogenase protein sequence may vary at the level of identity and similarity within different sub-groups of *Proteobacteria*. For example, *Herbaspirillum seropedicae* belonging to the β-subgroup of *Proteobacteria*, has α-type nitrogenase (Machado *et al*., 1996). So analysis of nitrogenase from *Azoarcus* sp. strain BH72, is crucial, as it provides a platform for the first "true β-type nitrogenase" to be analysed.

Characterization of *nifHDK* and *nifLA* transcripts reveals unusual as well as common features in *Azoarcus* sp. strain BH72.

The genetic organization of nitrogenase of *Azoarcus* sp. BH72 shows certain unique features. Downstream of the *nif* genes, a sequence encoding for 2[4Fe-4S] cluster carrying protein is present, having 59% amino acid identity to ferredoxin FdxN from *Rhodobacter capsulatus*. This organization is quite unlike to the *nifH* organization of *K. pneumoniae* and *A. vinelandii* where a *nifT* gene of unknown and nonessential function is present downstream of nitrogenase genes (Simon *et al*., 1996). In diazotrophs such as proteobacteria of the α-subgroup or *H. seropedicae* belonging to the β-subgroup, NifA proteins act as essential transcription activators of nitrogenase and are directly responsive to nitrogen and O_2. Strangely enough, *Azoarcus* sp. BH72 possesses NifL / NifA two-component regulatory system, with NifL inhibiting NifA activity, in response to O_2, very similar to that of diazotrophs of the γ subgroup of the *Proteobacteria*. The promoters for both *nifHDK* as well as *nifLA*, have been well characterized in *Azoarcus* sp. strain BH72 (Egener *et al*., 2001, Egener *et al*., 2002). About 80% of the bases of the putative promoter region of *nifHDK* upstream of the start codon match with the standard consensus defined for sigma-54 type promoters; for the *nifLA* promoter region, the match is 73.3%. This speaks in favour of an RpoN-dependent transcription from these promoters. Furthermore, typical NtrC-binding palindrome motifs are present upstream from the consensus in both cases,

suggesting an interaction of NtrC with the σ^{54}-type RNA polymerase, to initiate transcription activation.

Interestingly, genes of N-regulatory pathways may possess two different types of promoters. For example, the *E. coli glnA* is transcribed from two different promoters: *glnA*p1 – a sigma(70) dependent promoter for expression during N excess and *glnA*p2 – a sigma(54) dependent N-regulated promoter, used during N-starvation (Tian *et al*., 2001). Similarly, the *Azorhizobium nifA* gene carries overlapping house keeping type and sigma (54) type promoters that interactively respond to different signals (Loroch *et al*., 1995). On the other hand in *Azospirillum brasilense*, two overlapping upstream activator sequences have differential effects on the *nifH* promoter activity (Passaglia *et al*., 1995); promoter mapping with S1 nuclease revealed two start sites of the *nifH* transcript located 10bp and 40 bp downstream of the NtrA-dependent promoter (de Zamaroczy *et al*., 1989). The situation can be more complicated as is the case e.g. in the *nifH* promoter region from *Rhizobium leguminosarum*, where a pseudo-promoter and a pseudo-upstream element are present along with a sigma(54) N-regulated promoter consensus (Roelvink *et al*., 1990). Therefore it is important to map the transcription start point in order to define the promoter of a gene.

Usually the *nifHDK* and *nifA* / *nifLA* in diazotrophic proteobacteria are characterized by σ^{54} – type promoters, but exceptions are also present. In *A. brasilense*, consensus sigma-54 and NifA binding sites are present only upstream of *nifENX* gene, and not in the upstream of *nifHDK* (*Potrich et al*., 2001) whereas sigma (70) recognition sites occur upstream of its *nifA* gene (Fadel-Picheth *et al*., 1999). In *R. capsulatus*, no typical RpoN-activated promoters are present, upstream of *nifA1* and *nifA2*, the transcription activators of *nifHDK* gene (Foster-Harnett *et al*., 1992) On the other hand, *R. japonicum* share two sigma54 promoters, upstream of *nifH* and *nifDK* respectively (Adams *et al*., 1984). Whereas *H. seropedicea*, shares a typical sigma (54) promoter consensus, similar to *K. pneumoniae*, and possesses typical NifA and integration host factor (IHF) binding sites (Machado *et al*., 1996). In contrast, methanogenic archaeons, like *M. maripaludis* are characterized by AT-rich Box-A promoter sequences, 24 bp upstream of *nifH* gene ((Kessler *et al*., 1998). Primer extension analysis carried out for *nifHDK* and *nifLA* of *Azoarcus* sp. BH72 revealed that, indeed one major transcriptional start point is used for each of the above mentioned genes when the bacteria are fixing nitrogen. Moreover the detected

transcriptional start point in each case matched precisely with respect to the location of the upstream –12 / -24 consensus, suggesting that this might represent an active promoter *for nifHDK* and *nifLA,* respectively. Usually primer extensions are carried out by radioactively labelled reverse primers, which might turn out to be much time consuming and hazardous. However in this study, all primer extension experiments have been carried out non-radioactively, using Cy5 labelled reverse primers and an ALFexpress automated sequencer (Pharmacia biotech). It is clear from the experimental results that the new methodology developed, is quite robust and well reproducible.

The structural genes of the nitrogenase enzyme complex in strain BH72 are genetically linked to a ferredoxin gene (*fdxN*) (Egener *et al*., 2001). Ferredoxins are believed to be electron donors for nitrogenase in bacteria. In *A. vinelandii*, *R. capsulatus* or *S. meliloti*, *nif*-specific ferredoxins have been identified that are not genetically linked to the structural genes of nitrogenase (Klipp *et al*., 1989; Schatt *et al*., 1989). In *A. vinelandii,* a ferredoxin like gene is localized downstream of *vnf* coding for the iron protein of vanadium nitrogenase (Robson *et al*., 1986). Cotranscription of *nifHDK* with *fdxN* was proved by RT PCR in strain BH72, by amplifying a region of the transcript spanning between *nifK* and *fdxN*. This unusual transcriptional linkage speaks in favour of a functional linkage of these two proteins in strain BH72. In a similar way, it has been shown that indeed *nifL* and *nifA* in strain BH72 are also cotranscribed. This situation is more similar to *γ-Proteobacteria*, where *nifL* is transcribed along with *nifA*, NifL being the sensor inhibiting NifA activity in response to O_2 (Dixon, 1998). One of the major significance of coordinated synthesis of antagonistic regulatory proteins, like NifL and NifA, is to maintain the correct stoichiometry for efficient regulation of N_2 fixation. In *K. pneumoniae*, a low translation rate, as a result of inhibitory secondary structures arising at the Shine-Dalgarno sequence of NifA has been reported, in the absence of NifL (Govantes *et al*., 1996).

The *nifLA* transcript was detectable in aerobically grown cells on combined nitrogen but was more abundant during N_2 fixation in Northern blot analysis. This differential expression pattern was confirmed by RT PCR amplification of the *nifA* transcript. Also, no *nifA* transcript was detected in the polar mutant of *nifA* (BHLAO), while growing in combined nitrogen, confirming cotranscription. In *A. vinelandii*, transcription of *nifLA* is not repressed by ammonium (Blanco *et al*., 1993). However in *Klebsiella* and *Enterobacter*, NifA expression is strongly enhanced (about 10-fold)

under anaerobic conditions mediated by DNA-gyrase dependent negative supercoiling. In this aspect strain BH72 shows similarities to *K. pneumoniae nifLA* regulation, since the expression is enhanced both by ammonium deficiency and by microaerobiosis, without the involvement of the FNR box (Hu *et al*., 2000).

The NtrBC proteins of strain BH72: the "third element" in the N-regulatory cascade.

The *ntrBC* two-component "sensor-response regulator" system has been identified in many different bacteria. It plays an important role in sensing and evoking N-regulatory cascades involved in N_2 fixation and N-assimilation. NtrB acts as a bifunctional histidine kinase (phosphatase) that in turn phosphorylates NtrC. NtrC-P acts as transcriptional activator of many N-regulated genes. In the context of N_2 fixation, NtrC-binding activates *nifA* transcription (second element), which in turn acts as an essential transcriptional activator of *nifHDK*, the structural genes for nitrogenase (first element). The concentration of NtrC is increased as cells become starved for ammonia, concurrent with the activation of the *ntr* genes that have less efficient NtrC binding sites like the *nac* and *glnK* promoters than does *glnA* (Atkinson *et al*., 2002). In this way, NtrC regulated operons could be temporally separated (with time) for expression, in the regulatory cascade.

In this study, *ntrB* and *ntrC* coding regions have been identified in strain BH72. The *ntrBC* genes of strain BH72 share strong identity with *Ralstonia solanacearum*, and *Herbaspirillum seropedicea* counterparts, both belonging to the β-subgroup of *Proteobacteria*. Analyses of the predicted NtrB and NtrC proteins by SMART (www.smart.embl-heidelberg.de) revealed the presence of typical PAS, histidine kinase and ATPases domains in NtrB, and phosphoacceptor, ATP-binding and "helix-turn-helix" motifs in NtrC. All these domains are correlated to the function of sensor and response regulator respectively. PAS domains constitute signal-receiving domains of sensor kinases in association with bound cofactors. Autokinase activity is exhibited by histidine kinase domain of NtrB like sensors whereas ATPase-like domain is responsible for the phosphatase activity of the sensor, which is essential for the down regulation of the cascade under nonfavourable conditions. The N-terminal phosphoacceptor domain of NtrC-like response regulators have the conserved aspartate residues, representing the active site for phosphotransfer. The C-terminal helix-turn-helix domains are typical, not only for NtrC, but in general, for

DNA-binding proteins. The central ATP binding domain is characteristic for NtrC-like transcription activators, where binding of ATP in this domain helps in oligomerization of NtrC and subsequent transcriptional activation of the sigma(54)-dependent RNA polymerase, by formation of functional transcriptional open-complex. RT PCR analyses revealed that both *ntrB* and *ntrC* transcripts of the expected size are expressed during N_2 fixation. Also an RT PCR, similar to that of *nifLA* in strain BH72 has also verified their cotranscription status.

Surprisingly, no proper ribosome-binding Shine-Dalgarno motif could be detected before the putative start codons of *ntrB* and *ntrC*. Shine-Dalgarno (SD) dependent translation initiation predominates in *E. coli*. However, this bacterium also has the capacity to initiate translation in a SD independent manner. In contrast, plant chloroplasts and their cyanobacterial ancestors appear to have adopted SD-independent mechanisms for most of their translation initiations (Fargo *et al*., 1998). In *E. coli*, the human calcitonin gene was more strongly expressed from a nonconventional SD translation initiator depending on the binding energy (Mironona *et al*., 1999). The +2 codon immediately following the start codon may sometimes act as critical translation determinant (Stenstrom *et al*., 2001). On the other hand, extended base pairing surrounding the start codon may compensate for the lack of a classical SD (Esposito *et al*., 2001). A/T rich sequences upstream of the start codon of *E. coli* K12 have been reported to correlate with translation initiation (Yamagishi *et al*., 2002). Interestingly, an A+T element represented by a “TATTA” stretch could be located 2 bases upstream of the NtrC start codon in strain BH72.

An attempt to find a standard bacterial promoter consensus (sigma-54 or sigma-70), upstream of the *ntrBC* coding region failed. In α-*Proteobacteria*, *ntrBC* is generally encoded as *ORF1-ntrBC* operon, *ORF1* being an upstream open reading frame of unknown function. In *Azospirillum brasilense*, *ORF1* has similarity to *nifR3* of *Rhodobacter capsulatus* and is transcribed from a vegetative sigma(70)-type promoter (Machado *et al*., 1995). However in enterics, *ntrBC* transcription is linked to a *glnA* gene located upstream of *ntrBC* in form of a *glnA-ntrBC* operon and transcribed from a N-regulated sigma(54)-type promoter (MacFarlane *et al*., 1985). Primer extension analysis to map the 5’ end of the transcript revealed in *Azoarcus* sp., the presence of a single transcript which mapped at a position, 150 bp upstream from the putative start codon of the *ntrB*, however also at the vicinity of this position a promoter sequence consensus could not be detected.

Surprisingly, transcriptional fusions of the GUS reporter gene with the *ntrBC* upstream region were not successful, as the expression levels were too low. However, expression could be detected in RT PCR studies in strain BH72. It was

shown that the *ntrB* is expressed both in presence of combined nitrogen and on N_2 in a differential manner. Under these circumstances transcript abundance was much higher when cells fix N_2, but not subjected to autoregulation. In *Klebsiella pneumoniae* the *ntrBC* operon has a structure like P1-*glnA*-P2-*ntrBC*, P1 and P2 representing two promoters. P1 promotes transcription under N-deficiency and P2 under N-excess. P1 is regulated both positively and negatively, while P2 is only negatively regulated by NtrC (Morales-Alvarez *et al.*, 1984). In contrast, in *Rhizobium leguminosarum*, the promoter is subjected to negative autoregulation by NtrC (Patriarca *et al*., 1993). In this context, it's quite unusual that NtrBC in strain BH72 is not subjected to autoregulation. Another interesting finding in this study, is that *ntrB* in strain BH72 is expressed in a N regulated manner, although a typical sigma(54) promoter consensus is lacking upstream of *ntrB*. It is quite tempting to speculate that *ntrBC* transcription in strain BH72 is probably regulated from some distantly located upstream element and that the major transcript is subjected to subsequent processing. On the other hand the possibility of transcription being driven by some novel promoter elements cannot be ruled out. Another speculation in this respect might be related to DNA-bending within the genome, which might mobilize distantly located sigma(54)-type promoter elements, facilitate the binding of the transcription machinery, and trigger transcription. Interestingly, a putative ORF having homology to RNA binding protein from *Homo sapiens* is present in the upstream of *ntrBC* of strain BH72 in the same orientation. So the possibility of unusual transcriptional linkage of *ntrBC* with this ORF cannot be ruled out.

Nitrate assimilation in strain BH72 is positively controlled by NtrBC

The BntrBsp (*ntrBC*-mutant) of *Azoarcus* was impaired during growth on nitrate as sole N-source. While growing on agar plates with nitrate as sole N-source, the colony morphology appeared drastically different from the wild type; the mutant colonies appeared more opaque and rounded. When viewed under phase contrast microscope, the cells of BntrBsp appeared smooth surfaced with accumulation of shining crystalline particles, centrally. When growth is limited by availability of an essential nutrient other than carbon and energy source, many bacteria accumulate reserve materials such as polyphosphate, PHB (poly-β-hydroxybutrate) or glycogen

(Dawes *et al*., 1973). A pronounced increase of PHB in culture of nitrogen-fixing *A. lipoferum* Rp5 and *A. halopraeferens* Au4, and to a lower extent in *Azoarcus* sp. BH72 have been reported at high O_2 concentrations when the electrons could not be channelled to nitrogenase and were used for PHB synthesis instead (Hurek *et al*., 1987). Also the unreleased data from the sequenced genome of *Azoarcus* sp. BH72 provide evidence for the existence of several enzymes involved in the metabolism of PHB. In *Azospirillum brasilense* Sp7, the *ntrBC* genes are involved in the regulation of PHB synthesis by ammonia (Sun *et al*., 2000). So it is quite likely to assume that these refractive particles represent PHB granules and NtrBC in strain BH72 either directly or indirectly regulates PHB synthesis. However, an increase of PHB biosynthesis in the *ntrBC* mutant could be a direct consequence from the down regulation of P_{II} proteins. The same has also been reported for *A. brasilense* Sp7 (Sun *et al*., 2002). The prolongation of the generation time (9.5 h) of the *ntrBC* mutant growing aerobically on nitrate provides further evidence that BntrBsp was impaired in growth on nitrate, similar to that has been reported for *A. brasilense* (Liang *et al*., 1993). In this respect, it is relevant to mention that NtrBC of *Azoarcus* sp. BH72 was found by complementation of *A. vinelandii* mutants unable to grow on nitrate as sole N-source. Thus all these observations point towards an essential role of NtrBC in nitrate assimilation in strain BH72. It can be recollected, that the *glnB*$^-$*K*$^-$ (double mutant) of strain BH72 fixes N_2 and exhibits prolonged generation time while growing on nitrate as sole N-source, a phenotype similar to that of *ntrBC* mutant. On the other hand, no impairment of *ntrB* nonpolar mutant to grow on nitrate is an unusual feature. Indeed, the nonpolar *ntrB* mutant shared phenotypes similar to that of the wild type strain. This can be interpreted in terms of another phosphorylation circuit, which might be still active in *ntrB* nonpolar mutant and is able phosphorylate NtrC in absence of NtrB.

From the study of these phenotypes, it becomes apparently clear that NtrBC in strain BH72 has a regulatory effect on the main enzyme of nitrate assimilation, nitrate reductase. *Enterobacteria* can use nitrate and nitrite both as electron acceptors and as source of nitrogen for biosynthesis. In *Klebsiella*, assimilatory nitrate and nitrite reductase convert nitrate through nitrite to ammonium. The enzymes and structural genes for nitrate/nitrite respiration and assimilation are distinct, and are subject to different patterns of regulation. Respiratory enzyme synthesis is indifferent to the availability of ammonium, and transcription is induced by anaerobiosis via the FNR protein. In *Enterobacteria*, the respiratory enzyme is further induced by nitrate or nitrite through a two-component system, where NarX and NarQ act as cognate sensors and NARL and NARP being the response regulators, respectively (Stewart,

V. 1994). In contrary, the assimilatory enzyme synthesis is indifferent to oxygen availability, induced by ammonium and nitrate/nitrite limitation via NTRC or via the transcriptional antiterminator protein NasR (Stewart, V. 1994). Assimilatory nitrate reductase catalyzes the rate-limiting step in nitrate reduction with molybdenum as cofactor (Campbell, W.H. 1999). The gene for assimilatory nitrate reductase was amplified using sequence information from unreleased data of the *Azoarcus* genome project. The corresponding *Azoarcus* counterpart shared high homology with other assimilatory nitrate reductase protein sequences, but low sequence similarity with dissimilatory nitrate reductase sequences. In *Klebsiella*, *nasA* and *nasB* (coding for nitrate and nitrite reductase respectively) are linked in form of an operon. Surprisingly, the *nasBA* operon is tightly linked to respiratory nitrate reductase although it serves a different physiological function (Lin, *et al*., 1993). As expected from the *ntrBC* mutant phenotype, the assimilatory nitrate reductase transcript was abundant in strain BH72 while growing on nitrate but was strongly down regulated in BntrBsp. This very weak expression of assimilatory nitrate reductase in the *ntrBC* mutant may account for its prolonged generation time, while growing aerobically on nitrate. Therefore it is likely to assume that assimilatory nitrate reductase in strain BH72 is NtrBC regulated but still exhibits very low constitutive expression, unlike in others. Interestingly, in *Azospirillum brasilense*, not NtrBC, but a novel NtrY/X two component system is involved in nitrate assimilation instead. A cross talk between these two components may add a fine-tuning to nitrate assimilation (Ishida, *et al*., 2002).

Twitching motility in strain BH72 is down regulated by NtrBC.

“Twitching motility”- a term coined by Lautrop, describes flagella–independent surface motility exhibited by a wide range of bacteria. It is equivalent to social gliding motility in *Myxococcus xanthus* and is important in host colonization by a wide range of plant and animal pathogens as well as for formation of biofilms. Type IV pili are known to be important for twitching motility (Mattick, J.S. 2002) and this has also been reported for *Azoarcus* sp. BH72 (Plessel, 2001). The *ntrBC*-mutant exhibited two times more twitching compared to the wild type in complex medium. This is quite surprising, as many type IV pilin genes (*pilA*) are known to be regulated by PilR, which has a high sequence similarity at the amino acid level to the NtrC family of response regulators (Wu *et al*., 1997). On the other hand, it has been shown that signal transduction in Ntr and Che systems involves a common phosphotransfer

mechanism; high intracellular concentration of NRII can suppress a smooth-swimming phenotype of a *cheA* mutant involved in chemotaxis (Ninfa, *et al*., 1988). Such cross-talks between N-assimilation and chemotaxis signals in bacterial signal transduction pathways help in fine tuning. Therefore, it is possible that NtrBC from strain BH72 directly or indirectly controls the twitching motility negatively. On the other hand, it can be argued that intriguing cross talks may be involved in regulating twitching. However such predictions are quite speculative and more evidence is required to support this hypothesis.

The *nifLA* expression is positively regulated by NtrBC in strain BH72.

It has been well established that in strain BH72, the NifA acts as an essential transcription activator for the *nifHDK*, coding for the structural genes of nitrogenase (Egener *et al*., 2002). While in *Azotobacter vinelandii*, transcription of *nifLA* is not repressed by ammonium and not dependent on NtrC or RpoN (Blanco *et al*., 1993), it is NtrC-dependent in *K. pneumoniae* (Minchin *et al*., 1988) and *Enterobacter agglomerans* (Siddavattam *et al*., 1995). In *Klebsiella* and *Enterobacter cloacae*, *nifLA* expression is also enhanced (approx. tenfold) under anaerobic conditions mediated by DNA gyrase dependent negative supercoiling (Hu *et al*., 2000). In *ß-Proteobacteria*, like *Herbaspirillum seropedicae,* concerted action of NtrC, NifA and IHF (Integration Host factor) is essential to fine-tune NifA expression in response to ammonium and oxygen levels (Wasem *et al*., 2002). Unlike in *Klebsiella*, transcription of *nifA* does not require NtrB/NtrC proteins in *A. brasilense*, and the expression of *nifHDK* is controlled by posttranslational regulation of NifA activity (Zhang *et al*., 1997). Indeed, *nifLA* gene expression in strain BH72 was found to be NtrBC regulated, similar to that of *γ-Proteobacteria*. This fact was further exemplified by a relative low *nifH* expression in strain BntrBsp (but *nif*+) compared to strain BH72 under derepression conditions and relative prolongation of generation time in strain BntrBsp during N_2 fixation. The question whether NtrC acts directly on the *nifH* promoter or indirectly via NifA is debatable. Since *nifA* is invariably necessary for *nifHDK* transcription, it is not likely that NtrC is an additional transcriptional activator of *nifHDK*. However NtrC-binding tandem activation sequences are present upstream of *nifHDK* promoter. Although binding of NtrC, upstream of *nifHDK* promoter has not been reported for *Klebsiella* or *A. vinelandii*, the promoter of the alternative

nitrogenase of *R. capsulatus* has been shown to be activated by NtrC binding (Kutsche *et al*., 1996). While in *A. brasilense*, NtrC is not absolutely required for *nifH* promoter activation (Broek *et al*., 1992), the *glnG (ntrC*) gene product might directly regulate the symbiotic nitrogen fixation genes in *Rhizobium* (Sundaresan *et al*., 1983). In *Klebsiella pneumoniae*, GlnK is required for relieving the inhibition of NifA activity in absence of combined nitrogen (Jack *et al*., 1999). It has been shown (this work), that *glnK* expression is down regulated in the *ntrBC* mutant along with the expression of *glnY*. In accordance with this observation, reduced *nifH* expression can be attributed to the indirect effect of NtrC via GlnK, affecting NifA activity. At the same time, it could be further speculated that the expressed GlnY in BntrBsp perhaps cannot complement GlnK function by interacting with NifL and release its inhibitory effect from NifA, pointing to distinct regulatory functions of each of the three P_{II} paralogues in strain BH72.

Transcriptional regulation of the *glnK* and the *glnB* in strain BH72: role of NtrBC

glnK is genetically linked to *amtB* in strain BH72 (Martin *et al*., 2000). The physical association of these two genes is found in a diverse range of *Bacteria* and *Archea*. The *glnK-amtB* operon is induced specifically during conditions of ammonium limitation except in *Azotobacter vinelandii*, where it is an essential gene and expressed constitutively (Meletzus *et al*., 1998). GlnK is required to regulate expression of *ntr* genes other than *glnA* in cells lacking GlnB. Generally the *glnK*-promoter belongs to the *ntr* family of promoters and is expressed by an RNA polymerase containing the minor sigma factor σ^{54} (Atkinson *et al*., 1994). Surprisingly, no sigma(54) type of promoter region could be detected, upstream of *Azoarcus glnK* gene; instead an ORF (*ugk*) having similarity to a hypothetical protein from *R. solanacearum* which can be putatively exported into the periplasm and possess a signal peptide sequence, is present. Indeed, the *ugk* and *glnK* genes were found to be cotranscribred in strain BH72. However, a typical sigma (54) promoter consensus could be detected upstream of *Azoarcus ugk* gene. Previous Northern blot experiments have detected a single transcript bearing *glnK* and *amtB* (Martin *et al*., 2000). Thus it is plausible to think of a single large transcript bearing *ugk*, *glnK* and *amtB*, which may be transcribed from the sigma (54)-type promoter and susceptible to a differential RNA processing event. Surprisingly, no common transcriptional start point could be detected by primer extension carried out

individually for *glnK* and *ugk*. This can once more be explained in terms of RNA processing. Differential termination of the *nifH* transcript is known to occur in *R. capsulatus* (Willison *et al*., 1993), which could explain why multiple transcripts of *nifH* have been detected in strain BH72 and the full-length transcript was less abundant (Egener *et al*., 2001). The genetic linkage of *ugk*, *glnK* and *amtB* might have a far reached functional significance. Ugk, harbouring a signal peptide sequence, could be secreted into the periplasmic space and AmtB may function as membrane-bound ammonium sensor and propagate N-signal transduction cascade within the cell, with the help of GlnK.

The *glnK* gene expression in wild type, checked by different methods revealed that it is suppressed under combined nitrogen and strongly induced under N_2 fixation. This observation is quite in accord with the previous Northern-blot experiments (Martin *et al.,* 2000) and similar to what has been reported for other organisms. Interestingly, *glnK* expression in strain BntrBsp, was severely down-regulated as shown by RT-PCR and GUS assays. NRI (NtrC) mediated activation of the *glnK* promoter has been reported for *E. coli* (Atkinson *et al*., 2002). In *Psudomonas stutzeri*, NtrC and RpoN recognition sites can be observed upstream of the *glnK* promoter (Vermeiren *et al*., 2002) and *Klebsiella, glnK-amtB* operon seems to be under NtrBC control too (Jack *et al*., 1999).

Nitrogenase inhibition by ammonium (switch-off) has been observed in wild type BH72 and it has been shown that GlnK is essential for this process (Martin *et al*., 2002). Although GlnK expression was found to be down regulated in the *ntrBC*-mutant, its “switch-off” response was not abolished and covalent modification of NifH still occurred after ammonium addition as detected by Western-blots using NifH-antiserum. In contrast, *ntrBC* mutants of *Azospirillum brasilense* and *Rhodospirillum rubrum* had no effect on the *nif* expression, but abolished post translational regulation of nitrogenase activity and “switch-off” response (Liang *et al*.,1993; Zhang *et al*., 1995). Thus it can be speculated, that very weak expression of GlnK in the *ntrBC* mutant may be sufficient to initiate and drive such posttranslational responses. It is interesting to point out that GlnY is expressed in *ntrBC* mutant of strain BH72, which can be thought to drive ammonium “switch off” response in BntrBsp, where *glnK* is down regulated. However such speculation could be ruled out from previous experimental observation where a *glnB*$^-$*K*$^-$ (double mutant) did not respond to ammonium switch off response in the presence of GlnY only (Martin *et al*., 2002) which once more highlights the distinct functions of the P_{II} paralogues in strain BH72.

As shown previously by Northern blots, the *glnB* transcript was not detectable in wild type cells, but only in a *glnK* knock-out mutant, both under ammonium and N_2 (Martin *et al*., 2000). However *glnB::gusA* reporter gene fusion studies in this work revealed a N-regulated differential expression pattern of its transcript, being strongly induced under N_2 fixation. *glnB* genes may be constitutively expressed (*glnB* in *E. coli*; van Heeswijk *et al*.,1993) or under the control of a σ^{70}–like promoter, such as *glnB* under conditions of ammonium excess in *A. brasilense* (De Zamaroczy *et al*., 1993). RT-PCR to further verify this regulatory pattern in strain BH72, indicated that the abundance of the *glnB* transcript was more or less comparable both under combined nitrogen and N_2 fixation. Results of Western blot analysis with protein extracts from strain BH72 were more similar to the RT PCR results, indicating more or less constitutive expression of GlnB both under ammonium and N_2 fixation. These contradictory results are often difficult to interpret. However speculations can be made on the basis of these observations.

glnB and *gusA* are linked on a common large transcript which may be more stable, and a selective degradation process is only active under a particular condition (may be under anaerobiosis and combined nitrogen). This may be an explanation for the apparent paradox encountered with GUS measurements versus RT-PCR / Western blot results. However, it may be important to note that transcription and translation events may not bear a one to one correspondence relation to each other. Moreover in *E. coli*, it has been recently shown that distinct functions of P_{II} and GlnK are correlated with the timing of expression and levels of accumulation, and that P_{II} can be functionally converted to GlnK by engineering its expression from nitrogen regulated promoter (Atkinson *et al*., 2002). Thus the possibility of *glnB::gusA* expression driven by a *glnK t*ype N-responsive promoter (not linked) in *Azoarcu*s sp. strain BH72, cannot be ruled out

GlnB expression in the *ntrBC* mutant showed a strong reduction of its transcript abundance under N_2 fixation. In *Rhizobium* l*eguminosarum*, the *glnB* promoter was shown to be strongly regulated by nitrogen and NtrC, the *glnB* promoter being down regulated during bacteroid differentiation and symbiosis (Ercolano *et al*., 2001). The *glnB* gene from *B. japonicum* is expressed from tandem promoters and its differential expression is strongly regulated by NtrC (Martin *et al*., 1989). So, the *glnB* regulation by NtrC in strain BH72, resembles more to that of *Rhizobia*. Surprisingly, the *glnB* expression in BntrBsp appears to remain unaltered in presence of combined nitrogen. Interestingly, in *R. capsulatus*, *glnB* is transcribed from two promoters one of which require NtrC but RpoN independent, while the other is repressed by NtrC (Foster-Harnet *et al*., 1994).

GlnY expressed in strain BntrBsp, along with other P_{II} proteins.

The expression of GlnY in strain BntrBsp along with other P_{II} proteins is a novel observation. This P_{II} paralogue of strain BH72 has until now been reported to be expressed only in absence of GlnB and GlnK (in double mutant of *glnB* and *glnK*) and lacks differential uridylylation in response to nitrogen availability (it is always uridylylated) (Martin *et al*., 2000). Indeed, the expression of all the three P_{II}-paralogues in the *ntrBC* mutant has been verified, both at the level of RNA as well as of protein. Surprisingly, *glnY::gusA* was expressed at a low level in the *ntrBC* mutant under all sources of nitrogen. Infact, the relative highest level of *glnY::gusA* was detected under N_2 fixation, which point towards a novel role of GlnY in nitrogen fixation. However, a comparison of *glnY* expression is difficult to make, since its expression is below the detection level in strain BH72. Nevertheless, it may be interesting to investigate the uridylylation status of this protein in strain BntrBsp in order to make further predictions about its nitrogen sensing functions

The N-assimilation genes of strain BH72: role of NtrBC

GS/GOGAT and GDH constitute the main enzymes for ammonium assimilation. During growth in ammonium-rich medium, ammonium is primarily assimilated through glutamate dehydrogenase (GDH) by NADPH dependent reactions. At ammonium concentrations below 1 mM, the affinity of GDH to ammonium is too low, and the reaction operates in a direction of ammonia liberation from glutamate. In this situation, the glutamine synthetase / glutamate synthetase (GS/GOGAT) system takes over, although at the cost of ATP hydrolysis. To prevent waste of energy, the GS/GOGAT system has to be down regulated at high ammonium concentrations. The flux through GS is subject to regulation at the level of enzyme activity and transcription of the GS encoding gene, *glnA*. The counterpart enzyme, glutamate synthetase (GOGAT), is encoded by *gltB* and *gltD* genes (coding for large and small subunits respectively) and is also subject of N-dependent regulation. The *Azoarcus* counterparts of GS and GOGAT were PCR amplified using sequence information from the raw (unreleased) data of the genome project. Genes were identified by high amino acid sequence similarity with known protein sequences. Interestingly, the

putative GS from strain BH72 had high identity to glutamine synthetase III (*glnIII*) from *M. loti*. Indeed, *glnIII* expression in strain BH72 was found to be nitrogen regulated but strangely enough, its expression was found to be independent from NtrBC control. Similarly, the expression of GOGAT (*glt*) was found to be N-regulated in strain BH72 but unlike *glnIII*, it was found to be moderately regulated by NtrBC. In *Klebsiella*, similar to *E. coli*, P1*glnA*P2*ntrBC* occurs in form of an operon with P1 and P2 being the two promoters. P1 is activated under N-deficiency and regulated positively and negatively by NtrBC (Alvarez-Morales *et al*., 1984) while in *Rhodospirillum rubrum*, *glnA* is cotranscribed with *glnB*, from a weak and strong promoter under NH_4^+ and N_2 respectively, but the activity of *glnA* is enhanced by NtrC (Cheng *et al*., 1999). *Herbaspirillum* resembles the enterics more in this aspect, the *glnA-ntrBC* operon being subjected to NtrBC dependent transcription and activity control (Persuhn *et al*., 2000). The situation is more complicated in *Rhizobium*, where out of three GS encoding genes; the *glnII* promoter is fully dependent on the positive control by the NtrC protein (Iaccarino *et al*., 2001). On the other hand, the *gltB* promoter of *A. brasilense* showed the presence of sigma(70) like recognition sites in addition to a potential NtrA-RNA polymerase, IHF, and NifA binding site (Mandal *et al*., 1993) while *gltBD* operon encoding for (GOGAT) is also under *ntr* control in *E. coli* and *Klebsiella aerogens* (Goss *et al*., 2001). So it can be quite likely to assume that GOGAT expression in strain BH72 is partially regulated by NtrC, at least at the level of transcription. Another interesting observation is the unusual high similarity of these assimilatory enzymes to rhizobial members, which may have a far-reaching significance, phylogenetically.

At this juncture, the basic points discussed may be summarized. Analyses of *nifH* and *nifLA* transcripts of strain BH72 reveal certain usual as well as unusual features. Although, both *nifH* and *nifLA* utilized their upstream σ^{54}-promoters for their transcription (verified by primer extension), *nifH* was unusually cotranscribed with its downstream ferredoxin gene (*fdxN*). In this respect, it may be important to mention that primer extensions were carried out for the first time, non-radioactively, with automated sequencing, and produced successful and reproducible results. Unusually, *nifA* of strain BH72 was also found to be cotranscribed with its upstream *nifL* gene, in a very similar way like that of γ-*Proteobacterial* members and its expression was found to be affected by nitrogen. Furthermore cloning and characterization of the NtrBC from strain BH72 revealed many interesting findings. Although *ntrB* and *ntrC* of strain BH72 were also found to be cotranscribed, neither typical Shine-Dalgarno sequences could be detected upstream of their respective

start codons nor putative promoter sequence could be found upstream of its transcriptional start. Strangely enough, expression of *ntrB* gene was found to be N-responsive but not likely to be auto-regulated. The *ntrBC* mutant of strain BH72 was impaired to grow on nitrate and accumulated PHB granules under such conditions; even its nitrogenase was not repressed by nitrate. Consistent with this, the expression of assimilatory nitrate reductase was found to be NtrBC regulated. Unusually, twitching motility was up regulated in *ntrBC* mutant; a trait which was up to now not known to be linked to these genes. As in enterics, the *nifLA* gene expression in strain BH72 was found to be under NtrBC control which indirecty affected N_2 fixation. Looking at the level of P_{II} proteins, an unusual transcriptional linkage was found between *ugk* with *glnK* although a common transcriptional start point for both these genes could not be detected. *glnK* and *glnB* expressions were found to be down regulated in the *ntrBC*-mutant, with the unusual retention of “switch off “ response to ammonium. Another novel observation was the expression of GlnY along with the other P_{II} proteins in the *ntrBC*-mutant albeit at low level. Analyses of the expression of assimilatory genes, encoding for GS and GOGAT in strain BH72 revealed that their expression was affected by nitrogen. Surprisingly, GS expression was found to be NtrBC independent although GOGAT seemed to be moderately regulated by NtrBC. Therefore, NtrBC was shown to be involved in regulating multiple cellular processes, in strain BH72. The summarized points are presented in form of a schematic diagram in Fig 5.1

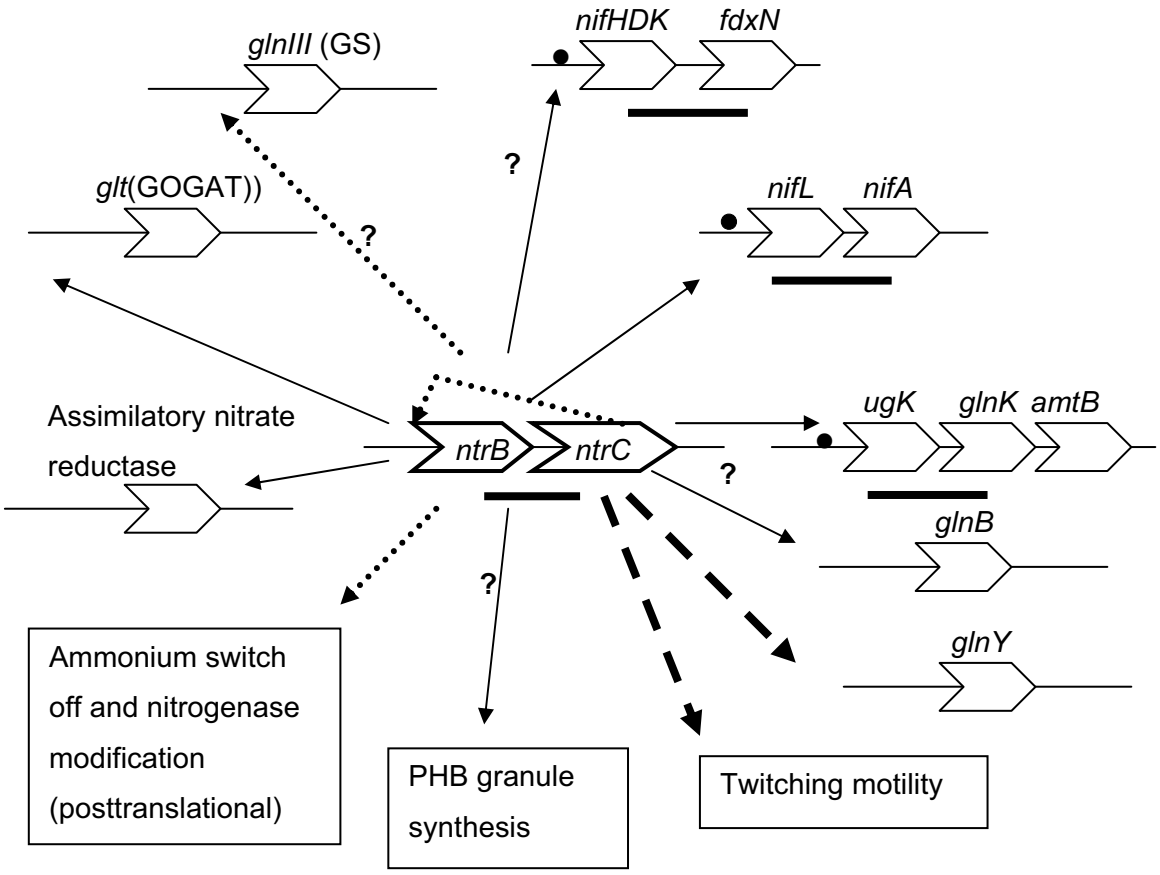

Fig 5.1 Schematic representation of the candidates of the N-regulatory cascade in strain BH72 studied in this work. The involvement of NtrBC in the process of N-regulation has been highlighted. (▬) represents the cotranscription status of several linked genes studied and (•) represents upstream σ^{54} promoter consensus. (⟶) depicts positive regulation and ▬▶ negative regulation mediated by the NtrBC on its putative targets while (·····▶) represents independent from NtrBC regulation. The symbol (**?**) represents situation where the role of the NtrBC is still not clear. All interpretations have been made from the results and observation of this study

Outlook for further studies

One of the challenging areas to explore in future is to search for more novel targets of NtrC in *Azoarcus* sp. BH72. Preliminary findings such as NtrC controlling twitching motility in strain BH72 already provides hint in this direction. Further progress in this area can be made utilizing the information from the *Azoarcus* genome project, using "microarrays" as detection tool. Although the domain organization of NtrB and NtrC

appear to be conserved, their function may vary in minute details among different subgroups of *Proteobacteria*. So an important area to explore in this direction is to study the function of the individual domains of NtrB and NtrC proteins in *Azoarcus* sp. BH72, by over expressing and purifying them in order to study their functions, both *in vitro* and *in vivo*. In this respect, it might be interesting to explore the nature of interaction of NtrC with enhancer sequences or NifA with *nifH* upstream region (by band shift assays). Side by side, study of protein-protein interactions of potential candidates of the N-regulatory cascade like NifL, GlnK. AmtB, NtrB, NtrC etc, by two-hybrid systems, may reveal novel interactions. On the other end, unravelling the protein translation mechanism lacking putative Shine-Dalgarno sequences, as have been reported for NtrB and NtrC, is itself challenging. Specific antisera against these purified proteins might help to answer these fundamental questions in a better way. Also by Northern blots, it is possible to verify the hypothesis whether *ugk*, *glnK* and *amtB,* are transcribed on a single large transcript and subsequently subjected to RNA processing event.

An interesting observation coming from this study is the effect of nitrate, where the growth of BntrBsp was found to be severely impaired. Surprisingly, *nifLA* expression was found to be much enhanced both in the wild type and *ntrBC* mutant on nitrate, as compared to its growth on N_2. In this respect, it is important to mention that a basal and constitutive *nifLA* expression is maintained in strain BH72 in presence of ammonium, which might involve a different regulatory mechanism. Although a similar effect of nitrate has been reported for the *glnB*$^-$*K*$^-$ double mutant strain (prolongation of generation time and non repression of nitrogenase), it differs from the BntrBsp by not accumulating PHB granules (data not shown) under such conditions. Interestingly, GlnY has been shown to be expressed in the mutant strains, *ntrBC*$^-$ and *glnB*$^-$*K*$^-$. Possibly, in presence of nitrate, another novel regulatory circuit might be activated in regulating the *nifLA* expression. Characterizing this putative regulation appears to be challenging. Side by side, with a better characterization of the GlnY protein in the *ntrBC* mutant (uridylylation status) may help in understanding the details of interaction of different N-regulatory cascades, in a better way.

Previous studies have already shown certain unique features related to N-metabolism in *Azoarcus* BH72. Important to mention in this respect, is the existence of a unique physiological switch off response in strain BH72, involving GlnK and Ferredoxin, which is functionally different from posttranslational covalent modification of nitrogenase. Along with this, the membrane association of GlnK, already point towards a novel N-regulation where putative UgK may be secreted into the periplasm, and AmtB might function as N-sensor and trigger a N-regulatory cascade

with the help of GlnK. Present finding, that physiological switch off is independent from NtrBC, further substantiate for the existence of this putative N-regulatory cascade in strain BH72, apart from NtrBC mediated general N-regulation. Trying to explore these novel N-regulatory circuits and interrelating them might be one of the potential challenges for the future work.

Last but certainly not least, few references have been cited to unfurl some novel aspects about NtrBC that may help in understanding the range of diverse functions, which it can mediate. In *E. coli*, all the operons known to be under NtrC control and a number of new ones could be detected. Many of these operons encode transport systems for nitrogen containing compounds including compounds recycled during cell wall synthesis, showing that scavenging appears to be a primary response to N-limitation. All together, 2% of all genes in the *E. coli* genome appear to be under NtrC control, although transcription of some depends on the nitrogen assimilation control protein acting as an adapter between NtrC and sigma(70) promoters (Zimmer *et al*., 2000). In a deep sea bacterium, *Shewanella violacea*, sigma(54)-type RNA poymerase was found to play an important role in pressure regulated transcription with the help of a NtrB-NtrC phosphorylation relay (Nakasone *et al*., 2002). The *ntrC* gene has been shown to be involved in colonization of rice roots by *Alcaligenes faecalis* (Cheng *et al*., 1998). In a *ntrB* deletion strain of *E. coli*, cross talk towards NtrC was observed upon joint activation of Pho and Uhp (Verhamme *et al*., 2002). Some new insights, regarding the modular organization of NtrB is the finding that all three domains of NRII are required for P_{II} activated NRII phosphatase activity, rather than distinct modular function and that two distinct surfaces of the C-terminal ATP-binding domain are involved in this activity (Pioszak *et al*., 2003).

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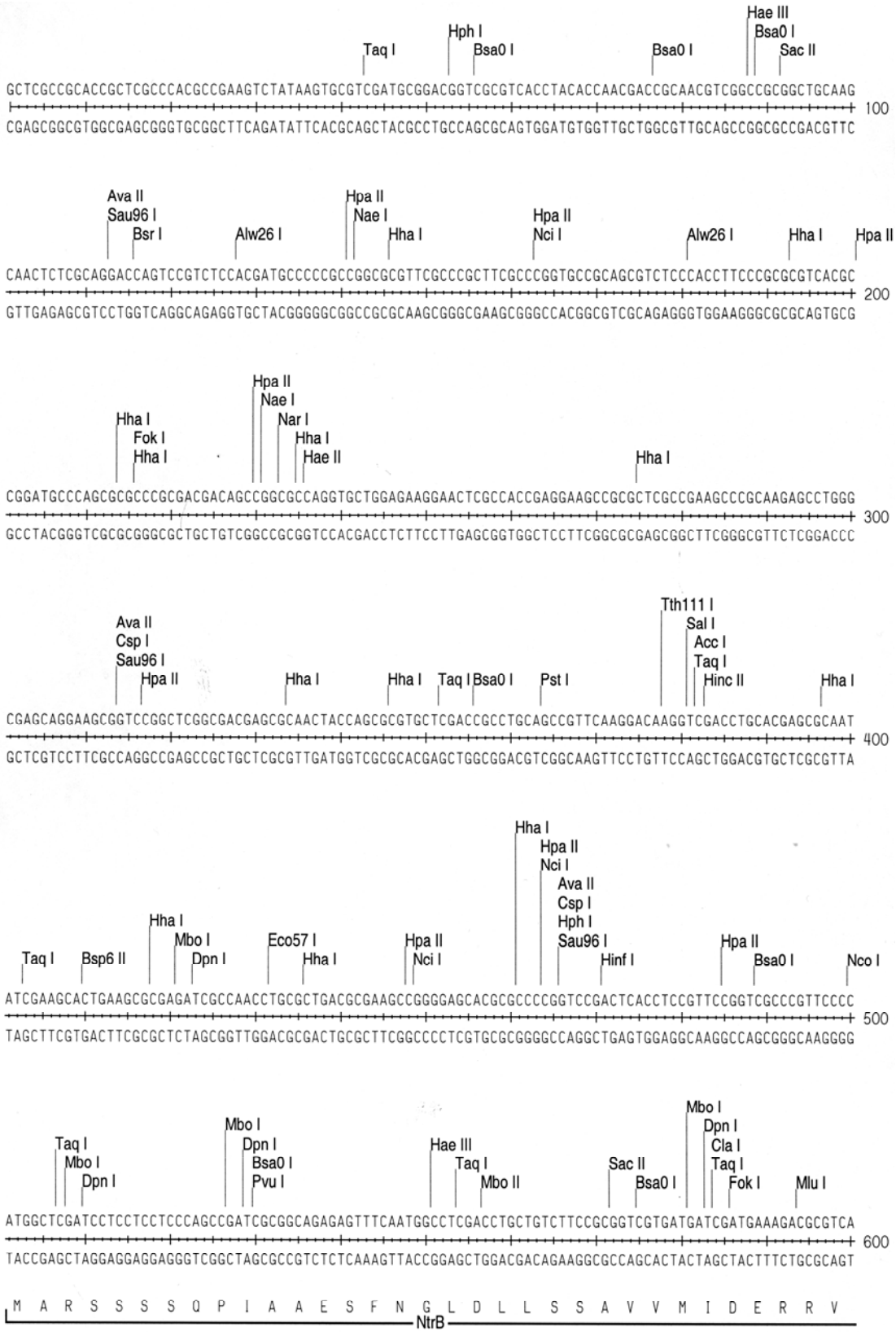
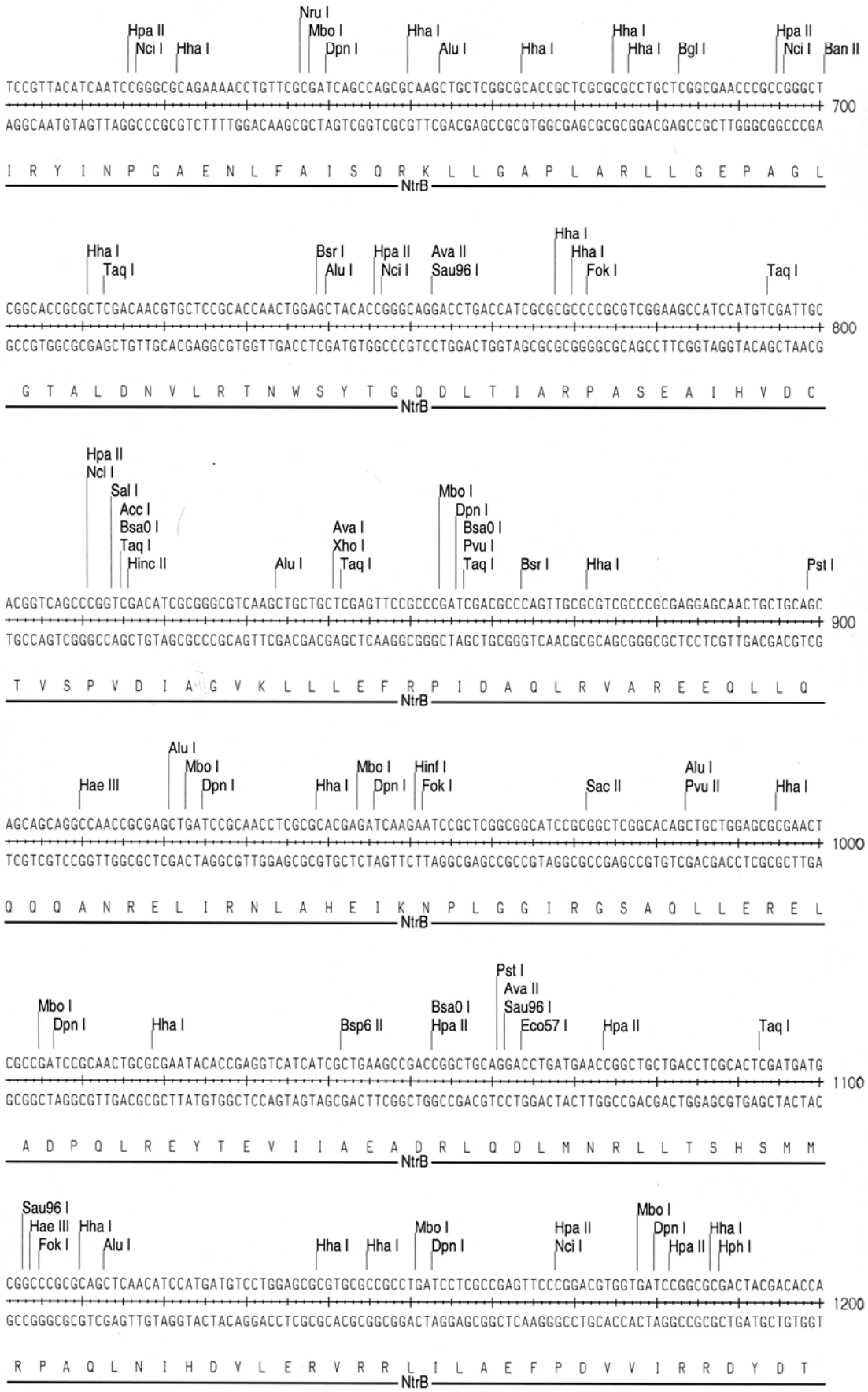
Attachment 4.5 DNA sequence encoding for *ntrB* and *ntrC* of *Azoarcus* sp. BH72 showing their respective open reading frames and restriction maps. In reference to section **4.2.1**

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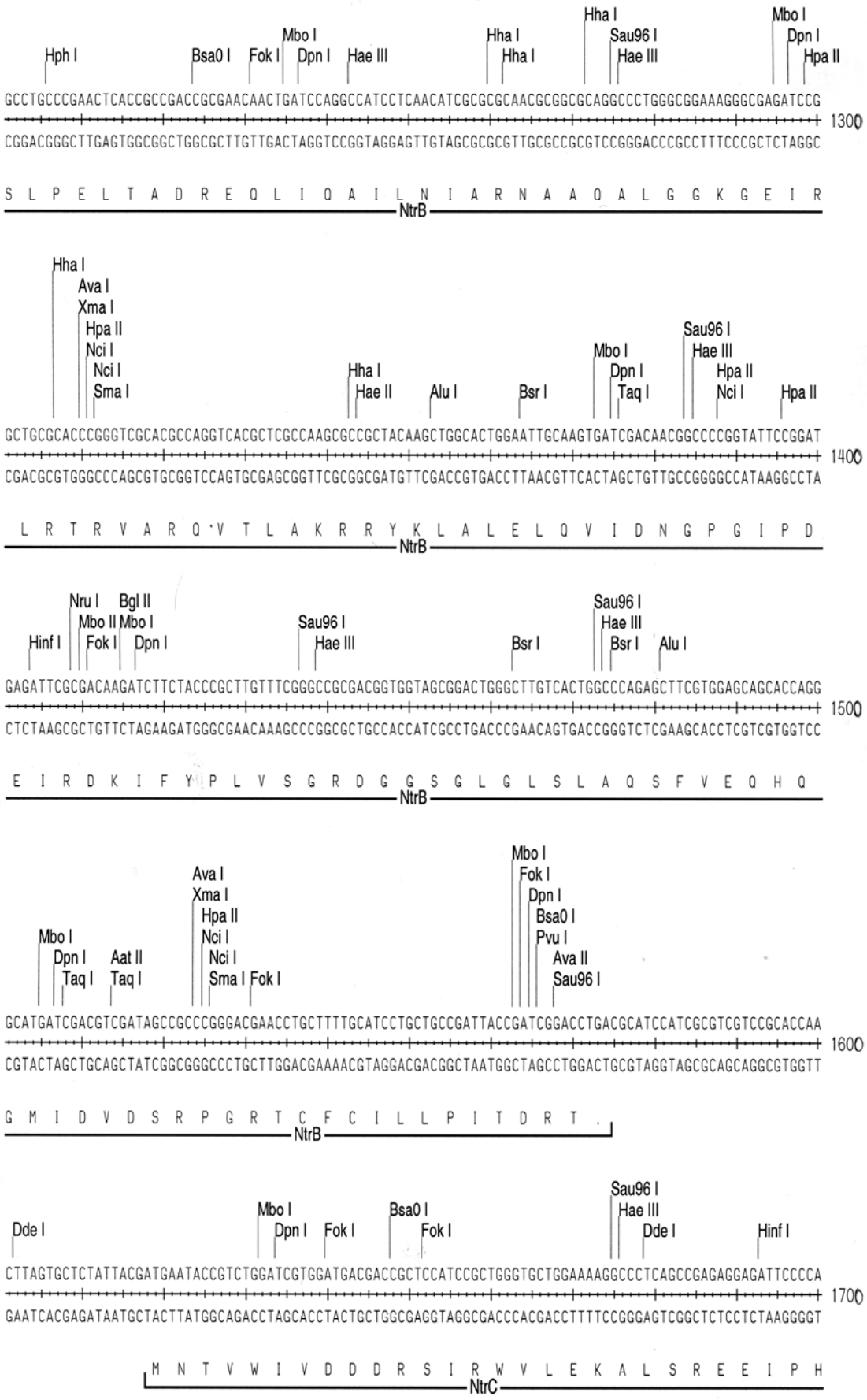
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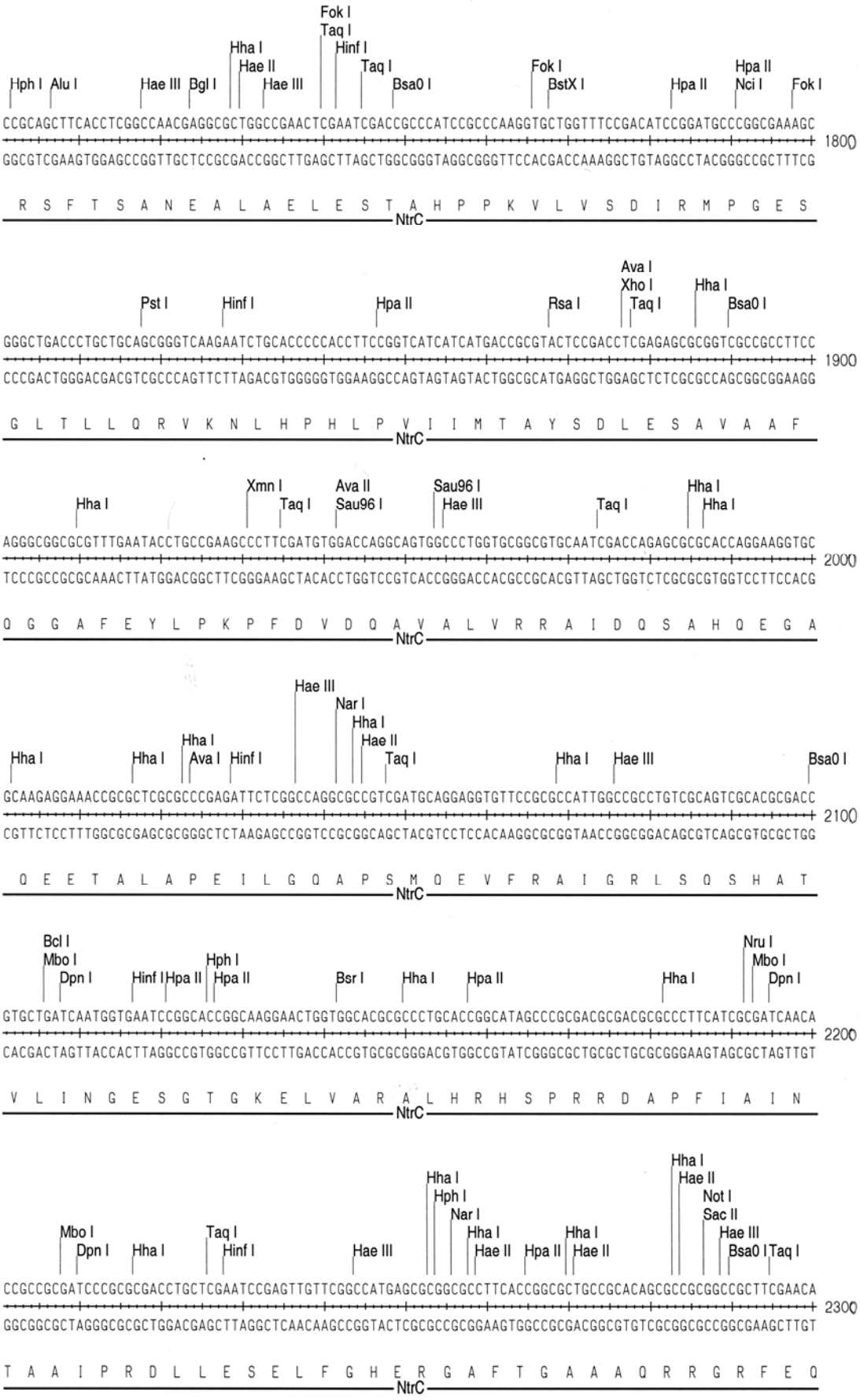
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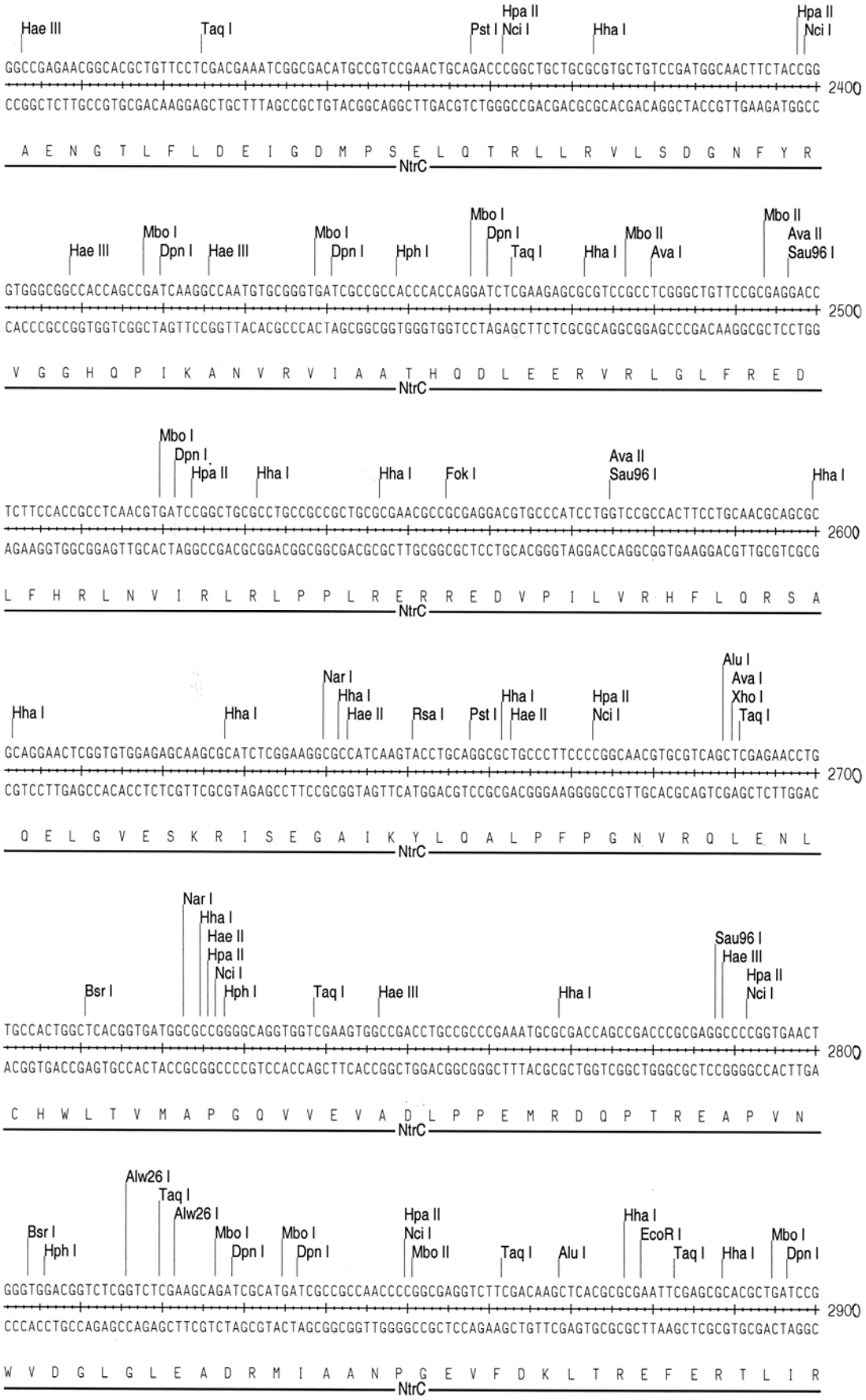
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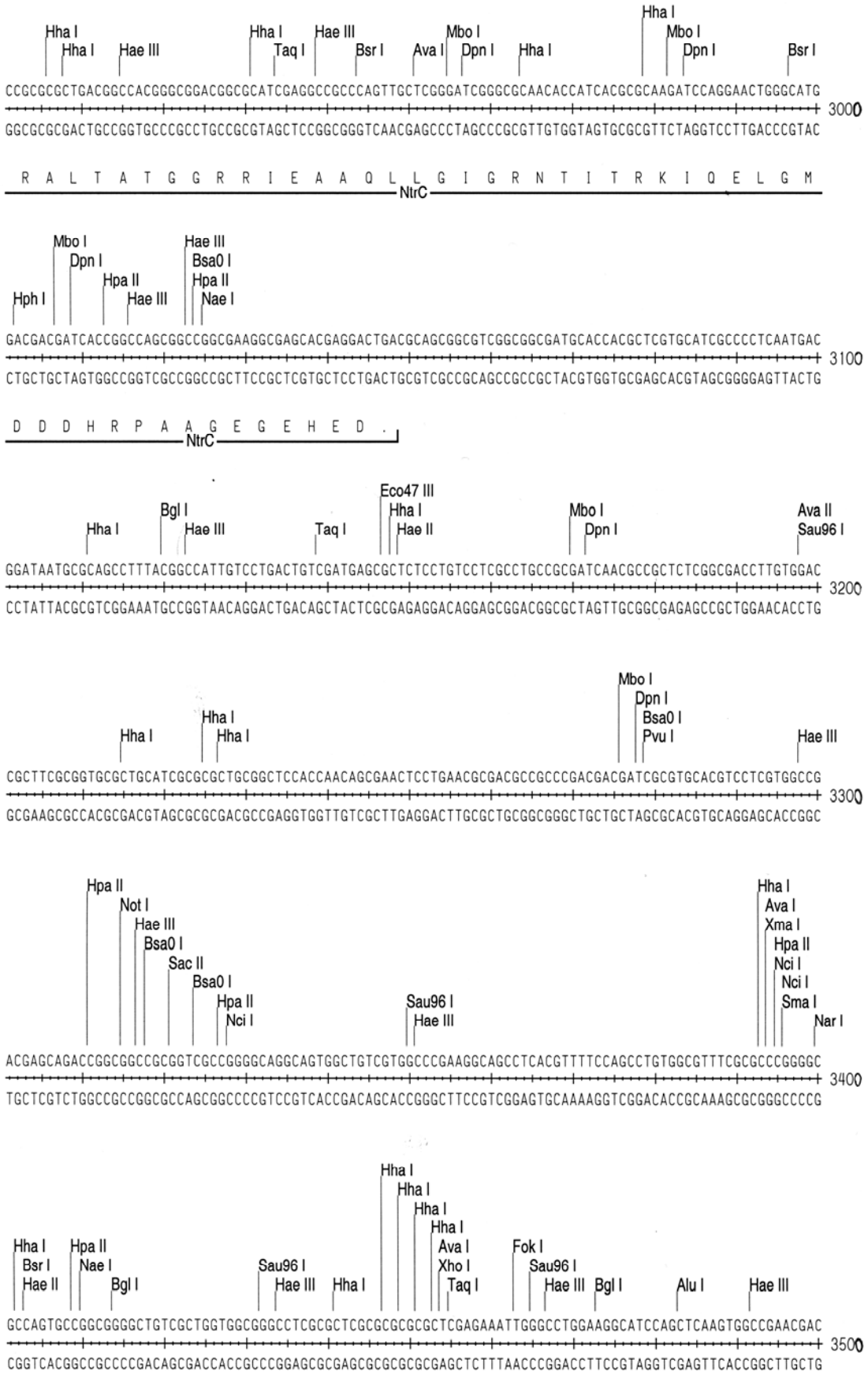
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Curriculum Vitae (C.V.)

Personal information

Name	Mr. Abhijit Sarkar
Date of birth	01.12.1973
Place of birth	Calcutta, India
Nationality	Indian
Family status	Bachelor

Education

1980-1990	Primary and secondary schooling, Julien Day School, Calcutta
1990	Passed secondary examination (+10)
1990-1992	Higher secondary schooling, Patha Bhawan, Calcutta.
1992	Passed higher secondary examination (+2)
1992-1995	College education, Presidency College, Calcutta University (+3)
1995	Achieved Bachelors Degree
1995-1997	Education for Masters Degree, Jawharlal Nehru University (JNU), New Delhi (+2)
1997	Achieved Masters degree
1997-1999	Attained M.Phil courses; Project , JNU , New Delhi
11/99-01/2000	Started preliminary Ph.D work at Max-Planck-Institut für Terrestrische Mikrobiologie in Marburg/Lahn under the supervision of Prof. B. Reinhold-Hurek
02/2000-04/03	Continuation and further completion of Ph.D work at Uni-Bremen, FB2, under the supervision of Prof. B. Reinhold-Hurek. Topic of dissertation, “Studies on the regulation of genes related to nitrogen fixation and N-assimilation in *Azoarcus* sp. strain BH72: The role of NtrBC.”

Acknowledgments

The probably most essential ingredient for a successful and enjoyable work as a Ph.D. student is the surrounding atmosphere experienced everyday. I had the luck to enjoy this truly stimulating and interdisciplinary environment in the working group of Prof. Dr. Barbara Reinhold-Hurek, who gave me a chance to work in her laboratory and made my stay in Germany very pleasant. I express my sincere gratitude to her for allowing me to work on such an interesting project. I thank her for making my dissertation successful with lot of helping discussions, new ideas and robust tips. Throughout my working period, she had always been approachable to open questions and tried to solve my problems.

I am also thankful to Prof. F. Koenig for sparing some time out from her busy schedule and evaluate my dissertation. At the same time I express my gratitude to Prof. S. Kelm and Prof. R. Stick, for agreeing to participate in the commission for my Ph. D. viva examination. I also thank “Universität Bremen” for funding this work.

My greatest thank go to Dr. Thomas Hurek, for critically reading my manuscript, and suggesting new ideas to give it a better shape. I also thank him, not only for helping me with the microscopy and other instruments, but also for making my wish of coming to Germany, fruitful.

I express my heartiest gratitude to all colleagues, students and other members of my working group. It is difficult to mention individually, but I thank all of them, not only for their enormous help and cooperation, but also for making my stay so enjoyable. I thank specially to Torsten and Lei for their help with computer work and Melanie for her generous help in time of great needs. I would also like to thank the members of other working groups of this building NW2, who were always so friendly to me and helped me whenever I approached them.

I am also grateful to my teachers and Professors in India, who had always inspired and motivated me for higher study. I also thank my Indian friends in Bremen, especially Subhasish, for helping to overcome my homesickness feeling.

I am also thankful to all my relatives and friends in India. Last, but certainly not least, I am grateful to my sister and parents for their cooperation and constant inspiration.

Declaration

I hereby assure that I prepared my dissertation entitled “Studies on regulation of genes related to nitrogen fixation and N-assimilation in *Azoarcus* sp. strain BH72: The role of NtrBC “ independently, without any prohibited aid and that I used no other but only the sources and aid, specifically designated for this work.
I also admit that this dissertation, in the present form or in a similar form was never submitted to another University and has never served for other examination purposes.

Bremen, date 14.04.2003

Erklärung

Ich versichere, daß ich meine Dissertation „Untersuchungen zur Regulation von Genen, die in der N_2-fixierung und N-Assimilation von Azoarcus sp. Stamm BH72 involviert sind: Die Rolle von NtrBC“ selbständig ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Bremen, den 14.04.2003