Physiologische und Proteomanalysen zur Untersuchung der Stickstofffixierungsmechanismen insbesondere in Hinblick auf die posttranslationale Regulation der Nitrogenase in *Azoarcus* sp. BH72

Physiological and proteome analyses to investigate the nitrogen fixation mechanism particularly with respect to the posttranslational regulation of nitrogenase in *Azoarcus* sp. BH72

Dissertation

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Erklärung

Ich versichere hiermit, dass ich meine Dissertation "Physiologische und Proteomanalysen zur Untersuchung der Stickstofffixierungsmechanismen insbesondere in Hinblick auf die posttranslationale Regulation der Nitrogenase in *Azoarcus* sp. BH72" selbständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet habe.

Bremen, April 2009

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Summary

Nitrogenase activity control in the grass-endophyte *Azoarcus* sp. BH72 is subjected to a posttranslational regulation, which is accompanied by covalent modification of its nitrogenase Fe-protein that was characterized here. Nitrogen-fixing cells of the betaproteobacterium *Azoarcus* sp. BH72 inactivate their nitrogenase after stimulation by addition of an external nitrogen source or energy depletion through ADP-ribosylation of the arginine residue 102 in one subunit of the Fe-protein. The modification was detected in the modified peptide sequence applying high-performance liquid-chromatography electrospray ionization/tandem mass spectrometry and was verified by site-directed mutagenesis of the respective arginine residue. In addition, analysis of the type of the modification led to the establishment of a MALDI-TOF MS (matrix assisted laser desorption/ionization-time of flight mass spectrometry) based method for the detection of a labile posttranslational modification.

The *Azoarcus* sp. BH72 genome harbors a gene that encodes a protein exhibiting sequence similarity to known dinitrogenase reductase ADP-ribosyltransferases (DraT). It possesses two genes, coding for proteins with sequence similarity to dinitrogenase reductase activating glycohydrolases (DraG). The expression of *draT*, which is co-transcribed with two genes encoding a hypothetical protein and a hemerythrin (DcrH1), was neither nitrogen nor oxygen regulated. Likewise, the expression of *draG2* was independent on the availability of fixed nitrogen, while transcription of *draG1* – probably located in an operon with *nifQ* - was nitrogen regulated. Physiological analysis of deletion mutants of the genes *draT*, *draG1* and *draG2* and of a *draG1draG2* double mutant led to the characterization of the DraT/DraG-system in *Azoarcus* sp. BH72. DraT was responsible for the inactivation of nitrogenase by ADP-ribosylation after ammonium addition or anaerobiosis. However, a DraT-independent, ammonium-mediated nitrogenase "switch-off" was also observed. In addition, physiological experiments of the *draG*-mutants as well as phylogenetic analysis of DraG amino acid sequences revealed that DraG1 mediates the reversibility of the nitrogenase inactivation by removal of the ADP-ribosylation, while DraG2 may have other functions as it only partially complemented a *draG1* deletion mutation. Moreover, a putative role of DcrH1 in nitrogenase protection against oxygen damage under conditions of slightly elevated oxygen concentrations is proposed.

Comparative proteomic studies led to the identification of proteins putatively important for the formation and function of intracytoplasmic membrane stacks (diazosomes) and for the interaction of *Azoarcus* sp. BH72 with a fungal endophyte of Kallar grass.

Zusammenfassung

Die Aktivitätskontrolle der Nitrogenase in dem Grass-Endophyten *Azoarcus* sp. BH72 unterliegt einer posttranslationalen Regulierung, die durch kovalente Modifikation der Eisen-Untereinheit der Nitrogenase gekennzeichnet ist und in dieser Arbeit charakterisiert wurde. Stickstofffixierende Zellen des Betaproteobakteriums *Azoarcus* sp. BH72 inaktivieren ihre Nitrogenase nach Stimulierung wie Zugabe einer externen Stickstoffquelle oder Energiebeschränkung durch APD-Ribosylierung des Argininrestes 102 in einer Untereinheit des Eisen-Proteins. Der Nachweis der Modifikation erfolgte über Sequenzierung des modifizierten Peptides anhand hochauflösender Flüssigkeits-Chromatographie Elektrospray-Ionisierungs/ Tandem-Massenspektrometrie und wurde durch Punktmutagenese des betroffenen Argininrestes verifiziert. Zudem führte die Untersuchung zur Art der kovalenten Modifikation zur Etablierung einer MALDI-TOF MS (Matrix-Assisted Laser Desorption/ Ionization-Time of Flight Mass Spectrometry) basierten Methode zum Nachweis labiler posttranslationaler Modifikationen an Proteinen.

Das *Azoarcus* sp. BH72 Genom enthält ein Gen, dessen Genprodukt Sequenzähnlichkeit zu bekannten Dinitrogenase-Reduktase ADP-ribosyltransferasen (DraT) aufweist. Es enthält zwei Gene, die für Proteine mit Sequenzähnlichkeit zu Dinitrogenase-Reduktase aktivierende Glykohydrolase (DraG) kodieren. Die Expression von *draT*, welches mit zwei Genen, die für ein hypothetisches Protein bzw. ein Hemerythrin (DcrH1) kodieren, cotranskribiert wird, war weder stickstoff- noch sauerstoffreguliert. Die Expression von *draG2* erfolgte ebenso unabhängig von der Verfügbarkeit bereits gebundenen Stickstoffs, während die Transkription von *draG1*, das möglicherweise in einem Operon mit *nifQ* liegt, stickstoffreguliert war. Die physiologische Analyse von Deletionsmutanten der Gene *draT*, *draG1* und *draG2* sowie einer *draG1draG2*-Doppelmutante führte zur Charakterisierung des DraT/DraG-Systems in *Azoarcus* sp. BH72. DraT war zuständig für die Inaktivierung der Nitrogenase durch ADP-Ribosylierung nach Ammoniumzugabe oder Anaerobiose. Es konnte zudem ein Ammonium-vermittelter DraT-unabhängiger Nitrogenase "Switch-Off" beobachtet werden. Des Weiteren haben sowohl physiologische Experimente der *draG*-Mutanten als auch phylogenetische Analysen von DraG-Aminosäure Sequenzen erwiesen, dass DraG1 die Reversibilität der Nitrogenase Inaktivierung durch Entfernung der ADP-Ribosylierung vermittelt, während DraG2 voraussichtlich eine andere Funktion zukommt, da es eine *draG1*-Deletion nur partiell kompementierte. Zudem konnte gezeigt werden, dass DcrH1 wahrscheinlich als Schutzprotein der Nitrogenase in Anwesenheit leicht erhöhter Sauerstoffkonzentrationen fungiert.

Vergleichende Proteomanalysen führten zur Identifizierung von Proteinen, die wichtig für die Bildung von intracytoplasmatischen Membranstaplen (Diazosomen), deren Funktion und der Interaktion von *Azoarcus* sp. BH72 mit einem Pilz-Endophyten aus Kallargrass-Wurzeln sind.

1. INTRODUCTION

Azoarcus sp. BH72 – a model organism as a nitrogen-fixing endophyte of grasses

Nitrogen fixation is the fundamental process, by which atmospheric nitrogen gas enters the nitrogen cycle and is thereby made accessible to all living organisms. The enzyme catalyzing the reduction of dinitrogen to ammonia – nitrogenase – is restricted to some prokaryotes called diazotrophs. Higher plants and animals profit from the process of biological nitrogen fixation which is carried out by diazotrophic bacteria and a few methanogenic archaea. Organisms unable to reduce the atmospheric nitrogen by their own can benefit from the fixed nitrogen provided by diazotrophs either directly for example in symbiotic interactions or indirectly through decomposition. Probably the most famous symbiotic interaction is the well studied nodule symbiosis between rhizobia and legumes. Some graminaceous plants obviously also benefit from biological nitrogen fixation. In contrast to the rhizobia-legume interaction, no specialized symbiotic structures like root nodules are detectable in grasses (Hurek *et al.*, 2002). Often, even the identification of key diazotrophs interacting with plant members of the grass family renders to be difficult due to a failure of isolating these microbes (Reinhold-Hurek & Hurek, 1998). However, some bacteria repeatedly isolated turned out to be the major contributors of biological nitrogen fixation in graminaceous plants (Reinhold-Hurek *et al.*, 1993). These bacteria can often penetrate deeply into the plant tissue causing no external sign of infection or negative effect on their host. According to a definition given by Quispel (Quispel, 1992) these bacteria where referred to as endophytes (reviewed in Hurek & Reinhold-Hurek, 2003).

Endophytic bacteria can colonize the root interior of virtually any plant (Ryan *et al.*, 2008). The biotechnological potential of this interaction is enormous. Endophytic bacteria can be used to promote plant growth and yield, to suppress pathogens and can be utilized to enhance biodegradation of pollutants in soil in the course of phytoremediation (Ryan et al., 2008). As a diazotrophic endophyte of grasses *Azoarcus* sp. BH72 is of great interest as a model organism due to its capability of colonizing the root interior of one of the most important crop plants – rice (Reinhold-Hurek & Hurek, 1998, Hurek & Reinhold-Hurek, 2003). Understanding the endophytic interaction of *Azoarcus* sp. BH72 with its host plant is important as in future this may lead to the development of a biological fertilizer. Studying the process of biological nitrogen fixation and its regulation in this organism is for that reason of great interest. In

particular, the regulation of nitrogenase activity might differ in this strain to the activity control of this enzyme in free-living diazotrophs, which fix nitrogen only for their own requirements. Some features of the endophytic interaction might be more similar to the characteristics of symbiotic interactions.

Azoarcus sp. BH72 was originally isolated from the rhizosphere of Kallar grass (*Leptochloa fusca* (L.) Kunth) (Reinhold *et al.*, 1986). This strain, taxonomically classified as a member of the class *Betaproteobacteria*, is capable of biological nitrogen fixation under microaerobic conditions (Reinhold et al., 1986, Reinhold-Hurek et al., 1993). More notably is its ability to fix nitrogen in the root interior of Kallar grass and deliver its host with assimilable nitrogen compounds as judged by $^{15}N_2$-incorporation (Hurek et al., 2002). *Azoarcus* sp. BH72 can invade roots of Kallar grass and rice inter- and intracellulary and has a growth promoting effect on rice seedlings (Hurek *et al.*, 1994b). Moreover, with reporter gene fusion experiments *Azoarcus* sp. BH72 was shown to express the nitrogenase structural genes *nifHDK* in the rice apoplast (Egener *et al.*, 1999). While conducting a proteomic approach on inoculated, compatible rice cultivars with *Azoarcus* sp. BH72 in comparison to plants treated with jasmonic acid (JA) known to initiate a plant defense response, it was concluded that JA-inducible stress responses are not important for the endophytic interaction (Miché *et al.*, 2006), thus indicating that the colonization is not harmful for the plant, as in pathogenic interactions.

In its natural ecosystem *Azoarcus* sp. BH72 may not only interact with Kallar grass. An interesting model system for studying the lifestyle of this diazotroph provides the investigation of *Azoarcus* sp. BH72 interacting with a fungal strain co-isolated from its natural habitat. During co-culture of *Azoarcus* sp. BH72 with this fungal strain 2003 related to *Acremonium alternatum*, the bacteria can reproducibly form intracytoplasmatic membrane stacks, termed diazosomes (Hurek *et al.*, 1995). These specialized structures have been as well detected under conditions of hyperinduction, a cellular state that was discovered by examining the influence of increasing oxygen deprivation on nitrogen fixation and respiration of strain BH72 (Hurek *et al.*, 1994a). As the nitrogen fixation rate did not decline with decreasing oxygen concentrations it was suggested that the bacteria direct the energy produced by respiration more efficiently into the nitrogen fixation reaction. Along with a more economical nitrogen fixation process and the production of diazosomes, it has been shown that the intracellular membrane stacks harbor the nitrogenase enzyme to a high extent as elucidated by immunogold labeling (Hurek et al., 1995).

In an attempt to identify changes in the protein profile of N_2-fixing *Azoarcus* sp. BH72 upon diazosome formation, protein patterns of two dimensional electrophoresis (2-DE) gels of bacteria fixing nitrogen under standard conditions were compared to diazosome containing cells (Karg & Reinhold-Hurek, 1996). The proteome of bacterial cells co-cultured with the fungus strain 2003 was represented by a relative lesser abundance of proteins indicating for a more specialized metabolism. Nine new proteins have been discovered under these conditions as well as five proteins that increased in concentration and five new major membrane proteins were detected (Karg & Reinhold-Hurek, 1996). Unfortunately, only the gene product of *glnB* and a putative ethanol dehydrogenase have been identified by N-terminal sequencing during that time (Karg & Reinhold-Hurek, 1996, Karg, 1996). However, the availability of the *Azoarcus* sp. BH72 genome sequence (Krause *et al.*, 2006) has made a more comprehensive analysis possible.

Biochemical aspects of nitrogenase

Information on structural features of nitrogenase – the enzyme catalyzing the reduction of N_2 to ammonia – is important for understanding the process of nitrogen fixation. Moreover, knowledge about nitrogenase biochemistry is helpful for understanding the mechanism underlying the posttranslational control of nitrogenase that is subject of this work. The enzyme nitrogenase consists of dinitrogenase and dinitrogenase reductase (Dean & Jacobson, 1992, Rees *et al.*, 2005). The active site for substrate recognition is present in dinitrogenase, also referred to as FeMo-protein due to its unique iron-molybdenum cofactor FeMoCo (Hawkes *et al.*, 1984, Shah & Brill, 1977, Kim & Rees, 1992). The ATP-driven reduction of N_2 to ammonia occurs at this metalloprotein, which is arranged in a $\alpha_2\beta_2$-symmetry (Hageman & Burris, 1978, Georgiadis *et al.*, 1992, Burgess & Lowe, 1996, Seefeldt *et al.*, 2004). The α-subunit is encoded by *nifD*, while *nifK* codes for the β-subunit (Roberts & Brill, 1980). Dinitrogenase reductase couples ATP-hydrolysis with electron transport to dinitrogenase, and it is encoded by the *nifH* gene (Seefeldt & Dean, 1997, Howard & Rees, 1994). Like dinitrogenase, dinitrogenase reductase contains a metallocluster as cofactor. The homodimeric protein possesses a single [4Fe:4S]-cluster at the dimer interface and is therefore also referred to as Fe-protein (Georgiadis et al., 1992). In addition to two molecules of NH_3 per molecule of dinitrogen reduced, hydrogen is produced as a by-product of this reaction (Simpson & Burris, 1984). The nitrogen fixation process is highly energy-demanding and therefore very costly for the cell. At least sixteen molecules of ATP are hydrolyzed during a complete reaction cycle by which minimum eight electrons have to be transferred

from the [4Fe:4S]-cluster of dinitrogenase reductase to the FeMoCo-bound substrate in dinitrogenase. Hence, for every transferred electron two ATP molecules are consumed (Burgess & Lowe, 1996, Rees & Howard, 1999, Halbleib & Ludden, 2000).

Transcriptional regulation of nitrogen fixation

Due to high energy requirements and oxygen sensitivity of the enzyme, the process of nitrogen fixation is regulated at different levels. This involves the nitrogenase posttranslational regulation and the transcriptional regulation of the *nif*-genes, an ingenious control system that is subject of this paragraph. Expression of the *nif* genes is tightly regulated in response to the cellular nitrogen status and oxygen concentration. Even transcriptional regulation is usually effected at two levels. This comprises the general nitrogen control system (Ntr) that coordinates nitrogen metabolism (Merrick & Edwards, 1995) and the NifL/NifA-system that regulates *nif*-gene expression in a specific manner (Martinez-Argudo *et al.*, 2004) (Figure 1). The Ntr-system is generally composed of four proteins: GlnB, GlnD, NtrB and NtrC (Merrick & Edwards, 1995). The P_{II}-protein GlnB is a small, trimeric signal transduction protein that senses the intracellular nitrogen status of the cell (Arcondéguy *et al.*, 2001). Under nitrogen-fixing conditions, when the intracellular glutamine to 2-oxoglutarate ratio is relatively low, P_{II}-proteins become uridylylated at a specific tyrosine residue (tyr51) located in the T-loop of each monomer (Brown *et al.*, 1971, Mangum *et al.*, 1973, Adler *et al.*, 1975, Kamberov *et al.*, 1995, Carr *et al.*, 1996, Atkinson & Ninfa, 1999). The T-loop is formed by eighteen amino acid residues extending above the surface of the barrel shaped protein (Carr et al., 1996, Xu *et al.*, 1998). The uridylylation reaction is catalyzed by the bifunctional uridylytransferase/uridyly-removing enzyme (UTase/UR), gene product of *glnD* (Jiang *et al.*, 1998). Depending on the uridylylation status of GlnB, the signal transduction molecule can interact with a variety of target proteins in order to regulate their activity. Among those is the two-component regulation system NtrB/NtrC. NtrB is a sensor histidine kinase initially autophosphorylated on histidine 139 in a ATP-dependent reaction (Ninfa & Bennett, 1991), while NtrC represents the response regulator that can bind to DNA with its C-terminal domain and acts as a typical σ^{54}-dependent transcriptional activator (Morett & Segovia, 1993). Under nitrogen excess conditions in the presence of GlnB and ATP, NtrB dephosphorylates NtrC (Kamberov *et al.*, 1994, Kamberov et al., 1995, Keener & Kustu, 1988, Liu & Kahn, 1995, Ninfa & Magasanik, 1986). Conversely, under conditions of nitrogen fixation, GlnB-UMP no longer interacts with NtrB. In this case the kinase activity of NtrB predominates and NtrB can in turn phosphorylate NtrC at an aspartic residue at position

54 (Merrick & Edwards, 1995). Phosphorylated NtrC oligomerizes and can then initiate transcription of particular genes in a σ^{54}-dependent manner (Reitzer & Magasanik, 1986, Porter *et al.*, 1993, Klose *et al.*, 1994).

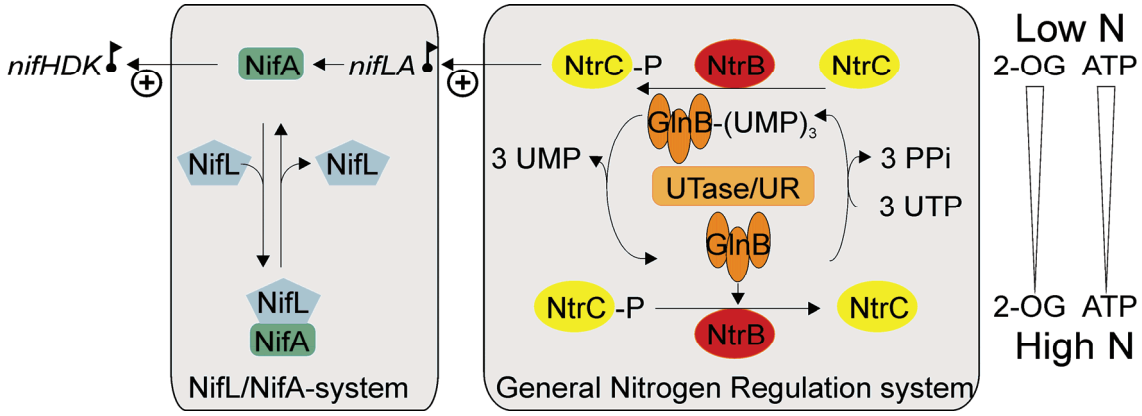

Figure 1: Model of the general nitrogen regulation and of the NifL/NifA-system. The general nitrogen regulation system is composed of UTase/UR (gene product of *glnD*) that – depending on the cellular fixed nitrogen status – modifies or demodifies P_{II}, the P_{II} protein GlnB, NtrB and NtrC. Under condition of nitrogen excess, interaction of deuridylylated GlnB with NtrB stimulates the phosphatase activity of NtrB which dephosphorylates NtrC. Conversely, under conditions of nitrogen fixation uridylylated GlnB no longer interacts with NtrB, whose kinase activity predominates now, leading to the phosphorylation of NtrC. The response regulator NtrC can in turn initiate the transcription of particular genes in a σ^{54}-dependent manner. In this way transcription of the *nifLA*-operon is initiated. NifA acts as the transcriptional activator of the *nif* structural genes, while NifL interaction with NifA in response to the fixed nitrogen or redox status signal leads to NifA inhibition (adapted from Merrick & Edwards, 1995).

Transcriptional regulation of the *nif*-genes through the NifL/NifA-system is controlled by the cellular redox and nitrogen status of the cell (Martinez-Argudo et al., 2004). Under nitrogen excess NifL inhibits the transcriptional activator NifA in diazotrophic gammaproteobacteria and the betaproteobacterium *Azoarcus* sp. BH72 (Egener *et al.*, 2002), while in alpha- and betaproteobacteria lacking NifL, NifA alone regulates *nif*-gene expression (Liang *et al.*, 1992, Liang *et al.*, 1993, Zhang *et al.*, 2000). Expression of *nifLA* in *Klebsiella pneumoniae* itself is activated by NtrBC, providing the link between both systems (He *et al.*, 1997). Like NtrC, NifA mediates transcriptional activation in a σ^{54}-dependent manner. NifA binding to enhancer-like elements upstream of the σ^{54}-dependent promoter and ATP hydrolysis results in a conformational change in σ^{54} allowing the RNA polymerase holoenzyme to undergo the conversion to the transcriptionally competent open promoter complex (Studholme & Dixon, 2003, Martinez-Argudo et al., 2004). NifA is composed of a three domain structure: an N-terminal GAF-domain (cGMP phosphodiesterase, adenylate cyclase, FhlA) (Ho *et al.*, 2000), a central domain characteristic for σ^{54}-dependent activators that belong to the AAA+ domain family (Neuwald *et al.*, 1999) and a C-terminal DNA-

binding domain. The GAF-domain is known to bind 2-oxoglutarate (2-OG), an important cellular carbon status signal (Little & Dixon, 2003). *Azotobacter vinelandii* NifL possesses two PAS-domains in the N-terminal part of the protein. PAS1 was shown to contain flavin adenine dinucleotide (FAD) as a prosthetic group, which can mediate the redox sensing (Zhulin & Taylor, 1997, Macheroux *et al.*, 1998, Hill *et al.*, 1996). The C-terminal GHKL-domain of NifL (gyrase, Hsp90, histidine kinase, MutL) shows sequence identity to histidine protein kinases, but phosphorylated NifL was never observed (Woodley & Drummond, 1994). Both parts are separated by a Q-linker (Wootton & Drummond, 1989).

The signal transduction cascade leading to the regulation of NifL-activity varies in the different diazotrophic organisms. However, in every case the P_{II} signal transduction proteins play a crucial role in conveying the signal for cellular fixed nitrogen status. In *A. vinelandii* for example deuridylylated GlnK interacts with the GHKL-domain of NifL, thereby stimulating its inhibitory activity (Little *et al.*, 2000, Little *et al.*, 2002, Woodley & Drummond, 1994). However, in *K. pneumoniae* relief of NifA from NifL inhibition under nitrogen fixation conditions was not dependent on the uridylylation status of GlnK (He *et al.*, 1998, Jack *et al.*, 1999, Arcondéguy *et al.*, 1999, Arcondéguy *et al.*, 2000). In contrast to *A. vinelandii* triplex formation of GlnK with the NifL/NifA inhibitory complex was suggested to stimulate complex dissociation under simultaneous nitrogen and oxygen limitation in *Klebsiella*, thereby activating NifA (Stips *et al.*, 2004). The binding of GlnK to NifA was decreased in response to 2-oxoglutarate. Upon 2-oxoglutarate binding, a conformational switch has been suggested to occur in GlnK leading to NifA release from the trimery complex (Glöer *et al.*, 2008). In addition, NifL has been shown to be sequestered to the cytoplasmic membrane upon reduction of the FAD cofactor under anaerobic conditions in *K. pneumoniae* (Stips et al., 2004, Grabbe & Schmitz, 2003). Therefore it has been proposed that *nif*-gene expression in *K. pneumoniae* is triggered by NifL localization in response to oxygen concentration and GlnK induced NifL/NifA complex dissociation in response to 2-oxoglutarate and probably ATP.

Posttranslational regulation of nitrogenase activity

The posttranslational regulation mechanism of nitrogenase comprises of the DraT/DraG-system responsible for covalent nitrogenase Fe-protein modification. This enzyme system provides a very rapid way to inhibit nitrogenase activity and its characterization is the main focus of this work. The existence of a posttranslational modification system was ascertained in *Rhodospirillum rubrum* by the discovery of an “activating factor” that was needed for the

activity stimulation of the purified nitrogenase components *in vitro* (Ludden & Burris, 1976). Subsequently, the activating factor has been purified in *R. rubrum* and has been described as a 32 kD protein (Saari *et al.*, 1984). Already in 1978 Ludden and Burris proposed the presence of phosphate, ribose and an adenine-like unit bound to one subunit of nitrogenase Fe-protein in *R. rubrum* (Ludden & Burris, 1978). This was suggested to lead to the two forms of this protein observed by SDS-PAGE analysis, with one subunit migrating slower. The incorporation of adenine has been demonstrated by labeling of *R. rubrum* cells with [8-^{3}H]adenine, which was detected in the Fe-protein "upper band" from extracts separated by SDS-PAGE and fluorography (Nordlund & Ludden, 1983). It appeared that one subunit of nitrogenase Fe-protein was modified by adenosine diphosphorylation of arginine residue 101 in *R. rubrum* (Pope *et al.*, 1985). Considering the three-dimensional structure of dinitrogenase reductase, R101 is situated in close proximity to the iron-sulfur cluster (Georgiadis et al., 1992). Therefore it was concluded that the ADP-ribosylation at this residue prevents the docking of dinitrogenase reductase to dinitrogenase and by this mean the electron transfer to FeMoCo is blocked (Murrell *et al.*, 1988, Seefeldt, 1994). The ADP-ribosylation on R101 in *R. rubrum* Fe-protein was then proven in 1985 by Pope *et al.* (Pope et al., 1985) by proton and phosphorus nuclear resonance spectroscopy, positive and negative-ion fast atom bombardment mass spectrometry, and fast atom bombardment/collisionally activated decomposition mass spectrometry and in 1989 for *Rhodobacter capsulatus* by using a similar methodology (Jouanneau *et al.*, 1989).

The enzyme responsible for the covalent modification was purified in *R. rubrum* and described as dinitrogenase reductase ADP-ribosyltransferse (DraT) (Lowery & Ludden, 1988). The discovery of the respective genes coding for DraT and DraG (dinitrogenase reductase activating glycohydrolase – or activating factor), was the basis for the study of the covalent modification system in other diazotrophs (Fitzmaurice *et al.*, 1989, Fu *et al.*, 1989, Masepohl *et al.*, 1993). DraT/DraG mediated posttranslational regulation of nitrogenase activity has been described at the molecular level for the alphaproteobacteria *R. rubrum*, *R. capsulatus*, *A. brasilense* and *A. lipoferum* (Fu et al., 1989, Kanemoto & Ludden, 1984, Liang *et al.*, 1991, Zhang *et al.*, 1993, Masepohl et al., 1993). By mutational analysis it was discovered that DraT catalyzes the covalent modification of dinitrogenase reductase after ammonium chloride addition or energy restraints to nitrogen-fixing bacteria leading to a fast inactivation of the enzyme called “switch-off” response. Reversely, under “switch on” conditions activation of nitrogenase is performed by DraG that can remove the ADP-ribose moiety. In *Azospirillum brasilense* ammonium-induced ADP-ribosylation of nitrogenase

Fe-protein was suggested to occur due to indirect detection through radioactively labeled ^{32}[P] that has been applied as $^{32}[PO_4^{3-}]$ to growing cells (Hartmann *et al.*, 1986).

In *A. brasilense* and *R. capsulatus* a second mechanism involved in the ammonium-mediated "switch-off" response was observed (Pierrard *et al.*, 1993, Zhang *et al.*, 1996). Interestingly, a NifH protein unable to serve as a substrate for DraT was able to inactivate nitrogenase. Contradictorily, *draT*-mutant strains that were as well defective in their capability to modify the nitrogenase Fe-protein did not perform a switch-off response (Zhang *et al.*, 1992, Masepohl et al., 1993). This would be in agreement with the assumption that DraT was involved in the switch-off response as a signal molecule in these bacteria. A DraT independent switch-off mechanism has been described for the betaproteobacterium *Herbaspirillum seropedicae* (Fu, 1989 #1634) and for the alphaproteobacterium *A. amazonense* (Hartmann et al., 1986). Both bacteria lack the *draT* and *draG* genes in their genomes. However, a fast ammonium switch-off has been observed, which was not explainable by transcriptional repression. The molecular mechanisms which are subjected to this DraT-independent inactivation are not known so far.

In the *Alphaproteobacteria draT* and *draG* are co-transcribed and in most cases the operon is located upstream of the *nifHDK*-structural genes. In *R. capsulatus* the *draTG*-operon is located widely separated from *nifHDK* (Masepohl et al., 1993). In *R. rubrum*, *A. brasilense* and *A. lipoferum draT* and *draG* are probably co-transcribed with an open reading frame (ORF) with unknown function (Fitzmaurice et al., 1989, Liang et al., 1991, Zhang et al., 1992, Inoue *et al.*, 1996). The expression of these genes is constitutive in *R. rubrum* (Triplett *et al.*, 1982), while in *A. lipoferum* expression was regulated in response to oxygen, but not to ammonium ions (Inoue et al., 1996).

Again, P_{II} proteins, known to transmit the signal for cellular nitrogen status, regulate DraT and DraG activity (Figure 2). In *A. brasilense* and *R. rubrum*, complex formation of deuridylylated P_{II}-proteins (GlnZ or GlnJ, respectively) with the ammonium transporter protein AmtB results in membrane sequestration of DraG, hereby preventing its interaction with modified Fe-protein (Wang *et al.*, 2005, Huergo *et al.*, 2006b, Huergo *et al.*, 2007). Ammonium-induced triplex formation of the AmtB-trimer with the deuridylylated GlnK-trimer and DraG as a regulatory mechanism of DraG activity has been also proposed for *R. capsulatus* (Tremblay *et al.*, 2007). The metabolite pools, in particular 2-oxoglutarate and/or the ATP/ADP ratio, appear to control the association/dissociation of Amt with P_{II} as shown in studies with a variety of bacteria, including *E. coli* (Durand & Merrick, 2006),

Azospirillum brasilense (Huergo et al., 2007, Huergo et al., 2009), *Rhodospirillum rubrum* (Wolfe *et al.*, 2007, Teixeira *et al.*, 2008) and *Bacillus subtilis* (Heinrich *et al.*, 2006).

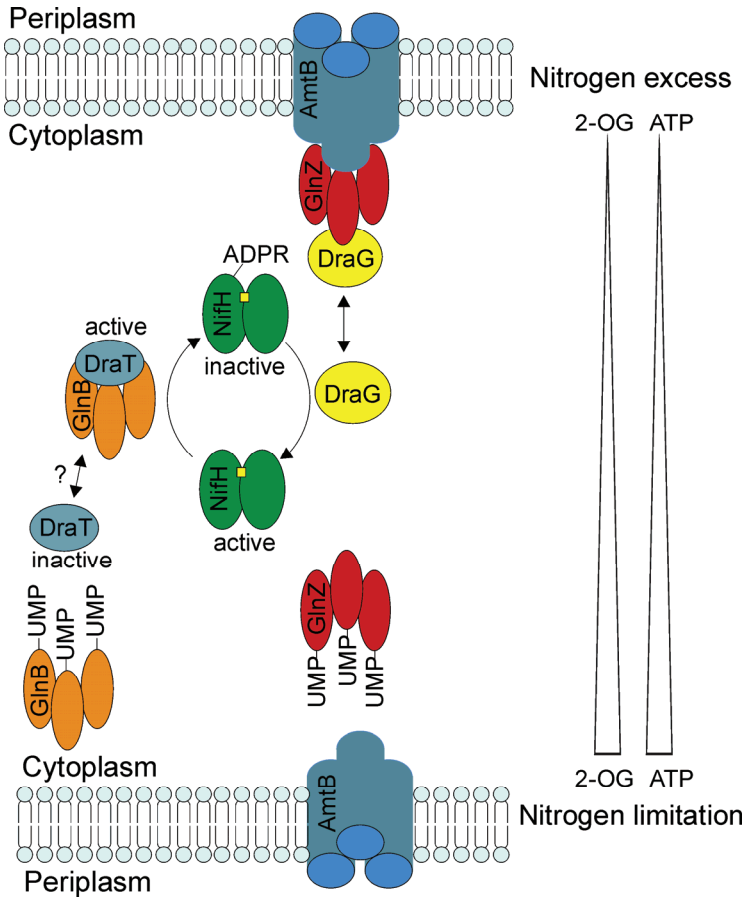

Figure 2: Model of the ammonium mediated posttranslational regulation of nitrogenase activity in *A. brasilense* (adapted from Huergo *et al.*, 2009). Under conditions of nitrogen excess, DraT-mediated ADP-ribosylation of the NifH-protein leads to nitrogenase inhibition. DraT activity is probably stimulated by interaction with the deuridylylated P_{II}-protein GlnB. At the same time triplex formation of DraG with the deuridylylated GlnZ-trimer and AmtB prevents NifH demodification. Under conditions of nitrogen fixation, GlnB becomes uridylylated and DraT is inactive, while upon uridylylation of GlnK DraG is free to remove the ADP-ribose on NifH.

Interestingly, in *R. capsulatus* GlnK sequestration to the membrane was independent from the uridylylation status of the P_{II} protein (Tremblay et al., 2007). Additionally, some transport-incompetent AmtB variants have been described in this bacterium, which form ammonia-induced complexes with GlnK but fail to regulate nitrogenase properly (Tremblay & Hallenbeck, 2008), thus indicating that complex formation by itself is not sufficient to regulate nitrogenase activity. Surprisingly, in *Azoarcus* sp. BH72 GlnK membrane association was not dependent on AmtB (Martin & Reinhold-Hurek, 2002). However, in total, four genes – namely *amtB*, *amtY*, *amtE* and *amtD* – encoding putative ammonium transporters, and therefore being putative binding partners of GlnK, are present in the *Azoarcus* sp. BH72 genome (Krause et al., 2006). This incident and the circumstance that *Azoarcus* sp. BH72

harbors three genes coding for P_{II}-like proteins makes the investigation of the nitrogen fixation regulation system particularly difficult in this strain. In *A. brasilense* DraT is able to interact with the deuridylylated form of the P_{II}-protein GlnB after ammonium chloride addition, suggesting that this interaction leads to the stimulation of DraT activity and subsequently ADP-ribosylation of dinitrogenase reductase (Huergo *et al.*, 2006a). The interaction of DraT and GlnB has been also demonstrated for *R. capsulatus* and *R. rubrum* in yeast-two-hybrid studies (Pawlowski *et al.*, 2003, Zhu *et al.*, 2006). Also the dinitrogenase reductase redox status has been shown to be involved in regulating DraT and DraG activity (Halbleib *et al.*, 2000). While DraG activity was higher with the reduced Fe-protein, DraT activity was stimulated with the oxidized form of the enzyme. A redox-dependent conformational change in the Fe-protein has been suggested to be responsible for the observed differences in activities.

Electron transport routes to nitrogenase

The involvement of the dinitrogenase reductase redox status for regulation of DraT and DraG activities suggests that the electron transport to nitrogenase might play a crucial role in this process. Therefore information on this topic is essential for a better understanding of the mechanisms underlying the activity control of nitrogenase. Electron transport routes to nitrogenase are diverse among diazotrophic organisms. In *Klebsiella pneumoniae* the pyruvate:flavodoxin oxidoreductase - gene product of *nifJ* – couples pyruvate oxidation with nitrogen reduction (Shah *et al.*, 1983). A flavodoxin encoded by *nifF* acts as the sole electron donor to dinitrogenase reductase in this bacterium (Nieva-Gomez *et al.*, 1980, Shah et al., 1983). However, such a non-branched electron transport pathway is an exception rather than the rule. In *Azotobacter vinelandii* the NifF flavodoxin is the major, but not the sole electron carrier (Bennett *et al.*, 1988). NifF as well as a ferredoxin (FdI) encoded by the *fdxA* gene support nitrogen fixation *in vitro*. However, *nifF*/*fdxA* double mutants can still grow diazotrophically, indicating that there must be one or more alternative electron carriers (Martin *et al.*, 1989). A possible candidate for this function could be FdxN, since disruption of the *fdxN* gene resulted in a decreased Mo-nitrogenase activity (Rodríguez-Quiñones *et al.*, 1993). In *R. rubrum* electron transport to nitrogenase of photoheterotrophically grown cells is established by a major pathway comprised of the *fixABCX* gene products (Edgren & Nordlund, 2006). FixA and FixB are similar to electron transfer flavoproteins, while FixC and FixX show similarity to electron transfer flavoprotein:ubiquinone oxidoreductases. Two

ferredoxins (FdxN and FdI) can serve as terminal electron carriers to nitrogenase in *R. rubrum*, but FdxN was shown to be the main donor (Edgren & Nordlund, 2005). In addition to the *fix* gene product dependent route, electrons generated through the oxidation of pyruvate by pyruvate:ferredoxin oxidoreductase can serve as an alternative electron transport pathway to *R. rubrum* nitrogenase (Edgren & Nordlund, 2006).

In *Azoarcus* sp. BH72 it was shown that FdxN is an important but not the essential electron donor to nitrogenase that is also involved in the ammonium induced switch-off of nitrogenase (Egener et al., 2002). This emphasizes the importance of the electron transport to nitrogenase for its activity regulation. Interestingly, a putative ion-translocating NADH:ferredoxine oxidoreductase was shown to be involved in electron transport to nitrogenase in *R. capsulatus* (Saeki *et al.*, 1993, Schmehl *et al.*, 1993). Seven of the genes implicated in electron transport to nitrogenase are organized in one operon designated *rnfABCDGEH* (Jouanneau *et al.*, 1998). Strains that carry mutated *rnf*-genes were unable to grow diazotrophically under illuminated anaerobic conditions (Saeki et al., 1993, Schmehl et al., 1993). RnfB and RnfC are potential iron-sulfur proteins and unlike the predicted transmembrane proteins RnfA, RnfD and RnfE, they are presumably situated at the cytosolic leaflet of the cell membrane in association with RnfADE (Jouanneau et al., 1998, Kumagai *et al.*, 1997). Another oxidoreductase involved in the electron transport pathway to nitrogenase was identified in *Anabaena* spp. PCC 7119. Electrons generated by the degradation of carbohydrates through the oxidative pentose-phosphate cycle are transferred by the action of the ferredoxine:$NADP^+$ oxidoreductase (FNR) from NADPH to FdxH (Neuer & Bothe, 1982). It has been proposed that FNR – whose expression was ten fold increased in heterocysts (Razquin *et al.*, 1996) – is involved in the main route that supplies reducing power to nitrogenase through the specific ferredoxin from heterocysts (FdxH) (Schrautemeier & Böhme, 1985, Böhme & Schrautemeier, 1987).

Objectives

The work presented in this study aimed to provide a deeper insight into the molecular mechanisms involved in the posttranslational control of nitrogenase activity in the betaproteobacterium *Azoarcus* sp. BH72. Previously, this system has been functionally characterized only in members of the class *Alphaproteobacteria*. Nitrogenase Fe-protein modification has been identified as ADP-ribosylation of arginine residue 102 in *Azoarcus* sp. BH72 by applying a mass spectrometric approach. Physiological analysis of deletion mutants of *draT*, *draG1* and *draG2* led to the characterization of the DraTG-system for posttranslational activity control of nitrogenase. Thus it was shown to be functionally

conserved among the *Proteobacteria*. Observed differences in *Azoarcus* with respect to nitrogenase activity regulation in comparison to the alphaproteobacterial mechanisms have been depicted. A DraT-independent ammonium mediated "switch-off" mechanism has been suggested to occur in *Azoarcus* sp. BH72. Putative mechanisms underlying such a DraT-independent regulation have been proposed. Furthermore, a putative function of DcrH1, whose gene is probably co-transcribed with *draT* and azo0536 in protecting nitrogenase against oxygen damage was suggested. In addition, proteins involved in the diazosome related nitrogen fixation and respiration process were identified by proteomics and their role in the cellular metabolism, diazosome formation and in the bacterium-fungus interaction has been discussed.

2. RESULTS AND DISCUSSION

Identification of ADP-ribose as the modifying group in the Fe-protein peptide sequence by applying a mass spectrometric approach

Covalent modification of the nitrogenase Fe-protein by ADP-ribosylation has been structurally characterized by mass spectrometry so far only for diazotrophic phototrophs of the class *Alphaproteobacteria* (Pope et al., 1985, Jouanneau et al., 1989). Other mechanisms than ADP-ribosylation have been proposed in different bacteria (Gallon *et al.*, 2000, Durner *et al.*, 1994, Hartmann et al., 1986). Even in *Azoarcus* sp. BH72 under conditions of diazosome formation, another type of Fe-protein modification was proposed (Hurek et al., 1995). Therefore, the structural characterization of the covalent modification was of particular interest in this endophyte.

The development of modern mass spectrometric techniques provided the possibility for an improved method for detection of ADP-ribosylations of proteins as shown by the detection of ADP-ribosylated peptides derived from nitrogenase Fe-protein of *Azoarcus* sp. BH72 (Oetjen *et al.*, 2009). Nitrogen-fixing cells of *Azoarcus* sp. BH72 were stimulated by ammonium addition to modify their nitrogenase Fe-protein and harvested. In a contemporary proteomic approach then total protein extracts were separated by two-dimensional gel electrophoresis in order to separate the two NifH isoforms. Spots containing modified or unmodified dinitrogenase reductase were excised and digested with sequencing-grade modified trypsin - cleaving C-terminal to arginine and lysine residues - and endoproteinase AspN, cleaving N-terminal to aspartate residues (Figure 3). Peptides were extracted, lyophilized and analyzed by tandem mass spectrometry (LC-MS/MS) or MALDI-TOF mass spectrometry. By performing high mass accuracy LC-MS/MS on modified Fe-protein samples, ADP-ribosylation was detected in the peptide sequence on arginine 102 (R102) in *Azoarcus* sp. BH72, representing the homologous arginine residue of *R. rubrum* R101. C18-LC-MS/MS spectra were searched against a dataset, consisting of all *Azoarcus* sp. BH72 proteins applying the Sequest algorithm (Yates *et al.*, 1995) implemented in the Bioworks 3.3 software (Thermo scientific) using an ADP-ribosylation specific mass change of 541.06 m/z on arginine as predefined search criterion. No ADP-ribosylation was detected when samples from the unmodified Fe-protein have been analyzed. The ADP-ribosylation on R102 was identified in the MS/MS result spectra of the triply charged precursor ion $[M+3H]^{+3}$ 1255.5 m/z ($[M+H]^{+}$ 3764.5 m/z)

representing the Fe-protein peptide 87-117 with the sequence CVESGGPEPGVGCAGR*GVITAINFLEEEGAY while R* represents the ADP-ribosylated arginine residue. Doubly charged and single charged b-type and y-type ion series specific for this peptide have been assigned (Fig. 4, in Oetjen et al., 2009) leading to the unambiguous detection of this type of modification on R102 in the *Azoarcus* sp. BH72 Fe-protein. In addition, collision-induced fragmentation resulted in doubly charged b-type ions of the intact peptide sequence 87-117 corresponding to different fragmentation levels of the ADP-ribose (Fig. 4, in Oetjen et al., 2009). The observed mass shift of 541 m/z is not explainable by any combination of amino acids adjacent to these peptides and it was only detected on this arginine residue.

```
1     MAKLRQCAIY GKGGIGKSTT TQNLVAALAE AGKKVMIVGC DPKADSTRLI
51    LHSKAQTTVM HLAAEAGSVE DLELDDVLSV GFGGVKCVES GGPEPGVGCA
101   GRGVITAINF LEEEGAYDDE LDFVFYDVLG DVVCGGFAMP IRENKAQEIY
151   IVCSGEMMAM YAANNIAKGI VKYANSGGVR LGGLICNSRN TDREDELIEA
201   LAAAMGTQMI HFVPRDNAVQ HAEIRRMTVI EYDPTHKQAD QYRQLAQKVL
251   NNKMLVIPTP IEMEQLEALL MEFGIMEQED ESIVGQTAAE LAAGAAA
```

Figure 3: Amino acid sequence of the *Azoarcus* sp. BH72 NifH-protein. Cleavage sites for trypsin (C-terminal to lysine or arginine residues) or for endoproteinase AspN (N-terminal to aspartate residues) are underlined. The large peptide 87-117 that contains the modified arginine residue (blue) is marked in red. In case of ADP-ribosylation on arginine 102 trypsin cleavage is blocked.

The basis for the success of this method was the finding that (i) digestion with two endoproteinases was necessary to obtain a peptide of a size feasible for mass spectrometric analysis since trypsin cleavage at R102 was blocked by the modification resulting in a large peptide (Figure 3) and (ii) arginine specific ADP-ribosylations are labile to trifluoroacetic acid (TFA) treatments, leading to the optimization of the peptide extraction protocol which was carried out in the absence of TFA. In preliminary attempts using trypsin cleavage alone and MALDI-TOF analysis, no modified peptide could be retrieved. However, the detection of the unmodified peptide 87-102 in samples of the modified Fe-protein that has been considered to be absent under these conditions, had initially arise uncertainties about the type of the Fe-protein modification in *Azoarcus* sp. BH72. Even the use of the two enzymes trypsin and endoproteinase AspN was not successful in the beginning, because standard methods have been applied for the peptide extraction that contained TFA which obviously led to the hydrolysis of the ADP-ribosylation. The lability of the ADP-ribosylation was confirmed by the detection of a truncated version of the formerly modified peptide with R102 exchanged by ornithine. Applying LC-MS/MS to the triply charged precursor ion with the monoisotopic mass $[M+H]^+$ of 3181.5 m/z, which was also observed in MALDI-TOF spectra, a nearly

complete doubly charged b-type and y-type ion series was obtained. The annotated amino acid sequence was specific for Fe-protein peptide stretch 87-117 assuming a delta mass change of -42.02 m/z for R102. This is consistent with an exchange of R102 by ornithine, which can be explained by a breakage of the guoanidino group leaving a primary amine attached to the ε-carbon atom of the former arginine residue (Figure 4). It is therefore assumed that this peptide version arose from the hydrolysis of the ADP-ribose moiety on R102. However, the case that this form represents a physiological Fe-protein state in the cell cannot be excluded but is unlikely.

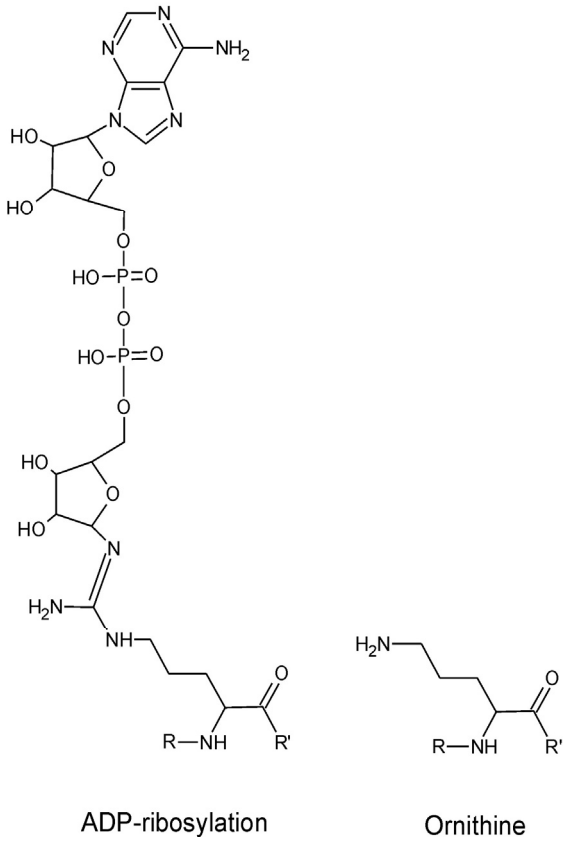

Figure 4: Chemical structure of the ADP-ribosylation on Fe-protein residue arginine 102 in comparison to an ornithine residue as indicated. The peptide chain is indicated by "R" or "R'", respectively.

The ADP-ribosylated peptide 87-117 with the monoisotopic mass of 3764.5 m/z has been also detected during MALDI-TOF MS in samples derived from the modified Fe-protein containing spots out of 2-DE gels. Due to the specific cleavage of trypsin C-terminal to lysine residue 86 and endoproteinase AspN cleavage N-terminal to aspartate residue 118 in the *Azoarcus* sp. BH72 Fe-protein, MALDI-TOF analysis emerged as a fast and reliable tool for the identification of ADP-ribosylations on this arginine residue in nitrogenase Fe-proteins. Since the NifH-protein amino acid sequence is highly conserved among prokaryotes in this

region, digestion with trypsin alone would in most cases not be sufficient to result in a peptide of an analyzable size for mass spectrometric examination of this modification. Due to the high specificity of the two endoproteinases used here, MALDI-TOF analysis is applicable for investigations of arginine specific ADP-ribosylations on Fe-proteins from other species.

Different types of covalent modifications on nitrogenase have been proposed in various reports, not necessarily leading to the inactivation of the enzyme. Gallon et al. (Gallon et al., 2000) for example suggested a palmitoylation of both dimers of nitrogenase Fe-protein in the cyanobacterium *Gloeothece*. Also *Anabaena variabilis* Fe-protein modification was supposed to deviate from ADP-ribosylation (Durner et al., 1994). In the diazotrophic bacterium *Azospirillum amazonense* two Fe-protein isoforms have been retrieved during SDS-PAGE; the proteins migrating closer together than the modified and unmodified Fe-protein subunits of *R. rubrum* (Hartmann et al., 1986, Song *et al.*, 1985). In this case both forms were active *in vitro* and no *draT* homolog could be identified using hybridization techniques. Therefore it is likely that *A. amazonense* dinitrogenase reductase is modified by another type of modification. The approach described by Oetjen *et al.* might help to characterize these putative other types of modifications on NifH-proteins.

In addition, the described method can be applied to detect ADP-ribosylations on other proteins or to identify this kind of modification in the course of whole proteome analyses. Like phosphorylations, reversible ADP-ribosylation emerges to be an important mechanism to regulate enzyme activity not only in bacteria. In eukaryotes, mono-ADP-ribosyltransferase reactions are involved in important cellular processes with substrates such as heterotrimeric G proteins, integrin, histones and even DNA (reviewed by Corda & Di Girolamo, 2003). As detection method, indirect identification by radioactive labeling of the donor molecule NAD was applied in most cases of the eukaryotic systems. However, the method for detection of ADP-ribosylation in proteins described by Oetjen *et al.* provides an easy, non-isotopic and applicable modern method implying state-off-the art mass spectrometric techniques for the analysis of ADP-ribosylations and other covalent modifications on proteins.

Genome organization and expression analysis of *draT*, *draG1* and *draG2* in *Azoarcus* sp. BH72 and putative function of DcrH1

Nitrogenase Fe-protein ADP-ribosylation is known to be a regulatory mechanism of enzyme activity in diazotrophic alphaproteobacteria (Ludden, 1994). Covalent ADP-

ribosylation of a specific arginine residue and thereby nitrogenase inactivation as a consequence of energy restraints or of ammonium addition to nitrogen-fixing cells is catalyzed by dinitrogenase reductase ADP-ribosyltransferase (DraT). The reverse mechanism – demodification and activation of nitrogenase – is accomplished by the action of dinitrogenase reductase activating glycohydrolase (DraG). The first functional characterization of the DraTG-system for posttranslational regulation of nitrogenase activity outside of the *Alphaproteobacteria* was provided by Oetjen *et al*. who studied the effect of deletion mutations of the respective genes in the betaproteobacterium *Azoarcus* sp. BH72 (Oetjen & Reinhold-Hurek, 2009). The *draT* gene was identified in the *Azoarcus* sp. BH72 genome. Its deduced amino acid sequence had 36% identity over the entire length to *R. rubrum* DraT. Different from the well-studied alphaproteobacterial system, the genome organization and expression analysis revealed co-transcription of *draT* with a gene encoding a hypothetical protein (azo0536) and with *dcrH1* (azo0535) whose gene product had sequence identity to a hemerythrin (Figure 5).

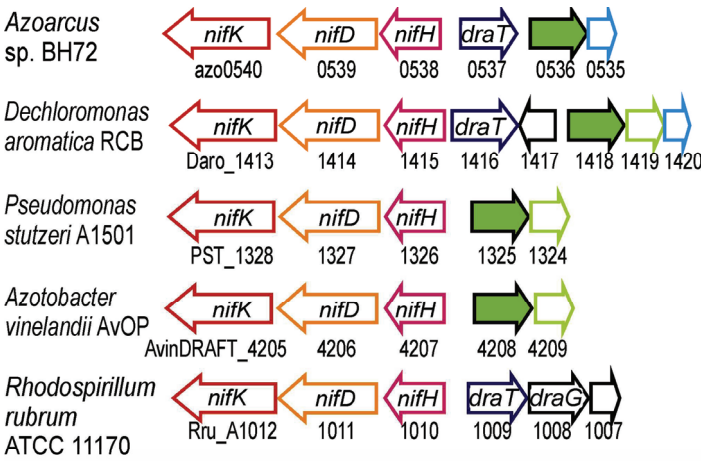

Figure 5. Genome organization of the *draT*- azo0536 region in *Azoarcus* sp. strain BH72 compared to strains bearing a putative azo0536 ortholog. For comparative purpose the *Rhodospirillum rubrum* gene organization around *draT* is shown. Numbers below the arrows represent the locus-tags for each strain.

Thus in contrast to the *Alphaproteobacteria* in *Azoarcus* sp. BH72 *draT* was not co-transcribed with *draG*. The gene product of azo0536 had similarity to a hypothetical protein (Daro_1418) of *Dechloromonas aromatica* RCB (55% identity), *Pseudomonas stutzeri* (PST_1325, 54% identity) and *Azotobacter vinelandii* (AvinDRAFT_4208, 52% identity), which showed a similar genomic organization upstream of the *nifHDK*-operon (Figure 5). However, the *Gammaproteobacteria A. vinelandii* and *P. stutzeri* do not harbor the DraT/DraG-system. So far a role of azo0536 in posttranslational regulation or covalent modification of nitrogenase in *Azoarcus* sp. BH72 could not unambiguously been shown (see

Chapter 2B). However, preliminary results of a azo0536 mutant that was polar on *dcrH1* revealed a diazotrophic growth deficiency of *Azoarcus* when cells have been grown in Erlenmeyer flasks without oxygen control and with relatively high starting oxygen concentration of ~1.4%. Due to sequence similarity of DcrH1 to hemerythrins a putative role of DcrH1 as an oxygen binding protein involved in protection of nitrogenase from oxygen damage was proposed (Chapter 2B).

Most of the known alphaproteobacterial genomes harbor only one *draG*-like sequence, while two paralogs coding for ADP-ribosylglycohydrolases were detected in the genome of strain BH72. One was in close proximity to the *nif*-structural genes (*draG1*), while the other was located in a different genomic context (*draG2*). Since *draG* mRNA could generally not be detected by northern blotting, it is not clear whether *draG1* or *draG2* are potentially co-transcribed with other genes. However, the genomic organization suggested co-transcription of *draG1* with *nifQ*. Reporter gene fusion experiments with *gusA* showed that expression of *draG1* was nitrogen regulated, while expression of *draT* and *draG2* was constitutive under the conditions studied. In comparison, expression of the *draTG* operon in *R. rubrum* is not N-regulated.

Phylogenetic analysis of DraG amino acid sequences

In order to allow speculations about the possible function of the two DraG paralogs, phylogenetic analysis was carried out on the DraG amino acid sequences. This analysis revealed that the phylogeny of DraG2 differed from DraG1 (supplemental Fig. S2, in Oetjen & Reinhold-Hurek, 2009). DraG1 clustered in a group together with dinitrogenase reductase ribosylglycohydrolases of the *Alphaproteobacteria*, where the system for nitrogenase posttranslational regulation is studied in molecular detail. These DraG sequences were defined by us as Group I sequences. Group I sequences probably consists of NifH specific ADP-ribosylglycohydrolases. Occurrence of *draT* orthologous sequences in these diazotrophs support this suggestion. In addition, our phylogenetic analysis indicated a more widespread distribution of the DraT/DraG-system in the *Proteobacteria* than previously suspected (Oetjen & Reinhold-Hurek, 2009). Group I ADP-ribosylglycohydrolase sequences could be detected mainly in alpha-, beta- and deltaproteobacteria. However, presence of Group I DraG-sequence in a member of the class *Gammaproteobacteria* or in a verrucomicrobium might be a result of horizontal gene transfer.

Azoarcus DraG2 clustered in Group IV together with putative ADP-ribosylhydrolases of unknown specificity. In comparison, as an example for *Alphaproteobacteria* harboring two DraG-like proteins, the second ADP-ribosylhydrolase sequence of two *Rhodospeudomonas* species clustered in Group II. This suggests that these proteins might also functionally be distinct from DraG1 or DraG2 of *Azoarcus* sp. BH72. In total four phylogenetically different groups of ADP-ribosylglycohydrolases could be distinguished. Groups II to IV harbored prokaryotes which lack *draT*. Even the presence of *nifH* was widely dispersed over the tree and it could not be assigned to a specific group except of Group I or Group III, which was composed of a cyanobacterium and members of the *Chlorobea*. However, species from the latter group did not possess *draT*. Assigning putative substrate specificity for the ADP-ribosylglycohydrolases of the groups II-IV is not possible due to the lack of functional information about ADP-ribosyltransferases in these bacteria. To shed more light into this question, application of a proteomic approach specific for the immunological detection of ADP-ribosylations using ethenoNAD as a substrate for the transferase reaction (Krebs *et al.*, 2003) combined with the mass spectrometric characterization presented as a part of this work would be helpful.

Role of DraT, DraG1 and DraG2 in regulation of nitrogenase activity

In order to characterize the role of DraT in the posttranslational control of nitrogenase activity, a deletion mutant of the *draT* gene was constructed. Previous work already indicated the occurrence of a modified Fe-protein in *Azoarcus* sp. BH72. By SDS-PAGE and Western blotting analysis with antiserum against NifH a higher molecular mass form of this protein has been obtained that suggested the existence of the covalently modified form of dinitrogenase reductase in *Azoarcus* sp. BH72 (Martin & Reinhold-Hurek, 2002, Hurek et al., 1995). SDS-PAGE and Western blotting analysis of deletion mutants revealed that in comparison to the wild-type strain the higher molecular mass form of the NifH-protein was absent in the *draT*-mutant. Therefore, DraT was responsible for the covalent modification of the nitrogenase Fe-protein by ADP-ribosylation in this bacterium. However, interestingly ammonium-mediated switch-off occurred even in the absence of DraT (Oetjen & Reinhold-Hurek, 2009). *Azoarcus* sp. BH72 seems to possess two nitrogenase regulatory mechanisms, the DraT/DraG mediated and a so far unexplained DraT-independent way of inactivation. This DraT-independent regulation was sufficiently efficient in *Azoarcus* sp. BH72 and its way

of regulation is probably similar to the mechanism discovered in *Azospirillum amazonense* or *Herbaspirillum seropedicae*; both bacteria lacking a *draT* ortholog but regulate their nitrogenase activity in response to fixed nitrogen (Hartmann et al., 1986, Song et al., 1985).

The reversibility of the DraT-mediated short term inhibition of nitrogenase is known to be accomplished by DraG in members of the *Alphaproteobacteria* (Zhang et al., 1992, Zhang *et al.*, 1997, Masepohl et al., 1993, Liang et al., 1991). To test whether Fe-protein demodification and reactivation is in *Azoarcus* sp. BH72 as well mediated by DraG, deletion mutants of *draG1*, *draG2* and a *draG1draG2* double mutant were constructed and functionally analyzed. Nitrogen-fixing cultures of the wild type and of *draG*-mutant strains cultured in an oxygen-controllable bioreactor were shifted to anaerobic conditions in order to induce Fe-protein modification. Growth of bacteria in the bioreactor for this experiment was crucial, as under these conditions a stable nitrogen fixation rate and a relatively high amount of modified dinitrogenase reductase dimers have been obtained when cells were flushed with nitrogen gas. A high degree of modification is necessary for a meaningful correlation of nitrogenase activity with the Fe-protein modification status. The analysis of deletions mutants in *Azoarcus* sp. BH72 revealed that DraG1 was clearly the main player responsible for Fe-protein demodification after transfer to anaerobiosis and subsequent adjustment to microaerobiosis. DraG2 only had an additive effect on the demodification (Oetjen & Reinhold-Hurek, 2009). A similar result with respect to the demodification has been obtained during addition of low ammonium chloride concentrations to nitrogen-fixing bacteria (Fig. 4, in Oetjen & Reinhold-Hurek, 2009). However, it was surprising to see that the Fe-protein modification status and nitrogenase activity were not strictly correlated in a *draG1* mutant or a *draG1draG2* double mutant during anaerobiosis experiments (Fig. 3, and Fig. 2 Oetjen & Reinhold-Hurek, 2009). An unusual nitrogenase reactivation has been observed in *draG*-mutants that was contrary to results from analogous experiments in the *Alphaproteobacteria*, where no reactivation took place in the respective mutants (Zhang et al., 1992, Liang et al., 1991). Recovery of approximately 50% of the initial nitrogenase activity in a *draG1* mutant was possible although only about 10% of dinitrogenase reductase dimers were unmodified (Figure 6). The physiological analysis of *draG*-mutants therefore suggests that *Azoarcus* synthesizes more NifH-molecules that are actually exploited, indicating that probably the electron transport to nitrogenase is the limiting factor for a maximum nitrogenase activity in this bacterium as a complete DraT-mediated ADP-ribosylation of dinitrogenase reductase was hardly observed during these studies. This circumstance again points towards the relatively low importance of the DraTG-system for posttranslational nitrogenase regulation in this

strain. Obviously, the reduced cellular pool of active, unmodified dinitrogenase reductase dimers – originated by the activity of DraT - was sufficient to recover high levels of initial nitrogenase activity rates. Moreover, the high number of unconsumed NifH-protein might go along with the formerly observed hyperinduction phenomenon in this endophyte (Hurek et al., 1994a). As previously proposed, a stable nitrogen fixation rate could be maintained with lower respiration rates in case of a putatively more efficient electron transport to nitrogenase.

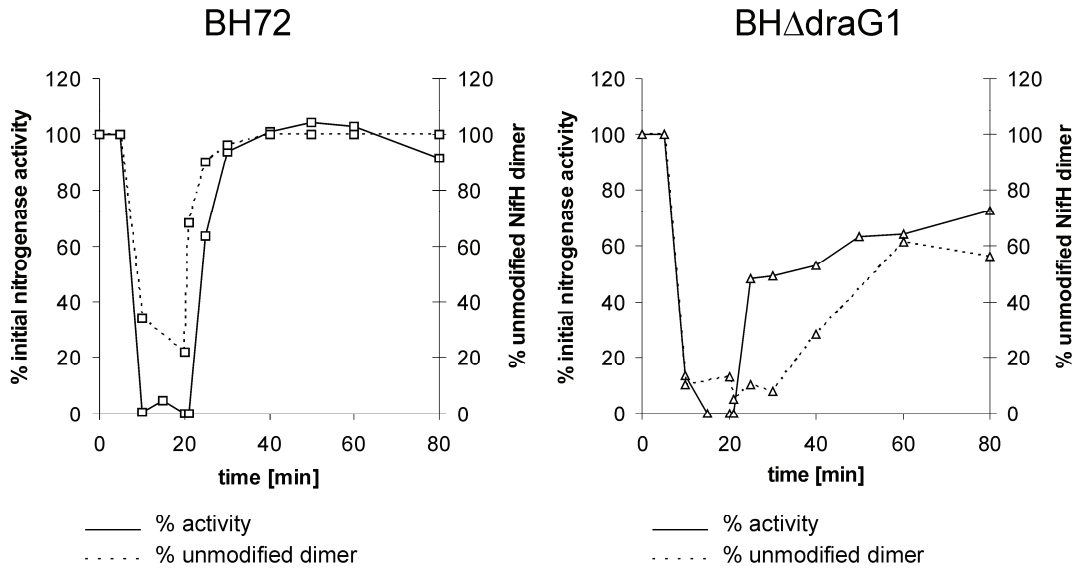

Figure 6: Correlation of nitrogenase activity with the modification state of dinitrogenase reductase of *Azoarcus* wild-type strain BH72 or *draG1* mutant BHΔdraG1 as indicated. For determination of the acetylene reduction rate, cells grown in an oxygen controllable bioreactor were transferred to microaerobic rubber-stopper sealed Erlenmeyer flasks with 10% acetylene. Cells were flushed with nitrogen gas for 30 min in the bioreactor, then transferred to anaerobic rubber-stopper sealed Erlenmeyer flasks and further incubated for 15 min. Hereafter, microaerobic conditions were adjusted in the flasks and ethylene production was determined. Diagrams display the percentage of initial nitrogenase activity (continuous line) and the percentage of unmodified dinitrogenase reductase dimer (dotted line).

As nitrogenase activity resumption was impaired in the *draG1* mutant or the *draG1draG2* double mutant it can be concluded that modified Fe-protein was inactive in *Azoarcus* sp. BH72. This finding was supported by results of nitrogenase *in-vitro* assays (see Chapter 2B, Fig. 6). Together with the results from phylogenetic analysis it was therefore suggested that DraG1 functions as dinitrogenase reductase ribosylglycohydrolase. The main cellular function of DraG2 was presumed to be different. However, its role is yet to be elucidated

DraT-independent nitrogenase inactivation

In correlation with previous results of a *glnB*-mutant (Martin & Reinhold-Hurek, 2002) our results have shown that *Azoarcus* sp. BH72 possesses an ammonium-induced nitrogenase switch-off mechanism which is independent of DraT (Oetjen & Reinhold-Hurek, 2009). Either a *glnB*-mutant (Martin & Reinhold-Hurek, 2002) or a *draT*-mutant was able to perform a switch-off response, but nitrogenase ADP-ribosylation was abolished (Table 1). The question arose which mechanism underlies such a DraT-independent nitrogenase inactivation. Previous results have shown that the electron transport to nitrogenase could be involved in this regulation, since a *fdxN*-mutant was effected in the ammonium-induced switch-off response (Egener *et al.*, 2001; Table 1). The gene *fdxN* codes for an important but not the sole electron carrier to nitrogenase in *Azoarcus* sp. BH72. A similar response has been observed in mutants of the genes encoding the putative membrane spanning Rnf-complex (Sarkar *et al.*, manuscript in preparation). In accordance, membrane association of the P_{II} protein GlnK - known to be involved in nitrogenase posttranslational regulation (Martin & Reinhold-Hurek, 2002) - was abolished in *rnf*-mutants (Sarkar *et al.*, manuscript in preparation). Interestingly, GlnK membrane association was not affected in mutants of the gene coding for the ammonium transporter AmtB (Martin & Reinhold-Hurek, 2002). This led to the suggestion about a potential protein-protein interaction of GlnK with the Rnf-complex dependent on the uridylylation status of GlnK thereby blocking electron transport to nitrogenase (Sarkar *et al.*, 2009).

Integrating the results of Sarkar *et al.* with the current findings on the DraT/DraG-system of *Azoarcus* sp. BH72, a hypothetical model for the nitrogenase regulation in *Azoarcus* sp. BH72 is proposed (Figure 7). Under conditions of fixed nitrogen deuridylylated GlnK could form a complex with Rnf1 thereby sequestering DraG1 to the membrane. In this way DraG1 would be sterically hindered to demodify dinitrogenase reductase. In addition, the electron transport to the NifH-protein via FdxN would be blocked. As in *A. brasilense*, interaction of GlnB with DraT could trigger ADP-ribosylation of NifH (Huergo et al., 2006a, Huergo et al., 2009).

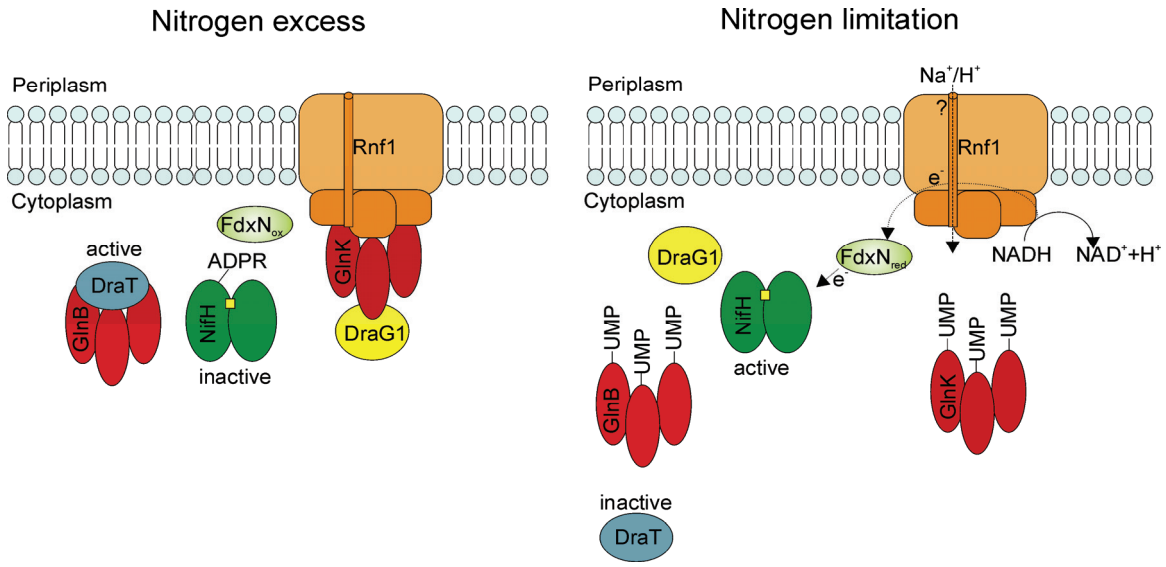

Figure 7: Putative model for nitrogenase regulation involving a blockage of the electron transport to nitrogenase based on interaction with the P_{II}-protein GlnK in response to ammonium. ADPR, ADP-ribosylation.

Conversely, under nitrogen fixation conditions the main route for electron transport to nitrogenase would be accomplished by oxidation of NADH through the Rnf-complex, thereby reducing FdxN, acting as the major terminal electron carrier to nitrogenase Fe-protein. Uridylylation of GlnB or GlnK would result in the dissociation of the corresponding complexes. Thereby DraG1 is free for nitrogenase Fe-protein demodification, while DraT is inactive. This mode of regulation would be independent of DraT, since nitrogenase inactivation would be performed as a consequence of a lack of electrons transferred to NifH. In *Azoarcus* sp. BH72, both, nitrogenase switch-off and Fe-protein ADP-ribosylation, are impaired by a mutation in *glnK* (Table 1), which is consistent with this hypothetical model as electron transport would not be blocked in a *glnK*-mutant and DraG1 would be free to demodify the NifH-protein.

Tabel 1: Impact of genes and their gene products on nitrogenase switch-off and Fe-protein modification in *Azoarcus* sp. BH72.

gene	gene product	ammonium "switch-off"[1]	Fe-protein modification[1]	reference
draT	dinitrogenase reductase ADP-ribosyltransferase	+	-	Oetjen and Reinhold-Hurek, 2009
glnB	P_{II}-like signal transmitter protein GlnB	+	-	Martin *et al.*, 2002
glnK	P_{II}-like signal transmitter protein GlnK	-	-	Martin *et al.*, 2002
glnY	P_{II}-like signal transmitter protein GlnY	n.d.	n.d.	
amtB	ammonium transporter protein AmtB	-	-	Martin *et al.*, 2002
amtY	ammonium transporter protein AmtY	-/+	n.d.	(Köhler, 2003)
amtE	ammonium permease	-/+	+	†[2]
amtD	ammonium permease	n.d	n.d.	
rnf	electron transport complex	-/+	+	A. Sarkar, manuscript in preparation
fdxN	ferredoxin N	-/+	+	Egener *et al.*, 2001 and results J. Oetjen

[1] + normal switch-off response or Fe-protein ADP-ribosylation; -/+ switch-off response impaired; - switch-off response or ADP-ribosylation abolished; †[2] A. Sarkar poster presentation 7th European Conference on Nitrogen Fixation, Aarhus (Denmark) ; n.d. not determined.

However, this hypothetical model is not sufficient to explain the phenotype observed in mutants of the ammonium transporter protein AmtB (Table 1). *Azoarcus amtB*-mutants were unable to modify their nitrogenase Fe-protein and are incapable of performing an ammonium "switch-off" response, indicating that this protein plays an important role in the posttranslational regulation of nitrogenase (Martin & Reinhold-Hurek, 2002). Four paralogous genes are located in the *Azoarcus* sp. BH72 genome coding for putative ammonium transporter, namely *amtB, amtD, amtE* and *amtY*. In addition to *amtB*, *amtE* and *amtY* have been studied for their potential role in nitrogenase posttranslational regulation (Table 1). The putative ammonium transporters AmtE and AmtY are involved in the fast ammonium induced nitrogenase inactivation, as mutants of either of the corresponding genes were impaired in the switch-off response (Sarkar, unpublished results; Köhler, 2003). The *amtE* gene codes for an unusual ammonium transporter; the C-terminal part of the proteins comprises of a PAS, GGDEF and an EAL domain. The GGDEF and EAL domains are probably involved in cyclic di-GMP signaling. The GGDEF-domain (Pfam entry PF00990) catalyzes the synthesis of cyclic di-GMP, while the EAL-domain (Pfam entry PF00563) has diguanylate phosphodiesterase function. Cyclic di-GMP is a second messenger that regulates

many processes including biofilm formation, adhesion and developmental transition (reviewed in Ryan *et al.*, 2006). It might be reasonable to speculate about a potential function of this molecule in nitrogen metabolism. The PAS-domain (Pfam entry PF00989) is implicated in various signaling cascades and it is known to play a role in oxygen and redox sensing. In addition to its role in ammonium switch-off in *Azoarcus* sp. BH72, AmtE was shown to mediate ammonium-dependent swarming facility in this bacterium (Sarkar, unpublished results). This finding as well as results from others (reviewed by Tremblay & Hallenbeck, 2009) indicates that ammonium transporters belonging to the family of Amt/Rh-proteins may act as ammonium sensors that regulate cellular metabolism in response to ammonium despite their function as ammonium transporters. However, it is not clear so far, how such an Amt-mediated signaling transduction might function.

Since all *amt*-genes analyzed so far in *Azoarcus* sp. BH72 are at least implicated in the ammonium mediated switch-off response (Table 1), it is likely that the signal leading to nitrogenase inactivation involves the ammonium transport itself. In *amtB*-mutants of *Azoarcus* ammonium transport was not affected with high ammonium chloride concentrations (Martin & Reinhold-Hurek, 2002). However, the transport was reduced, when low ammonium chloride concentrations such as 200 µM have been used (Martin & Reinhold-Hurek, 2002). It is unlikely that the ammonium ion itself serves as the signal leading to the physiological switch-off, because *amtB*-mutants have been affected in the switch-off after addition of high ammonium chloride concentrations, which could enter the cell independent of AmtB (Martin & Reinhold-Hurek, 2002). The current model of ammonium transport through Amt proteins emphasizes a mechanism in which the NH_4^+ ion is deprotonated at the external site; NH_3 is conducted through the hydrophobic pore of the protein and reprotonated at the cytoplasmic site (Javelle *et al.*, 2008). The question whether a proton is co-transferred through the Amt-protein still needs to be elucidated (Figure 8).

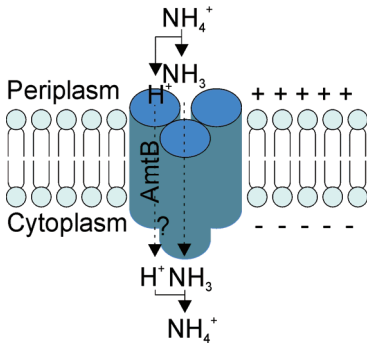

Figure 8: Putative model of ammonium transport through AmtB. NH_4^+ is probably deprotonated at the periplasmic site and NH_3 can pass the hydrophobic pore of the protein. A proton might be co-transferred through AmtB. At the cytoplasmic site NH_3 can be reprotonated to NH_4^+ (adapted from Tremblay & Hallenbeck, 2009).

Concomitant transfer of a proton with NH_3 would lead to a net electrogenic transport. It might be possible that the net charge transfer could result in a signal mediating nitrogenase inactivation. In case of NH_4^+-deprotonation at the periplasmic site and sole NH_3 conduction the cytoplasma membrane electrochemical gradient would increase upon ammonium transport. In this model, the increase of the electrochemical potential might trigger a signal leading to nitrogenase switch-off. However, DraT-independent ammonium switch-off might involve putative unidentified proteins.

Nitrogenase Fe-protein modification in diazosome containing cells of *Azoarcus* sp. BH72 and nitrogenase membrane association

An interesting physiological state of the *Azoarcus* sp. BH72 metabolism is characterized by the presence of membrane stacks (diazosomes) involved in an efficient nitrogen fixation process (hyperinduction) (Hurek et al., 1994a, Hurek et al., 1995). This state is reproducibly achieved by co-culturing *Azoarcus* sp. BH72 with the fungal endophyte strain 2003 (Hurek et al., 1995). The bacteria can adhere to the fungal mycelium and experience probably a stable microoxic environment under these conditions, which is a prerequisite for hyperinduction and diazosome formation. The nitrogenase protein was mainly located in the internal membrane stacks in diazosome containing cells (Hurek et al., 1995). It was suggested that the close proximity of nitrogenase to the energy and redox generating systems is the basis for the observed high efficient nitrogen fixation process. In addition, a higher molecular mass form of the nitrogenase Fe-protein has been identified. This form was not detectable by ^{32}[P]-labeling

and autoradiography. Therefore it was suggested that under these conditions nitrogenase Fe-protein was modified by a different kind of modification than ADP-ribosylation. For this reason, it is interesting to investigate whether this type of modification is detectable under co-culture conditions of the *draT*-mutant strain. However, no second band of lower electrophoretic mobility could be detected during Western blot analysis (Fig. 1, in Oetjen & Reinhold-Hurek, 2009).

Confirming previous result (Hurek et al., 1995), during comparative proteome studies the Fe-protein was down-regulated in co-culture cytoplasmic fractions of wild-type strain BH72 with the fungus isolate 2003, while it was always present in membrane fractions (Battistoni *et al.*, 2009). However, in the recent study a high proportion of modified Fe-protein could not be detected in membrane fractions of diazosome containing cells (see Figure 1, Battistoni et al., 2009). A weak spot characterized by a slower migration behavior and a shift towards a more acidic pI probably displays the ADP-ribosylated form of this protein. The ADP-ribosylation reaction might have been induced during the harvest, as cells most likely get anaerobic during centrifugation steps.

Membrane association of the NifH-protein in *Azoarcus* sp. BH72 does not exclusively occur in diazosome containing cells of this bacterium. Fe-protein has been also detected in membrane fractions under standard nitrogen fixation conditions (Battistoni et al., 2009). Interestingly, under pure culture conditions when cells have been grown in a bioreactor with controlled oxygen concentrations of 0.6%, the unmodified Fe-protein derived from the *draT*-mutant has been discovered to a high extent in membrane fractions (Chapter 1B). In comparison, the modified protein from the *draG1draG2* double mutant could not be identified in membrane fractions. In summary, these results imply that the active unmodified nitrogenase Fe-protein is partially membrane associated even under standard nitrogen fixation conditions. It is reasonable to assume that it interacts with a membrane protein and that this interaction supports the electron transport to the FeMo-protein.

Differentially displayed proteins during co-culture of *Azoarcus* sp. BH72 with a fungal partner

Proteome studies allow the identification of distinct sets of proteins present under a specific metabolic status of an organism. Nitrogen fixation of *Azoarcus* sp. BH72 during co-culture with the endophytic fungal strain 2003 might simulate more precisely the natural

environmental conditions for this bacterium. The formation of diazosomes, which was postulated to take place concomitant with an efficient and economic nitrogen fixation process, makes this state particularly attractive for proteome studies.

The availability of the *Azoarcus* sp. BH72 genome sequence (Krause et al., 2006) was the basis for a more comprehensive analysis of the diazosome-related proteome of this strain (Battistoni et al., 2009). Two-dimensional polyacrylamide gel electrophoresis (2-DE) was applied on the membrane or cytoplasmic fraction of bacterial cells derived from co-culture experiments of the fungus strain 2003 with the wild-type strain BH72 or a mutant strain BH1599 unable to produce type IV pili (Dörr *et al.*, 1998). Strain BH1599 was therefore impaired in its ability to attach to the fungal mycelium and to produce diazosomes (Dörr et al., 1998). This comparison allowed for the specific detection of proteins involved in diazosome structure and function. 2-DE protein patterns of membrane or cytoplasmic fractions derived from the different conditions were compared to the control, strain BH72 grown under nitrogen fixation conditions in pure culture. Differentially regulated proteins have been analyzed by MALDI-TOF MS as well as MALDI-TOF TOF analysis and were subsequently identified by a combined database search of the MS and MS/MS spectra (Battistoni et al., 2009). Generally, the metabolism of *Azoarcus* sp. BH72 in co-culture was characterized by an extensive down-regulation of proteins (Table 2) confirming previous results (Karg & Reinhold-Hurek, 1996).

Table 2. Differentially regulated proteins in *Azoarcus* sp. BH72 during co-culture with the fungus strain 2003 in comparison to standard nitrogen fixation conditions or to strain BH1599 grown under co-culture conditions.

	BH72 co-culture *versus* BH72 standard N-Fix
down-regulated in membrane fractions	53%
up-regulated in membrane fractions	18%
down-regulated in cytoplasmic fractions	46%
up-regulated in cytoplasmic fractions	10%
	BH72 co-culture *versus* BH1599 co-culture
differentially regulated	7%

In order to investigate the metabolism of *Azoarcus* under co-culture growth condition with a fungal partner, metabolic pathways were reconstructed and compared in the different conditions studied by searching the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Figure 2, Battistoni et al., 2009). As a carbon source for the fungal endophyte strain 2003 glucose was included in the co-culture medium which can not be metabolized by *Azoarcus* sp. BH72. In addition, the malate concentration of this medium was relatively low in order to promote bacterial growth in the beginning and to bypass the lag-phase. After consumption of

the malate, *Azoarcus* was dependent on fungal exudates as carbon sources. This was in accordance with the up-regulation of specific proteins from the acetate and ethanol assimilation during co-culture growth. The ability of *Azoarcus* sp. BH72 to utilize ethanol as carbon source has been described earlier (Reinhold-Hurek et al., 1993) and was confirmed here by the up-regulation of the alcohol dehydrogenases ExaA2 and ExaA3. Some proteins belonging to the amino acid metabolism have been differentially expressed during co-culture growth conditions. Most of these proteins were down-regulated or constitutively expressed, confirming the previous observation that diazosome-containing cells were arrested in growth (Hurek et al., 1995). Also the expression of chaperones and enzymes involved in protein synthesis and processing has been changed under co-culture growth conditions, indicating an adaptation to the different growth conditions (Battistoni et al., 2009).

Regulation of proteins involved in nitrogen fixation during co-culture growth are of great importance, since they might be involved in hyperinduction, a condition possibly reflecting a naturally occurring physiological state of *Azoarcus* sp. BH72. Surprisingly, expression of some *nif*-genes involved in cofactor biosynthesis (*nifN*, *nifU*) or nitrogenase maturation (*nifM*, *nifY*) was obviously down-regulated under co-culture conditions, while the cellular expression level of the nitrogenase structural components (NifHDK) remained equal (Battistoni et al., 2009). This finding is consistent with the conclusion that diazosome containing cells are arrested in growth (Hurek et al., 1995) and it indicates that nitrogenase is stable under these conditions.

Diazosome-related proteins

The identification of up-regulated proteins in diazosome containing cells of the wild type and not in strain BH1599 has been of special interest, because BH1599 is unable to attach to the fungal mycelium and to produce internal membrane stacks (Table 2). Therefore, these proteins might be involved in diazosome structure or function. WbnF, a putative nucleoside-diphosphate-sugar epimerase protein probably involved in exopolysaccharide synthesis and therefore likely to be important for the bacterium-fungus interaction, belonged to this group (Battistoni et al., 2009). Interestingly, the cytochrome c-type biogenesis protein CycH was specifically induced in *Azoarcus* sp. BH72 during co-culture growth (Battistoni et al., 2009). In *Sinorhizobium meliloti* a four-gene cluster (*cycHJKL*) is required for the production of all c-type cytochromes (Kereszt *et al.*, 1995). The encoded gene products are located in, or bound to, the cytoplasmic membrane, and oriented periplasmically in *S. meliloti*. They form a heme

lyase complex which is responsible for the maturation of cytochrome c. Mutations in any of the *cyc* genes resulted in the loss of c-type cytochromes, and blocked symbiotic nitrogen fixation (Kereszt et al., 1995). Consistent with the induction of CycH, the gene product of *ccmA* (also termed *helA*) was induced under co-culture conditions. The *ccmABCD* genes encode an ABC transporter specific for heme (Goldman *et al.*, 1997). This complex is a component of the system I for cytochrome c biogenesis that is usually found in alpha- and gammaproteobacteria (Goldman & Kranz, 2001). It might therefore be assumed that cytochrome c plays an important role in an efficient respiration process associated with an economic nitrogen fixation reaction during hyperinduction in *Azoarcus* sp. BH72. Noteworthy has been also the induction of polynucleotide phosphorylase (PNPase). PNPase is encoded by the *pnp* gene and it is induced at low temperatures (Jones *et al.*, 1987, Zangrossi *et al.*, 2000). PNPase has 3'-to 5' exoribonuclease activity and is involved in messenger RNA degradation in *Escherichia coli* (Donovan & Kushner, 1986). Therefore, the induction of PNPase in *Azoarcus* sp. BH72 under co-culture growth conditions might influence the stability of a pool of different mRNA species. Interestingly, in *E. coli* PNPase has been also shown to be responsible for residual *in vivo* polyadenylation of RNA in the absence of Poly(A) polymerase and for the incorporation of non-A residues into poly(A) tails (Mohanty & Kushner, 2000). A polyadenylated *nifH* mRNA could be isolated from uninoculated Kallar grass roots whose cDNA was identical to the *Azoarcus* sp. BH72 *nifH* gene (Hurek et al., 2002). The observed tail was heteropolymeric, suggesting other enzymes than Poly(A) polymerase for its synthesis. In addition, polyadenylated mRNA was detected in *Azoarcus* sp. BH72 by Northern blot analysis using a poly (dT) oligonucleotide as probe (Hurek et al., 2002). It might be reasonable to speculate that under specific conditions PNPase accounts for such mRNA processing in *Azoarcus* sp. BH72. Oligopeptidase A, encoded by *prlC* in *Azoarcus* sp. BH72, has been also induced in the wild-type under co-culture conditions (Battistoni et al., 2009). The *E. coli prlC* gene is homologous to the *Salmonella typhimurium opdA* gene (Conlin *et al.*, 1992). OpdA cleaves medium sized peptides generated by ATP-dependent proteases (Jain & Chan, 2007). Its induction in *Azoarcus* might result in changes of the protein composition under these conditions.

Several membrane proteins, conserved hypothetical and hypothetical secreted proteins have been identified in strain BH72 specifically induced under co-culture growth conditions (Battistoni et al., 2009). The function of these proteins still needs to be elucidated. Interestingly, the gene products of two genes probably located in one operon have been differentially regulated (locus tags: azo0244; encoding a membrane fusion protein, azo0246;

encoding a probable outer membrane efflux protein). In addition, a putative peptidoglycan associated lipoprotein – gene product of *pal* – was down-regulated in diazosome containing cells of the wild-type. Pal is anchored to the outer membrane through an N-terminal lipid attachment and was believed to stabilize the outer membrane by providing a noncovalent link to the peptidoglycan layer (Parsons *et al.*, 2006). The differential regulation of membrane proteins in cells containing diazosomes might display that these proteins play a role in the formation or function of these specialized structures. The gene azo2696, coding for a conserved hypothetical protein, has been also induced specifically in wild-type cells under co-culture growth conditions. The chromosomal organization suggested that this gene is located in an operon with *fliC2*, a gene coding for flagellin. Since it is well known that bacterial flagella despite of their role in bacterial motility are also involved in diverse processes like biofilm formation or host microbe interaction, the induction of the gene product of azo2696 might indicate that this protein is implicated in the bacterium-fungus interaction.

Nitrogen fixation and metabolism in *Azoarcus* sp. BH72 as an endophyte of grasses

As demonstrated in this work, the protein composition of co-cultured cells of *Azoarcus* sp. BH72 had changed significantly indicating a specialized metabolism under the influence of the fungus. As depicted before, growth of *Azoarcus* sp. BH72 in co-culture with the fungus isolate 2003 might reflect more precisely the conditions of its natural habitat and might serve as a model system for studying the *Azoarcus* lifestyle. In comparison to the well studied nodule symbiosis, so far not much is known about the characteristics of endophytic interactions. However, it is possible that some features, in particular regulation of the nitrogen metabolism and energy generation, might be controlled differentially in this bacterium in comparison to free-living organisms and that some aspects might resemble characteristics of symbiotic interactions. In general, the *S. meliloti* bacteroid metabolism is characterized by the absence of ammonium assimilatory proteins, a substantial change in the amino acid transport, a shutdown of the protein synthesis machinery as well as of polyhydroxybutyrate (PHB) synthesis and an induction of some stress response related proteins (Djordjevic, 2004). In agreement to this, the overall metabolism of *Azoarcus* sp. BH72 was shown to be down-regulated under co-culture growth conditions, while the nitrogenase content of the cell has been stable, ensuring a constant ammonium production. Since diazosome-containing cells were proposed to be arrested in growth, the continual ammonium production implies that the

produced nitrogen source might in addition be assimilated by the partner organism. Unlike in *S. meliloti* bacteroides (Djordjevic, 2004), proteins involved in ammonium assimilation like glutamine synthethase (GlnA) were not down-regulated, when *Azoarcus* sp. BH72 was grown in co-culture. Surprisingly, even the alpha subunit of the urease complex that catalyzes the hydrolysis of urea to ammonium and carbon dioxide was present under co-culture conditions. No known ammonium transporters could be detected during the proteome studies. However, in *Rhizobium etli* AmtB was down-regulated during bacterioid differentiation (Taté *et al.*, 1998). In fact, in bacteroids ammonium assimilation to alanine takes place and amino acid transport is essential for effective nitrogen fixation in nodules (White *et al.*, 2007). This might be an explanation why ammonium assimilation during co-culture growth of *Azoarcus* sp. BH72 occurs, although cells are probably not dividing. A considerable change in amino acid transporters could not be detected in co-cultured cells of *Azoarcus* sp. BH72 probably because these proteins are generally difficult to identify by proteomics, but some proteins involved in amino acid synthesis were differentially regulated suggesting that the amino acid metabolism plays an important role in the bacterium-fungus interaction. In agreement to results from symbionts co-culture growth seems to be a stressful event for *Azoarcus* sp. BH72 as some proteins involved in detoxification were up-regulated in presence of the fungus. It is generally accepted that dicarboxylic acids and not sugars are transported to bacteroids in order to drive nitrogen fixation (White et al., 2007). In contrast, in *Azoarcus* sp. BH72 it seems that pyruvate production from alcohols such as ethanol is important for the fungus interaction.

A constant nitrogen fixation reaction must be maintained by a steady respiration process. As proposed a distinct set of cytochromes established by cytochrome biogenesis proteins like CycH and CcmA might be involved in such a specialized respiration reaction, which can be important for *Azoarcus* sp. BH72 and the bacterium-fungus or even bacterium-plant interaction.

In summary, the proteomic analysis revealed many differences and some similarities of the *Azoarcus* – fungus interaction to characteristics of the well studied root nodule symbiosis.

GENERAL CONCLUSIONS AND OUTLOOK

The work presented here addresses the posttranslational regulation of nitrogenase in the endophytic bacterium *Azoarcus* sp. BH72 and deals with the relationship of the specialized protein composition during co-culture growth on the *Azoarcus* metabolism.

The characterization of the DraT/DraG-system for posttranslational regulation of nitrogenase in *Azoarcus* sp. BH72 was found to be functionally conserved in this betaproteobacterium. DraT-dependent nitrogenase inactivation occurred through ADP-ribosylation of arginine residue 102. Covalent modification of Fe-protein was triggered by environmental signals like nitrogen excess or oxygen deprivation. The reversibility of the process was mediated by mainly DraG1, acting as dinitrogenase reductase activating glycohydrolase. The role of DraG2 was presumed to be different. To gain more information on the function of this protein, it would be interesting to perform a proteomic approach combining immunological and mass spectrometric analyses specific for the detection of ADP-ribosylations on proteins. This could help to detect putative other targets that are regulated by reversible ADP-ribosylation.

Although many similarities to the posttranslational regulation system of the well-studied alphaproteobacterial systems could be depicted, some aspects with respect to regulation deviated in this endophytic betaproteobacterium. The physiological, DraT-independent ammonium induced nitrogenase "switch-off" mechanism seems to be more important for the regulation of nitrogenase activity, while the DraT/DraG-machinery probably operates as a backup system. Even the "switch-on"-mechanism was only partially dependent on DraG1 as after addition of switch-off effectors a residual cellular pool of unmodified dinitrogenase reductase dimers were available. The requirement of *Azoarcus* sp. BH72 for a fast activation of nitrogenase which – if necessary – occurs via protein *de-novo* synthesis might be a typical requisite of the endophytic lifestyle of this bacterium. The functionality of the DraT-independent switch-off could not be clarified so far, but a blockage of the electron transport to nitrogenase is most likely involved in this process. In order to validate this model, carrying out protein interaction studies of the component proteins might be beneficial. In addition, some arguments speak for an implication of the ammonium transporters of the Amt/Rh-family as sensory proteins.

Preliminary results indicated that DcrH1 probably protects nitrogenase from oxygen damage. However, it is necessary to generate a mutant of the *dcrH1* gene to ascertain that the

observed effects were due to the absence of this protein. Spectrometric analyses might assist in providing evidence that DcrH1 is a hemerythrin that can bind oxygen.

Adopting a comparative proteomic approach on diazosome containing and standard nitrogen-fixing cells of *Azoarcus* sp. BH72, proteins probably involved in diazosome formation, function or playing a role in the bacterium-fungus interaction could be identified. These proteins are interesting candidates for mutagenesis and functional analyses. The induction of proteins involved in electron transport or redox-reactions under co-culture conditions indicates that these processes play an important role in diazosome containing cells as well. A diazosome specific electron transport system might go along with an economic nitrogen fixation reaction under these conditions, with cells containing probably the active nitrogenase enzyme in close association to these structures. The adaptation of the *Azoarcus* metabolism during co-culture growth with a fungus isolate strain 2003 towards fungal exudates clarifies the importance of this interaction in the *Azoarcus* ecology.

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CHAPTER
1A

Mass spectrometric characterization of the covalent modification of the nitrogenase Fe-protein in *Azoarcus* sp. BH72

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- Majority of experiments:
  - Experimental design and development
  - Bacterial cell growth
  - Construction of mutants by site-directed mutagenesis and analysis
  - Two dimensional gel electrophoresis
  - Endoproteolytic digestion of NifH and peptide extraction
  - MALDI-TOF experiments and MALDI-TOF data analysis
- Writing of the manuscript except for the ESI-LC-MS part

Contribution S. Rexroth:

- ESI-LC-MS analysis and data processing
- Writing of the ESI-LC-MS part of the manuscript

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Mass spectrometric characterization of the covalent modification of the nitrogenase Fe-protein in *Azoarcus* sp. BH72

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Nitrogenase Fe-protein modification was analyzed in the endophytic β-proteobacterium *Azoarcus* sp. BH72. Application of modern MS techniques localized the modification in the peptide sequence and revealed it to be an ADP-ribosylation on Arg102 of one subunit of nitrogenase Fe-protein. A double digest with trypsin and endoproteinase Asp-N was necessary to obtain an analyzable peptide because the modification blocked the trypsin cleavage site at this residue. Furthermore, a peptide extraction protocol without trifluoroacetic acid was crucial to acquire the modified peptide, indicating an acid lability of the ADP-ribosylation. This finding was supported by the presence of a truncated version of the original peptide with Arg102 exchanged by ornithine. Site-directed mutagenesis verified that the ADP-ribosylation occurred on Arg102. With our approach, we were able to localize a labile modification within a large peptide of 31 amino acid residues. The present study provides a method suitable for the identification of so far unknown protein modifications on nitrogenases or other proteins. It represents a new tool for the MS analysis of protein mono-ADP-ribosylations.

Catalyzing the reduction approximately 300×10^{12} g nitrogen to ammonia per year, nitrogenase is one of the most abundant enzymes in the biosphere [1,2]. It consists of the Fe-protein (dinitrogenase reductase, also referred to as NifH), an α_2 dimer of the *nifH* gene product and of the MoFe-protein (dinitrogenase) with an $\alpha_2\beta_2$ symmetry [3]. ADP-ribosylation of a specific arginine residue of one subunit of dinitrogenase reductase represents one mechanism to inactivate the enzyme [4]. By this means, diazotrophic bacteria can rapidly adapt their metabolic demand to changing environmental conditions, such as energy depletion or nitrogen sufficiency [5–9]. A well-studied example for this post-translational modification is the NifH specific ADP-ribosylation system in the photosynthetic purple bacterium *Rhodospirillum rubrum*, although this system also operates in other members of the α-*Proteobacteria*. In the case of *R. rubrum* and *Rhodobacter capsulatus*, it has been demonstrated that the modifying group is an ADP-ribose moiety on amino acid residue Arg101 or Arg102 (R102), respectively [10,11]. The method applied by Pope *et al.* [10] involved Fe-protein purification, the preparation and purification of a modified hexapeptide or tripeptide, and structural analysis by NMR and MS.

The ADP-ribosyltransferase was identified as dinitrogenase reductase ADP-ribosyltransferase (DraT) in *R. rubrum* [12] and the respective ribosylhydrolase as dinitrogenase reductase activating glycohydrolase (DraG) [13,14]. This system has been studied in

Abbreviations
ACN, acetonitrile; CBB, Coomassie brilliant blue; DraG, dinitrogenase reductase activating glycohydrolase; DraT, dinitrogenase reductase ADP-ribosyltransferase; TFA, trifluoroacetic acid.

various α-*Proteobacteria* [7–9,15,16], both physiologically and by analysis of knockout or deletion mutants, showing that the nitrogenase Fe-protein modification leads to the inactivation of the enzyme and, vice versa, demodification leads to activation.

Recently, we demonstrated that a post-translational modification system also occurs in the β-proteobacterium *Azoarcus* sp. BH72 [17]. This model endophyte of grasses was originally isolated from Kallar grass [18,19]. It is able to express the *nif*-genes in roots of rice [20] and Kallar grass [21], provides fixed nitrogen to its host plant [22], and is thus an interesting candidate for studies of the nitrogenase regulatory mechanism. Phylogenetic analysis indicated that the system for the post-translational modification of nitrogenase Fe-protein is probably also present in the δ- and γ-subdivision of the *Proteobacteria* [17]; however, it has not yet been analyzed in detail outside the α-subdivision of *Proteobacteria*.

Studies have indicated other types of post-translational modifications on nitrogenase that do not necessarily lead to the inactivation of the enzyme. Gallon *et al*. [23] proposed a palmitoylation of both dimers of nitrogenase Fe-protein in the cyanobacterium *Gloeothece*. In addition, *Anabaena variabilis* Fe-protein modification was assumed to deviate from ADP-ribosylation [24]. Migration differences of the NifH protein during SDS/PAGE (i.e. indicating a post-translational modification) were also observed in the diazotrophic bacterium *Azospirillum amazonense* [16,25]. In this case, both forms were active *in vitro*, and no *draT* homolog could be detected by Southern hybridization, suggesting another type of modification.

Protein inactivation by APD-ribosylation is widespread among all domains of life. Examples for mono-ADP-ribosyltransferase reactions occur in *Archaea* [26], prokaryotes, eukaryotes, and even viruses, most likely as a result of horizontal gene transfer [27]. Other examples of prokaryotic ADP-ribosyltransferases are the bacterial toxins, such as *Clostridium botulimum* C2 and C3 or *Pseudomonas aeroginosa* ExoS [28]. In eukaryotes, mono-ADP-ribosyltransferase reactions are involved in important cellular processes, with substrates such as heterotrimeric G proteins, integrin, histones, and even DNA, as a regulatory process [27]. Detection of ADP-ribosylation on proteins is often accomplished by radioactive labeling of the donor molecule NAD^+ and autoradiography. A protocol for the immunological detection of ADP-ribosylated proteins via ethenoNAD has been described elsewhere [29].

In the present study, we present a fast and nonradioactive proteomic approach involving MS techniques, which allowed the identification of the arginine-specific ADP-ribosylation on the nitrogenase Fe-protein in the β-proteobacterium *Azoarcus* sp. strain BH72. Our approach involved 2D gel electrophoresis, an optimized peptide-extraction protocol to retain the labile ADP-ribosylation, and MALDI-TOF MS or tandem MS (LC-MS/MS). Moreover, the present study provides the technical basis for the identification of so far unknown post-translational modifications on nitrogenase Fe-proteins or other proteins.

Results and Discussion

Site-directed mutagenesis of the target arginine of dinitrogenase reductase

An indication for the covalent modification of one subunit of dinitrogenase reductase in *Azoarcus* sp. BH72 has already been observed by SDS/PAGE and western blotting, where a protein of lower electrophoretic mobility was detected [30,31]. Treatments with phosphodiesterase I or neutral hydroxylamine resulted in the disappearance of the modified form, indicating an arginine-specific ADP-ribosylation [31]. Recently, we showed that Fe-protein modification in *Azoarcus* was dependent on DraT [17], as in other bacteria such as *R. rubrum* [6,7], *Azospirillum brasilense* [5], *Azospirillum lipoferum* [5,16,32] or *R. capsulatus* [8], where the system for the post-translational modification of nitrogenase is well studied. DraT was shown to catalyze ADP-ribosylation of nitrogenase Fe-protein on a specific arginine residue in these bacteria. This suggested that nitrogenase Fe-protein was modified by ADP-ribosylation of R102 also in *Azoarcus* sp. BH72. Further support was obtained by site-directed mutagenesis of the target arginine of dinitrogenase reductase in *Azoarcus* sp. BH72. In an *Azoarcus* point mutation strain BHnifH_R102A, no modified NifH protein was observed during a western blot analysis of total protein extracts after induction of Fe-protein modification by the addition of 2 mM ammonium chloride to nitrogen fixing cells, in contrast to wild-type strain BH72 (Fig. 1). The exchange of R102 by alanine led to a shift of the protein during SDS/PAGE, which was observed previously in *R. capsulatus* [33].

Optimization of protein processing for MS analysis of the modified peptide

Because modern state-of-the-art MS techniques provide currently the best tool for a direct proof of a post-translational modification, we investigated both *Azoarcus* sp. BH72 dinitrogenase reductase isoforms by MS. Therefore, total protein from nitrogen fixing

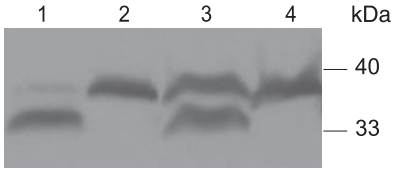


Fig. 1. Effect of site-directed mutagenesis of the target arginine residue R102 on modification of the NifH protein. Western blot analysis of *Azoarcus* wild-type strain BH72 (lanes 1 and 3) and isogenic point mutation strain BHnifH_R102A (lanes 2 and 4) using antiserum against the *Azoarcus* NifH-protein under nitrogen fixation conditions without (lanes 1 and 2) and after induction of NifH-protein modification by incubation with 2 mM NH_4Cl for 20 min (lanes 3 and 4).

cells treated with 2 mM ammonium chloride was separated by 2D gel electrophoresis. Proteins were stained with Coomassie brilliant blue (CBB) R-250 (Fig. 2, upper left panel), Fe-protein specific spots were excised and analyzed by MALDI-TOF MS; however, initial attempts using standard methods were not successful. Unexpectedly, an ADP-ribosylation specific shift of $[M+H]^+$ 541 *m/z* of the tryptic peptide 87–102 could not be observed by trypsin in-gel digestion and MALDI-TOF analysis (data not shown). However, NifH (accession number AAG35586 in the NCBI non-redundant database) could be identified by mass finger prints using the PROFOUND search engine (National Center for Research Resources, The Rockefeller University, New York, NY, USA) with a coverage of 40% and an *E*-value of 2.5×10^{-3}. Eight matching peptides assigned to the *Azoarcus* sp. BH72 NifH protein out of fourteen could be detected. As already discussed [34], the modification of R102 would block trypsin cleavage at this position and hence result in a peptide of > 6000 Da. Because peptides of this size are generally difficult to analyze by MS [35], we choose to perform double digestions of the NifH protein with trypsin and endoproteinase Asp-N. A peak of 3764.5 *m/z* corresponding to the ADP-ribosylated peptide 87–117 could not be observed in MALDI-TOF

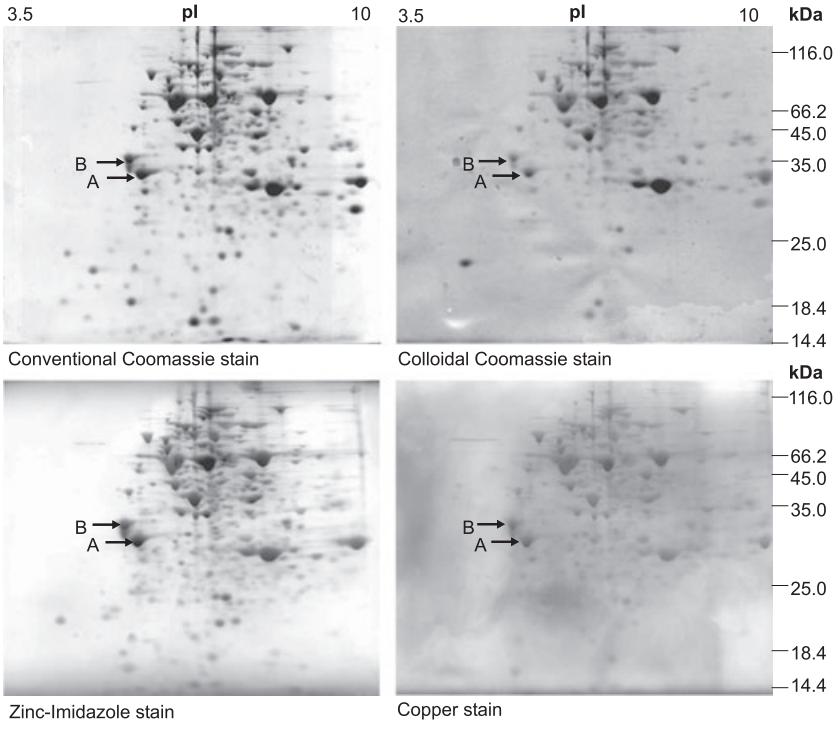
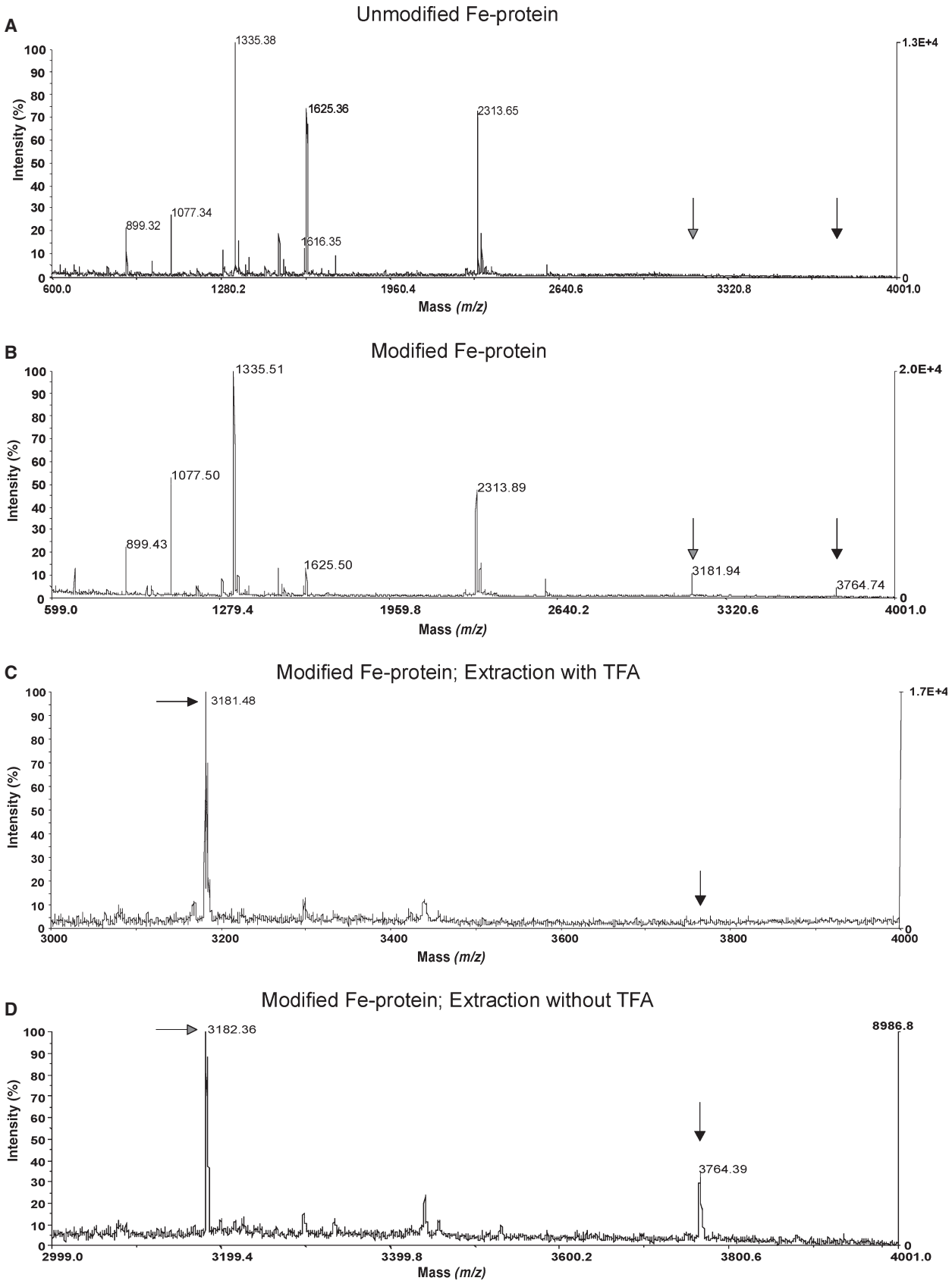


Fig. 2. Comparison of different protein staining methods conducted on 2D PAGE gels as indicated. Total protein (600 μg) was initially loaded onto IEF tube gels for each experimental condition. Spots containing nitrogenase Fe-protein are marked by arrows. A, Unmodified Fe-protein; B, modified Fe-protein.

Fig. 3. Analysis of both nitrogenase Fe-protein isoforms from conventional Coomassie stained SDS/PAGE gels by MALDI-TOF MS. MALDI-TOF spectrum of the unmodified Fe-protein (A) compared to the modified form (B). Peptide extraction was performed in the absence of TFA. A peak corresponding to the ADP-ribosylated peptide 87–117 of theoretically MH^+ 3764.5 *m/z* was only present in spectra of the modified protein (arrow), as well as a peak corresponding to the ornithine variant (open arrow). (C,D) Spectra are shown from the modified Fe-protein, with a detailed view for the mass range 3000–4000 *m/z*. A peak corresponding to the ADP-ribosylated peptide (arrow) is absent in the case of peptide extraction with TFA (C), whereas it is present when peptide extraction is performed without TFA (D). The ornithine species (open arrow) could be detected under both conditions.


A
Unmodified Fe-protein
1335.38
1.3E+4
1625.36
2313.65
1077.34
899.32
1616.35
Intensity (%)
600.0
1280.2
1960.4
2640.6
3320.8
4001.0
Mass (m/z)
B
Modified Fe-protein
1335.51
2.0E+4
1077.50
2313.89
899.43
1625.50
3181.94
3764.74
Intensity (%)
599.0
1279.4
1959.8
2640.2
3320.6
4001.0
Mass (m/z)
C
Modified Fe-protein; Extraction with TFA
3181.48
1.7E+4
Intensity (%)
3000
3200
3400
3600
3800
4000
Mass (m/z)
D
Modified Fe-protein; Extraction without TFA
3182.36
8986.8
3764.39
Intensity (%)
2999.0
3199.4
3399.8
3600.2
3800.6
4001.0
Mass (m/z)

spectra from trypsin and endoproteinase Asp-N digested modified Fe-protein, when peptides had been extracted with 0.1% trifluoroacetic acid (TFA)-containing solutions (Fig. 3C). Because we were considering arginine-specific ADP-ribosylation to be acid labile, we aimed to avoid acid treatments in further experiments.

Already during staining procedures, proteins are often exposed to a very low pH of approximately 1. Therefore, we analyzed four different staining procedures: (a) a conventional Coomassie staining protocol; (b) a colloidal Coomassie staining solution [36]; (c) a zinc-imidazole stain [37]; and (d) a copper stain [38], as well as their impact on the further processing of proteins by MS. In all staining methods, except for the conventional Coomassie stain, the pH was kept nearly neutral. Most protein spots were detectable using a conventional Coomassie staining protocol or the zinc-imidazole stain, respectively, whereas the copper stain and the colloidal Coomassie stain were less sensitive (Fig. 2). In the latter case especially, small proteins were scarcely detectable. This might have been the result of diffusion during overnight staining because proteins were not fixed by this method. However, both nitrogenase Fe-protein isoforms were visible with all staining methods applied [Fig. 2; unmodified Fe-protein (A); modified Fe-protein (B)]. Furthermore, peptide extraction was performed in the absence of TFA to avoid acidic conditions. A peak corresponding to the ADP-ribosylated peptide 87–117 (theoretical monoisotopic mass $[M+H]^+$ 3764.56; observed masses 3764.74 *m/z* in Fig. 3B and 3764.39 *m/z* in Fig. 3D) was only detected in MALDI-TOF spectra of the modified Fe-protein, providing evidence that nitrogenase Fe-protein indeed is modified by ADP-ribosylation, resulting in the observed migration difference during 2D gel electrophoresis.

Another striking difference of the MALDI-TOF spectra from the modified Fe-protein in comparison to the unmodified Fe-protein is the decreased intensity of peak 1625.4 *m/z* and the absence of peak 1616.3 *m/z* (Fig. 3A,B). These peaks correspond to native peptide 87–102 ($[M+H]^+$ 1616.7156 *m/z*) or peptide 103–117 ($[M+H]^+$ 1625.8057 *m/z*), respectively. The decrease of peak 1625.4 *m/z* and absence of peak 1616.3 *m/z* can be explained again by the inability of trypsin to cleave C-terminal to R102 due to the modification at this residue. However, the presence of peak 1625.5 *m/z* in the spectrum of the modified Fe-protein indicated that the ADP-ribose moiety was partially hydrolyzed before trypsin digestion, leading to the cleavage at this site.

The staining procedure did not have an effect on the presence of the ADP-ribosylated peptide during MALDI-TOF analysis because it was detectable under all studied conditions. Even after conventional Coomassie staining in the presence of acetic acid, the modified peptide could be retrieved. However, analysis of modified Fe-protein electroeluted from excised spots from conventional Coomassie stained 2D gels suggested lability. Both forms were detected by SDS/PAGE analysis, indicating hydrolysis of the modification under these conditions even in the absence of TFA (see Supporting information, Fig. S1 and Doc. S1). The LC liquid phase which contained formic acid still allowed the detection of the ADP-ribosylation. Cervantes-Laurean *et al.* [39] reported a half-time of more than 10 h for ADP-ribose linked to arginine in 44% formic acid. However, the detection of the ornithine variant during LC-ESI-MS analysis indicated a partial hydrolysis under these conditions. The strong effect of TFA on the arginine-specific ADP-ribosylation might be caused by the high degree of acidity of this acid with its pK_a value of 0.26 compared to the other acids used in the present study.

Characterization of the covalently modified peptide by tandem MS analysis

To demonstrate that peak 3764 *m/z* indeed represented the ADP-ribosylated peptide 87–117 with R102 as the modified residue, we performed tandem MS analysis (LC-ESI-MS/MS) on trypsin/endoproteinase Asp-N double digested modified Fe-protein. Applying C18 LC-MS/MS analysis to the peptide sample and performing a database search using the SEQUEST algorithm [40] for protein identification resulted in an unambiguous identification of the nitrogenase Fe-protein; the sequence coverage was 74% with more than 6000 independent MS/MS spectra of the LC-MS run being assigned to this protein by the SEQUEST algorithm, when peptide matches were limited to $P > 10^{-4}$ and a mass accuracy below 5 p.p.m. Only two minor contaminants, the selenophosphat synthetase and the phosphoribosylaminoimidazole synthetase, have been detected within the sample. Only 24 MS/MS spectra could be assigned to theses contaminations.

Applying the mass shift for the ADP-ribosylation of 541.06 *m/z* as a predefined differential mass shift for arginine, two peptides, CVESGGPEPGVGCAGR*GVITAINFLEEEGAY and CVESGGPEPGVGCAGR*GVIT, displaying the ADP-ribosylation on R102 were identified by LC-MS/MS analysis. In total during the LC-MS run, 18 MS/MS spectra of triply charged parent ions have been assigned to these peptides with *P*-values of approximately 10^{-8} and mass accuracies of

2 p.p.m. The observed mass shift of 541 *m/z* cannot be explained by any combination of amino acids adjacent to these peptides, nor has this mass shift been observed for any other arginine residue within the sample.

Figure 4 displays a LC-ESI-MS/MS spectrum assigned to the ADP-ribosylated peptide with the complex fragmentation pattern typical for triply charged ions. All significant signals in the spectrum can be assigned to singly and doubly charged ions of the b- and y-ion series, as well as to fragmentation of the post-translational modification. The most intense signal in the spectrum is a loss of 134 Da, corresponding to the dissociation of the adenosyl-residue at the post-translational modification.

Although the unmodified variant of the peptide lacking the post-translational modification was generally not detectable using our approach as a result of cleavage at the unmodified argine residue, a species of the peptide with a substitution of the arginine by ornithine with the theoretical monoisotopic mass $[M+H]^+$ of 3181.4816 *m/z* was observed by LC-ESI-MS. A peak corresponding to this ornithine-substituted peptide has been also observed in MALDI-TOF spectra (Fig. 3B,C,D, open arrow). This variant is probably attributed to the end product of an *ex vivo* decay of the ADP-ribosylation and its presence again demonstrated the lability of the arginine-specific ADP-ribosylation. Applying LC-ESI-MS, the ornithine and the ADP-ribosylated species, which were eluted at retention times of 56.3 and 62 min, respectively, were used to determine the accurate mass shift of the post-translational modification with high mass-accuracy from the FT-MS spectra of the parent ions. The masses for the triply charged parent ions for the ADP-ribosylated or the ornithine substituted species were observed at 1255.531 *m/z* and 1061.169 *m/z*, respectively. The observed mass difference for these two peptides of 583.084 Da was within 1.8 p.p.m. of the calculated mass difference.

In summary, our MS approach led to the unequivocal detection of the ADP-ribosylation on Arg102 in the *Azoarcus* sp. BH72 Fe-protein. Taken together with the results of our previous study [17], the data indicate that DraT catalyzes the ADP-ribosylation reaction in this β-proteobacterium on one subunit of the nitrogenase Fe-protein, leading to the inactivation of the enzyme. Thus, the results obtained in the present study extend our knowledge of the nitrogenase post-translational modification system outside of the α-class to other members of the *Proteobacteria*.

Conclusion

The analysis of post-translational modifications on proteins still represents a challenging task, especially in the case of labile covalent modifications, as shown in the present study for arginine-specific ADP-ribosylations. Although we were unable to demonstrate that different staining methods are crucial for the detection of this modification, it might be helpful for the investigation of other labile modifications (e.g. phosphorylations). In the present study, we demonstrated that TFA-treatments should be omitted during MS examination of arginine-specific ADP-ribosylations. Our

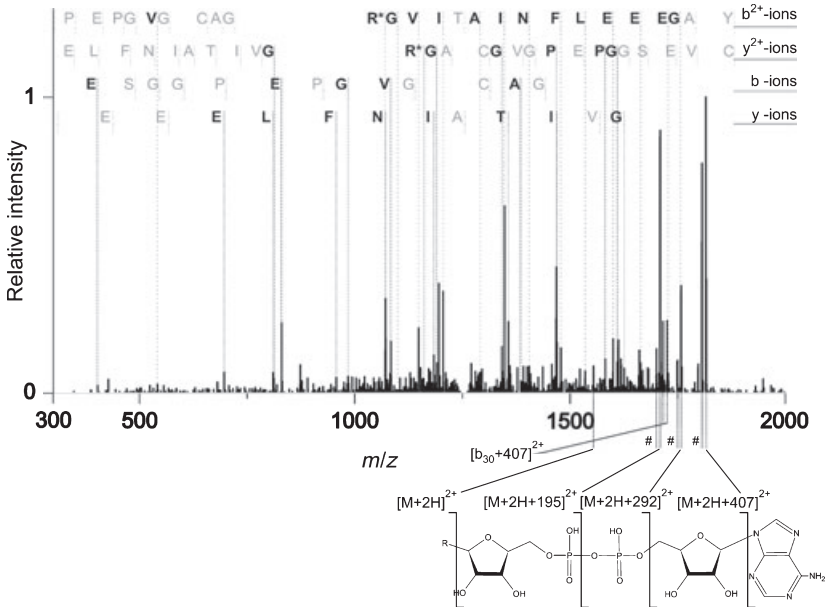


Fig. 4. Tandem MS analysis of the triply charged precursor ion $[M + 3H]^{+3}$ 1255.5 *m/z* by LC-ESI-MS/MS. The MS/MS spectrum is shown for the modified peptide, CVESGGPEPGVGCAGR*GVITAINFLEEE-GAY. R*, ADP-ribosylated Arg102, with a mass shift of 541.06 *m/z*. Signals from the singly and doubly charged b- and y-ion series, as well as ions from the fragmentation of the post-translational modification, are indicated. The range of detection is limited to 300–2000 *m/z* by the ion trap used.

study describes a valuable method by which protein (mono)-ADP-ribosylations can be analyzed using 2D gel electrophoresis and MS. In addition, the approach employed might be effective for the analysis of other types of modifications on nitrogenase Fe-proteins. Probably, it also provides a new method for the investigation of other labile modifications on proteins.

Experimental procedures

Bacterial strains, media and growth conditions

Azoarcus sp. BH72 was grown under conditions of nitrogen fixation in an oxygen-controlled bioreactor (Biostat B; B. Braun Melsungen AG, Melsungen, Germany) [41] in N-free SM-medium [18] at 37 °C, stirring at 600 r.p.m., and an oxygen concentration of 0.6%. Cells were harvested when D_{578} of 0.8 was reached. To induce nitrogenase Fe-protein modification, cells were supplemented with 2 mM ammonium chloride 15 min prior to harvesting. Cells were collected by centrifugation and washed with NaCl/Pi at 4 °C, and aliquots of approximately 150 mg were stored at −80 °C until further processing. For western blot analysis of the R102A point mutant and wild-type strain, bacteria were grown microaerobically in 100 mL SM-medium containing 5 mM glutamate in 1 L rubber stopper-sealed Erlenmeyer flasks with rotary shaking at 150 r.p.m. and 37 °C. Before the addition of 2 mM NH_4Cl, 2 mL of culture was processed by SDS extraction. After 20 min of incubation with NH_4Cl, cells were harvested and total protein was extracted by SDS extraction.

DNA analysis and site-directed mutagenesis

Chromosomal DNA was isolated as described previously [42]. Additional DNA techniques were carried out in accordance with standard protocols [43]. For construction of an Arg102 point mutation of NifH, plasmid pEN322d, a derivative of pEN322 [20] containing a *Hinc*II-fragment of the *Azoarcus* sp. BH72 *nifH* gene, was used. By amplification with *pfuTurbo*® DNA polymerase (Stratagene Europe, Amsterdam, the Netherlands) using the sense primer MutNifHR102A (5′-GGCGTCGGCTGCGCCGGC**GCC**GGC GTTATCACCGCCATCAACTT-3′) and the antisense primer MutNifHR102A-r (5′-AAGTTGATGGCGGTGAT AACGCC**GGC**GCCGGCGCAGCCGACGCC-3′), the original codon for R102 'CGT' was exchanged to 'GCC' (primer sequences shown in bold). A *Btg*I restriction site was thereby eliminated. After amplification, parental DNA was digested with *Dpn*I [44] for 1 h at 37 °C. Mutated plasmid DNA was transformed into *Escherichia coli* DH5αF′ and the success of mutation was verified by *Btg*I digestion and sequencing. The *Hinc*II/*Eco*RI-fragment of the mutated *nifH* (bp 53–814) was subcloned into pK18mobsacB [45], resulting in pK18_R102A. Conjugation into *Azoarcus* was carried out by triparental mating, and sucrose selection after recombination carried out according to the method previously described by Schäfer *et al*. [45]. Genomic DNA of the mutant strain BHnifH_R102A was analyzed by PCR of *nifH* using primers Z114 and Z307 [46] and *Btg*I-digestion.

Protein extraction

For 2D gel electrophoresis, total protein was extracted essentially as described previously [47]. Cells of approximately 150 mg fresh weight were resuspended in 700 μL of extraction buffer [0.7 M sucrose, 0.5 M Tris, 30 mM HCl, 0.1 M KCl, 2% (v/v) 2-mercaptoethanol]. Cell disruption was carried out by sonication (4 × 45 s with 50 W output and 60 s breaks on ice using a Branson sonifier 250; Branson, Danbury, CT, USA). Phenylmethanesulfonyl fluoride was added to a final concentration of 0.5 mM. Cells were incubated on ice for 30 min. Then, cell debris was removed by centrifugation (16 200 ***g*** for 5 min at 4 °C) and proteins were extracted with Tris Cl-buffered phenol (pH 8.0), precipitated and resuspended in 700 μL of 2D sample solution as described previously [47]. Determination of protein concentration was carried out using the RC DC protein assay (Bio-Rad, Hercules, California, USA) according to manufacturer's instructions. SDS extraction of proteins for SDS/PAGE and western blotting was performed as described previously [48].

Electrophoresis and western blotting

SDS/PAGE and western blotting were carried out as described previously [17]. IEF for 2D gel electrophoresis was essentially performed as described previously [30] but in glass tubes with an inner diameter of 2.5 mm. Gels contained 3.5% acryl-bisacrylamide (30 : 1), 7.1 M urea, 1.6% Chaps, 2.5% ampholytes 4–6, 1.25% ampholytes 5–8 and 1.25% ampholytes 3–10 (Serva, Heidelberg, Germany). Total protein (600 μg) was loaded on top of the IEF gels. Before conducting the second dimension, extruded IEF gels were equilibrated for 30 min in 60 mM Tris Cl, pH 6.8, 1% SDS, 20% glycerol and 50 mM dithiothreitol. Vertical gel electrophoresis in 13 × 16 cm SDS/PAGE gels was carried out with a 10% (w/v) polyacrylamide gel as described previously by Laemmli [49].

Gel staining and processing

Conventional CBB staining was performed using standard conditions. The staining solution contained 45% (v/v) ethanol, 9% (v/v) acetic acid and 0.25% (w/v) CBB R-250. Destaining was carried out using a solution of 30% (v/v) ethanol and 10% (v/v) acetic acid. Gels were stored in

18% (v/v) ethanol, 3% glycerol (v/v). Colloidal Coomassie staining was performed as described by Candiano *et al.* [36], except that the staining solution was titrated with 25% ammonium hydroxide to a pH of 7.0. When staining was completed, gels were washed with distilled H_2O and, if necessary, destained using protein storage solution. Copper staining or zinc-imidazole staining was performed exactly as described previously [37,38]. For documentation, gels were scanned at 600 dots per inch on a UMAX Power Look III scanner (UMAX, Data Systems, Inc., Taipei, Taiwan). Dinitrogenase reductase-containing protein spots were excised with a clean, sharp scalpel, 1 day after staining of the gels at the latest, and were stored at 4 °C. Pieces of approximately 1 mm^3 were stored in 1.5 mL Protein LoBind Tubes (Eppendorf, Hamburg, Germany) at −80 °C until in-gel digestion.

In-gel digestion and peptide extraction

Protein-containing gel pieces from copper-stained or zinc-imidazole-stained gels, respectively, were washed twice for 8 min in 1 mL of 50 mM Tris buffer, 0.3 M glycine, pH 8.3, containing 30% acetonitrile (ACN) [37]. Gel pieces emerging from all staining techniques were washed, reduced and alkylated using standard conditions [50], with slight modification. Gel pieces were again washed, dehydrated and dried in a vacuum concentrator. Digestion was carried out overnight in trypsin digestion solution containing 5 $ng{\cdot}\mu L^{-1}$ modified sequencing-grade trypsin (Roche, Mannheim, Germany) in 25 mM NH_4HCO_3 at 37 °C. For double digestions, gel pieces were dried in a vacuum centrifuge and dehydrated in digestion solution containing 2 $ng{\cdot}\mu L^{-1}$ endoproteinase Asp-N (Roche) in 50 mM NH_4HCO_3 and incubated overnight at 37 °C. Peptide extraction was performed in the absence of TFA using 50% ACN, 30% ACN, and again 50% successively. Samples were treated for 15 min in a sonication bath to facilitate extraction between each step. Combined peptide extracts were centrifuged to dryness in a vacuum concentrator and stored for no longer than 2 weeks at –20 °C until analysis by MS.

MALDI-TOF analysis

For MALDI-TOF analysis, peptides were resuspended in 10 μL of 50% ACN, diluted 1 : 10 with ultrapure bidest H_2O and mixed with an equal volume of matrix solution containing saturated 2,5-dihydroxybenzoic acid in 100% ACN. Of this solution, 0.5 μL was spotted on a 96 × 2-position, hydrophobic plastic surface plate (Applied Biosystems, Foster City, CA, USA) and dried. Average spectra were acquired with 100 laser shots per spectrum using a Voyager DE-Pro MALDI-TOF mass spectrometer (Applied Biosystems) operated in the reflector mode. Instrument settings were optimized for peptides in the range 2000–3500 Da with a guidewire set to 0.005% and a delay time of 200 ns. Accelerating voltage was set to 20 kV, grid voltage to 74% and the mirror voltage ratio to 1.12. Calibration was performed by acquiring the Peptide Calibration Mix 2 (Applied Biosystems) as an external standard.

LC-MS analysis

Lyophylized peptide samples were dissolved in 50 μL of buffer A (95% H_2O, 5% ACN, 0.1% formic acid) and analyzed on a 15 cm analytical C18 column [inner diameter 100 μm, Phenomenex Luna (Phenomenex, Torrance, CA, USA), 3 μm, C18(2), 100 Å], which had been pulled to a 5 μm emitter tip. For reverse phase chromatography, a gradient of 120 min from buffer A (95% H_2O, 5% ACN, 0.1% formic acid) to buffer B (10% H_2O, 85% ACN, 5% isopropanol, 0.1% formic acid) was used with a flow rate split to 200 $nL{\cdot}min^{-1}$ (Thermo Accela; Thermo Fisher Scientific Inc., Waltham, MA, USA), resulting in a peak capacity of approximately 130. For MS analysis, a Thermo LTQ Orbitrap mass spectrometer was operated in a duty cycle consisting of one 300–2000 *m/z* FT-MS and four MS/MS LTQ scans.

Data analysis

For analysis of the LC-MS/MS data, the SEQUEST algorithm [40] implemented in the BIOWORKS 3.3.1 software (Thermo Fisher Scientific) was applied for peptide identification versus a database, consisting of all 3989 proteins listed in the NCBI database for *Azoarcus* sp. BH72, using a mass tolerance of 10 p.p.m. for the precursor-ion and 1 amu for the fragment-ions, no enzyme specificity for the cleavage, and acrylamide modified cysteins as fixed modification. For detection of modified peptides a potential arginine modification of 541.0611 *m/z* was used as a parameter during the search.

MALDI-TOF raw data were processed with the DATA EXPLORER software (Applied Biosystems). A peak list for peptide mass fingerprints was prepared after baseline correction, noise filtering (correlation factor = 0.7) and de-isotoping. For protein identification, the NCBI nonredundant database was searched with peptide mass finger prints using the PROFOUND search engine (National Center for Research Resources). Complete modification was set to acrylamide-modified cysteins, and methionine oxidation was used as partial modification. Charge state was fixed to MH + and the mass tolerance for monoisotopic masses was fixed to 0.05%. All other parameters were set as predetermined.

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Supporting information

The following supplementary material is available:
Fig. S1. Effect of conventional Coomassie staining on the Fe-protein ADP-ribosylation.
Doc. S1. Electroelution of proteins from acrylamide gels.

This supplementary material can be found in the online version of this article.

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SUPPLEMENTAL MATERIAL

Additional experimental procedures

Electroelution of proteins from acylamide gels

Nitrogenase Fe-protein was eluted from acrylamide gel pieces using the Model 422 Electro Eluter from Biorad (Biorad, Hercules, CA, USA) according to manufacturer's instruction in 50 mM NH_4HCO_3 with 0.1% SDS for 2.5 hours at 10 mA per tube and successively in 50 mM NH_4HCO_3 for 2 hours at room temperature. Before SDS-PAGE analysis the volume of the eluted protein solution was reduced in a vacuum concentrator.

SUPPLEMENTAL DATA

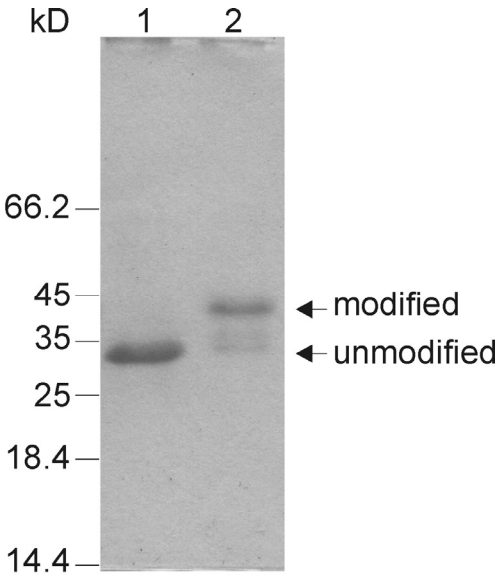

Fig. S1: Effect of conventional Coomassie staining on the Fe-protein ADP-ribosylation. Unmodified (lane 1) and modified (lane 2) Fe-protein containing spots were excised from 2D-gels stained with conventional Coomassie brilliant blue method, electroeluted and separated by SDS-PAGE. Occurrence of a "lower band" in lane 2 with modified Fe-protein indicates that the modification was partially hydrolyzed.

CHAPTER
1B

Cellular localization of the nitrogenase Fe-protein in dependence on its modification and effect of dinitrogenase reductase variants on nitrogen fixation and ADP-ribosylation

Cellular localization of the nitrogenase Fe-protein in dependence on its modification and effect of dinitrogenase reductase variants on nitrogen fixation and ADP-ribosylation

Abstract

Nitrogenase Fe-protein was shown to be partially membrane associated in diazosome-containing cells of *Azoarcus* sp. BH72. In this study, membrane association under standard nitrogen fixation conditions was demonstrated to be dependant on its ADP-ribosylation. Although most of the cellular NifH-protein was found to be located in the cytoplasmic fraction, a relatively high extent was membrane associated when Fe-protein was in its unmodified form. In comparison, the ADP-ribosylated Fe-protein could not be detected in membrane fractions. In addition, it was shown here that the Fe-protein arginine residues 102 and 142 are essential for the nitrogen fixing capability of nitrogenase. Moreover, arginine residue 142 was important for the Fe-protein with respect to its ability in serving as a substrate for ADP-ribosylation. Furthermore, this article describes a nitrogenase Fe-protein enrichment protocol which could be used for the size determination of the unmodified protein by MALDI-TOF MS.

INTRODUCTION

The two component metalloprotein nitrogenase catalyzes the reduction of dinitrogen to ammonia. It consists of the molybdenum-iron protein (dinitrogenase) and the iron-protein (dinitrogenase reductase) (Dean & Jacobson, 1992, Rees *et al.*, 2005). Under ideal condition the overall reaction of nitrogenase can be summarized

$$N_2 + 8e^- + 16MgATP + 8H^+ \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$$

Substrate binding and reduction occurs at the unique molybdenum-iron-sulfur-homocitrate containing cofactor (FeMoCo) of FeMo-protein (Hawkes *et al.*, 1984, Shah & Brill, 1977, Kim & Rees, 1992), which consist of a $\alpha_2\beta_2$-symmetry (Hageman & Burris, 1978, Georgiadis *et al.*, 1992, Burgess & Lowe, 1996, Seefeldt *et al.*, 2004). The MoFe-protein obtains in addition two [8Fe:7S] cluster, termed the P-cluster, which serve as a reservoir of electrons for transfer to FeMoCo during substrate reduction (Kim & Rees, 1992). A single [4Fe:4S] cluster

is ligated between the two subunits of the Fe-protein (Georgiadis et al., 1992). This metalloprotein donates electrons from its reduced [4Fe:4S] cluster to the P-cluster of MoFe-protein from where the electrons are subsequently delivered to FeMoCo (Einsle *et al.*, 2002, Lee *et al.*, 2003, Lovell *et al.*, 2003, Hinnemann & Nørskov, 2003). Each single electron transfer from the Fe-protein docked with the MoFe-protein requires hydrolysis of at least two MgATP molecules (Seefeldt & Dean, 1997). The catalytic cycle demands several rounds of Fe-protein – MoFe-protein association and dissociation, since multiple electrons are necessary for substrate reduction (Fig. 1). Therefore, docking of Fe-protein to MoFe-protein is a critical step in the nitrogen fixation reaction. Two charged amino acid residues – arginine 140 and lysine 143 - were shown to be involved in the docking process in *Azotobacter vinelandii* Fe-protein to MoFe-protein (Seefeldt, 1994). In addition, arginine 140 in *A. vinelandii* was shown to be essential for Fe-protein ADP-ribosylation by dinitrogenase reductase ADP-ribosyltransferase (DraT) from *Rhodospirillum rubrum* (Grunwald *et al.*, 2000).

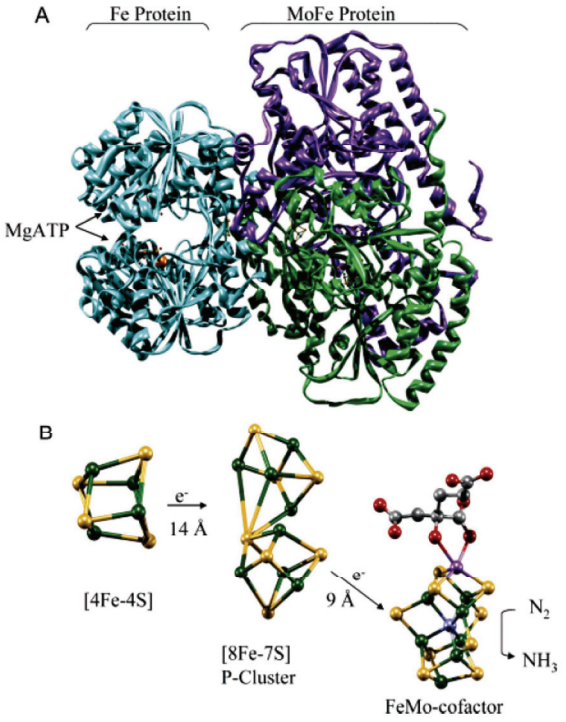

Fig. 1: Nitrogenase Fe-protein and one αβ-unit of the MoFe-protein (A). The Fe-protein (blue), with the two sites for MgATP binding indicated, is docked to one αβ-unit (β-subunit in purple and α-subunit in green) of the MoFe-protein. (B) The flow of electrons from the [4Fe:4S] cluster of Fe-protein to the P-cluster and FeMo-cofacter of MoFe-protein. This illustration was taken from Seefeldt et al., 2004.

ADP-ribosylation of a specific arginine residue of one subunit of nitrogenase Fe-protein is a mechanism to reversibly inactivate this enzyme (Ludden, 1994, Halbleib & Ludden, 2000). This process has been described in molecular detail for a few diazotrophic alphaproteobacteria (Fu *et al.*, 1989, Kanemoto & Ludden, 1984, Liang *et al.*, 1991, Zhang *et al.*, 1993, Masepohl *et al.*, 1993) and the betaproteobacterium *Azoarcus* sp. BH72 (Oetjen & Reinhold-Hurek, 2009), but phylogenetic analysis suggested a more widespread distribution of the enzyme system for posttranslational nitrogenase activity control within the *Proteobacteria* (Oetjen & Reinhold-Hurek, 2009). Previous studies suggested other mechanism than ADP-ribosylation for covalent Fe-protein modification in *Azoarcus* sp. BH72, which specifically occurs in diazosome containing cells of this grass endophyte (Hurek *et al.*, 1995). Under these conditions, nitrogenase was shown to be located to a high extent in the formed membrane stacks - termed diazosomes.

In this study results regarding the influence of the *Azoarcus* sp. BH72 Fe-protein residue arginine 142 on the nitrogen fixation capability and on its function as substrate for ADP-ribosylation are presented. The membrane association of dinitrogenase reductase under standard nitrogen fixation conditions was investigated in dependence on its modification status by ADP-ribosylation. In addition, a Fe-protein enrichment protocol and results from the MALDI-MS analysis of the undigested Fe-protein are documented.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1.

Azoarcus point mutation strains BHnifH_R102A and BHnifH_R142A have been obtained by site-directed mutagenesis. To check the diazotrophic growth ability of the point mutation strains, cells were first precultured in 20 ml SM+N medium containing 10 mM NH_4Cl and 0.1 g/l yeast extract (Reinhold *et al.*, 1986). Cells were washed with nitrogen-free SM-medium and then transferred in semisolid SM medium as described (Reinhold et al., 1986). To obtain nitrogenase Fe-protein of these strains, cells have been grown microaerobically in 100 ml SM medium with 5 mM glutamine as sole nitrogen source in 1 l rubber stopper sealed Erlenmeyer flasks as described before (Martin & Reinhold-Hurek, 2002). For nitrogenase enrichment *Azoarcus* sp. BH72 wild type was grown in a 2 l bioreactor under controlled oxygen conditions as previously described (Oetjen *et al.*, 2009) and shocked by addition of 2 mM NH_4Cl 15 min prior to harvesting.

Table 1: Bacterial strains and plasmids.

Strain or plasmid	Description[1]	Reference
Strains		
Escherichia coli		
DH5αF`	F`*recA1 endA1 hsdR17*(rK$^-$, mK$^+$) *supE44* (λ-thi-1 relA1 ϕ80d*lacZΔM15* Δ(*lacZYA-argF*)U169	Hanahan, 1983
Azoarcus		
BH72	wild-type	Reinhold et al., 1986
BHnifH_R102A	BH72, *nifH* bp 304-306 (CGT) changed to GCC leading to a conversion of arginine residue 102 to alanine.	Oetjen et al. 2009
BHnifH_R142A	BH72, *nifH* bp 424-425 (CG) changed to GC leading to a conversion of arginine residue 142 to alanine	this study
BHΔdraG1ΔdraG2	BH72, Δ*draG1*, in-frame deletion bp 217-654, ΔdraG2, in-frame deletion bp 318-764	Oetjen & Reinhold-Hurek, 2009
BHΔdraT	BH72, Δ*draT*, in-frame deletion bp 33-741	Oetjen & Reinhold-Hurek, 2009
Plasmids		
pBGVN3	Ap^r, 9.0 kb Sau3A1 fragment containing the *nif*-region in pUC19	Hurek et al., 1995
pEN322	Ap^r, EcoR1/HindIII fragment from pBGVN3 carrying 800 bp of the *nifH* gene and 640 bp of the upstream sequences.	Egener et al., 1998
pEN322d	Ap^r, containing a 761 bp *nifH* fragment	†[2]
pK18mobsacB	Km^r, RP4 mob region, containing *sacB* gene	Schäfer et al., 1994
pK18_R102A	Km^r, pK18mobsacB derivate bearing the *nifH* fragment of bp 54-814 with the site-directed mutagenesis of bp 304-306 (CGT) changed to GCC	Oetjen et al., 2009
pK18_R142A	Km^r, pK18mobsacB derivate bearing the *nifH* fragment of bp 54-814 with the site-directed mutagenesis of bp 424-425 (CG) changed to GC	this study
pRK2013	Km^r **,** RK2 *tra* gene cloned in ColE1 replicon	Figurski & Helinski, 1979

[1] Ap^r, Km^r: ampicillin or kanamycin resistence, respectively. †[2] unpublished work Prajwal

DNA analysis and site directed mutagenesis. DNA methods have been carried out following standard protocols (Ausubel *et al.*, 1987). Construction of the *Azoarcus* strain BHnifH_R102A has been described elsewhere (Oetjen et al., 2009). For construction of the arginine142 point mutation in an analogous strategy, plasmid pEN322d, a derivative of pEN322 (Egener *et al.*, 1998) containing a HincII-fragment of the *Azoarcus* sp. BH72 *nifH*-gene, was used for amplification with *pfuTurbo*® DNA polymerase (Stratagene Europe, Amsterdam, The Netherlands) using the sense primer MutNifHR142A (5'-GGCTTCGCGATGCCGATT**GCC**GAAAACAAGGCCCAGGAAATCTA-3') and the antisense primer MutNifHR142A-r (5'-TAGATTTCCTGGGCCTTGTTTTC**GGC**AATCGGCATCGCGAAGCC-3'). The original codon for R142 "CGC" was exchanged to "GCC" (bold in the primer sequences). A HinfI restriction site was thereby eliminated. After amplification parental DNA was digested with DpnI (Nelson and McClelland, 1992) for 1 h at 37°C. Mutated plasmid DNA was transformed into *E. coli* DH5αF' and the success of mutation was verified by HinfI digestion and sequencing. For conjugation, a HincII/EcoRI-fragment of the mutated *nifH* (bp 54-814) was subcloned into pK18mobsacB (Schäfer *et al.*, 1994) resulting in pK18_R142A. Conjugation and

recombination in *Azoarcus* was carried out by triparental mating using an *E. coli* strain bearing plasmid pK18_R142A and the helper strain bearing plasmid pRK2013 and sucrose selection according to Schäfer *et al*. (Schäfer et al., 1994). Genomic DNA of point mutation strain BHnifH_R142A was analyzed by amplification of *nifH* using primers PolF and AQER (Poly *et al.*, 2001, Demba Diallo *et al.*, 2008) and HinfI-digestion.

Protein extraction, fractionation and Fe-protein enrichment. SDS extraction of total proteins for Western blot analysis was described before (Hurek *et al.*, 1994). Membrane and cytoplasmic protein fractionation was carried out as described in Chapter 2B. Nitrogenase Fe-protein enrichment has been performed according to Ludden (Ludden & Burris, 1978) with modifications. Cell lysis of approximately 5 g wet cell paste of *Azoarcus* sp. BH72 wild-type was carried out by incubation for 30 min on ice with 1 mg/ml lysozyme in 50 mM Tris-Cl, pH 7.6 containing 100 mM NaCl and sonication (3x 45 sec with 60 sec breaks on ice). After removal of unbroken cells by centrifugation, cell fractionation was performed by ultracentrifugation at 175 000 xg and 4°C for 60 min. The supernatant was used for polyethylene glycol 6000 (PEG 6000) precipitation with 10% PEG 6000 (w/v) for 2 h at 4°C during end-over-end incubation. After centrifugation at 9000 xg and 4°C for 20 min the supernatant has been processed with 30% PEG 6000 (w/v) over night. The precipitated protein was collected by centrifugation at 9000 xg and 4°C for 30 min and resuspended in 50 mM Tris-Cl, pH 7.6. The protein suspension was passed through a DEAE-cellulose column (1.5 cm diameter x 15 cm) and washed as described (Ludden & Burris, 1978). The brown band containing the Fe-protein was eluted with 400 mM NaCl in 50 mM Tris-Cl, pH 7.6 and concentrated by a second passage through a DEAE-column (0.5 cm diameter x 4 cm). After elution with 400 mM NaCl in 50 mM Tris-Cl, pH 7.6 protein was precipitated over night at -20°C with 0.1 M ammonium acetate in methanol. Hereafter, protein was collected by centrifugation at 3200 xg and 4°C for 10 min, washed two times and the pellet was stored at -20°C until use.

SDS-PAGE, Western blot analysis and 2D gel electrophoresis. To separate the modified and unmodified form of the nitrogenase Fe-protein, SDS-PAGE was carried out in a low crosslinker gels and proteins were blotted onto a polyvinylidene difluoride (PVDF)-membrane as described (Oetjen & Reinhold-Hurek, 2009). For electroelution and MALDI-TOF analysis of the undigested NifH-protein, enriched Fe-protein samples were resuspended in 2D sample buffer and separated by 2D gel electrophoresis as previously described (Oetjen et al., 2009). Approximately four Fe-protein containing spots were excised with a scalpel, pooled, cut in pieces of about 1 mm^3 and stored at -80°C.

Electroelution of proteins from acylamide gels. Nitrogenase Fe-protein was eluted from acrylamide gel pieces using the Model 422 Electro Eluter from Biorad (Biorad, Hercules, CA, USA) according to manufacturer's instruction in 50 mM NH_4HCO_3 with 0.1% SDS for 2.5 hours at 10 mA per tube and successively in 50 mM NH_4HCO_3 for 2 hours at room temperature. The volume of the eluted protein solution was reduced to approximately 100 µl in a vacuum concentrator and Fe-protein was precipitated in 0.1 M ammonium acetate in methanol over night at -20°C. After centrifugation for 30 min at 12000 xg and 4°C, the protein pellet was dried under a stream of nitrogen and resuspended in 10 µl bidest H_20 for MALDI-TOF analysis or in trypsin digestion solution, respectively.

Trypsin In-Solution digestion. In-Solution digestion of electroeluted Fe-protein derived from four spots from 2D polyacrylamide gels with 300 µg of total extracts was carried out over night at 37°C in 30 µl trypsin digestion solution (50 mM NH_4HCO_3 containing 15 ng/µl sequencing grade trypsin, [Roche Applied Science, Indianopolis, IN, USA]).

MALDI-TOF analysis. MALDI-TOF analysis of peptides was performed as described (Oetjen et al., 2009). For analysis of the undigested protein purified nitrogenase Fe-protein in bidest H_2O was mixed 1:1 with matrix solution (2,5-dihydroxybenzoic acid saturated in 100% acetonitrile) and 1 µl was spotted onto a 96 × 2-position, hydrophobic plastic surface plate (Applied Biosystems, Foster City, CA, USA) and dried. The Voyager DE-Pro MALDI-TOF mass spectrometer (Applied Biosystems) was operated in the linear mode and polarity was set to positive. Spectra were acquired with 250 shots per spectrum with an acceleration voltage set to 25 kV, a grid voltage of 93%, a guide wire of 0.3% and a delay time of 600 nsec. One point external calibration was done by acquisition of a bovine serum albumin solution using the doubly protonated species with a mass $[M+2H]^{+2}$ of 33215.5 m/z as standard.

RESULTS AND DISCUSSION

This chapter aims to summarize results obtained during the optimization of methods suitable for the analysis of the *Azoarcus* sp. BH72 Fe-protein modification. In addition, unpublished results regarding Fe-protein modification and Fe-protein localization are discussed.

Fe-protein membrane association. Nitrogenase membrane association was pronounced in cells of *Azoarcus* sp. BH72 containing intracytoplasmatic membrane stacks (Hurek et al., 1995). Fe-protein modification was said to be a prerequisite for diazosome formation and was proposed to be a mechanism for membrane association (Hurek et al., 1995). Therefore membrane association of modified or unmodified Fe-protein derived from a *draG1draG2* double mutant or from a *draT* mutant, respectively, grown in pure culture under standard nitrogen fixation conditions was assessed. Nitrogenase ADP-ribosylation has been induced in strain BHΔdraG1ΔdraG2 by anaerobiosis treatments. With the aim to compare nitrogenase Fe-protein localization, protein derived from the different fractions was analyzed by Western blotting. Protein samples were applied onto the SDS-PAGE gel according to the volume of the cytoplasmic fraction; however, in order to allow detection of the Fe-protein, membrane fractions have been resuspended in a smaller volume leading to a 5x concentration.

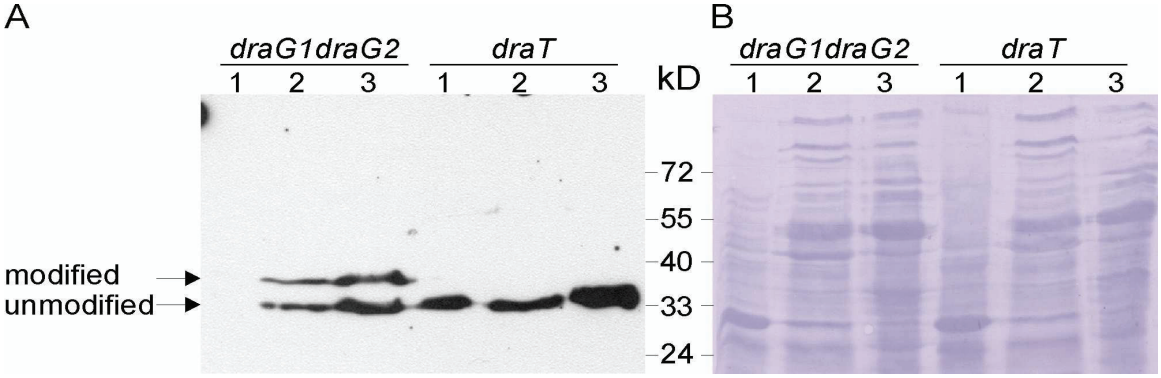

Fig. 2. Cellular localization of the NifH-protein in dependence on the modification status. Western blot with antiserum against the *Azoarcus* nitrogenase Fe-protein (A). Coomassie stained blot from experiment A showing that equal amounts have been loaded in the respective lanes (B). Protein samples derived from a *draG1draG2* double mutant or a *draT*-mutant as indicated to obtain modified or unmodified Fe-protein. Cells were exposed to anaerobiosis and harvested anaerobically. Membrane fraction (1), salt wash fraction (2) and cytoplasmic fraction (3). One representative result out of two repetitions is shown.

The result from the Western blot shows that (i) most of the protein was detected in the cytoplasmic fraction under both tested conditions and (ii) only in strain BHΔdraT the unmodified Fe-protein could be detected in the membrane fraction (Fig. 2). This is in correlation with the observed result seen in wild-type strain BH72 grown under standard nitrogen fixation conditions (Hurek et al., 1995), where the NifH-protein could only be detected in the cytoplasmic fraction. However, it was shown here that Fe-protein ADP-ribosylation – which is a prerequisite for the putative other type of modification in diazosome containing cells of *Azoarcus* (Oetjen & Reinhold-Hurek, 2009) – is probably inhibitory for the membrane association. This would be in agreement with a hypothesis in which the unmodified active nitrogenase is in loose association of the inner leaflet of the cytoplasma membrane, probably in contact to components of the electron and redox equivalents generating respiratory chain.

Role of arginine residues R102 and R142 for Fe-protein function and its modification.

Localization of the modification on R102 in *Azoarcus* sp. BH72 was proven by mass spectrometric examination and was verified by site-directed mutagenesis of the target arginine residue R102 (Oetjen et al., 2009). However, to investigate the influence of arginine residue R142 on the nitrogen fixation capability and on the Fe-protein modification, a point mutant was constructed by site-directed mutagenesis of this residue. Both point mutation strains, *Azoarcus* strain BHnifH_R102A as well as *Azoarcus* strain BHnifH_R142A, were unable to fix nitrogen in batch-culture or in semi-solid agar tubes (Fig. 3). In *Azotobacter vinelandii* it

was concluded that arginine residue 140 - homologous to R142 in the *Azoarcus* sp. BH72 Fe-protein amino acid sequence - is important for the docking of dinitrogenase reductase to dinitrogenase (Seefeldt, 1994). Hence a mutation at this site, as already concluded for R101 in *R. rubrum* (see above), prevents electron transport to FeMo-protein. This would explain why the corresponding mutants in *Azoarcus* were unable fix nitrogen.

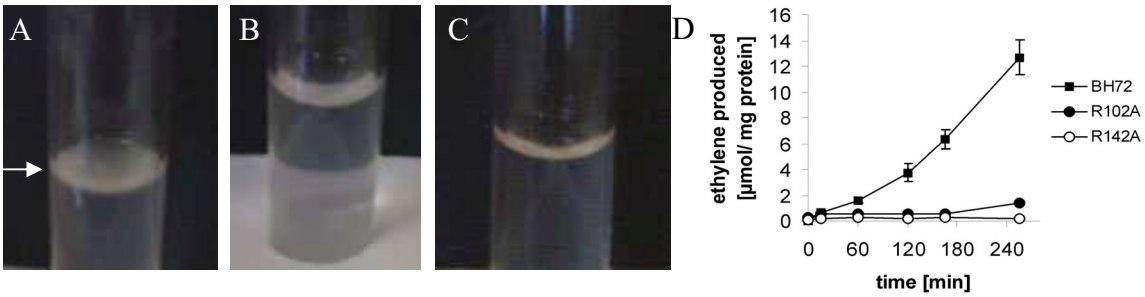

Fig. 3. Nitrogen fixation is abolished in point mutation strains BHnifH_R102A (B) and BHnifH_R142A (C). Cells were transferred in semi-solid agar tubes with nitrogen free SM medium and incubated for 4 days at 37°C. A pelicular shaped growth zone typical for nitrogen fixing cells could only be observed for wild-type strain BH72 (arrow in A). No acetylene reduction was observed in the point mutation strains in comparison to the wild-type in batch-cultures (D). Error bars derive from two parallels.

Strain BHnifH_R142A like strain BHnifH_R102A was also impaired in its ability to modify dinitrogenase reductase (Fig.4). Apparently, the exchanges of arginine 142 by alanine changes the overall three-dimensional structure of the NifH-protein sufficiently to preclude Fe-protein modification on R102 in *Azoarcus* sp. BH72. A similar result has been obtained for a R140Q variant of the *A. vinelandii* dinitrogenase reductase during *in vitro* ADP-ribosylation assays with *R. rubrum* DraT (Grunwald et al., 2000). Site-directed mutagenesis of this residue resulted in a protein not serving as a substrate for ADP-ribosylation. This Fe-protein variant could form a cross-linkable complex with DraT; however, complex formation was reduced to 14% of the wild-type level clearly showing that this residue is crucial for the ADP-ribosylation reaction, but not for the interaction with DraT.

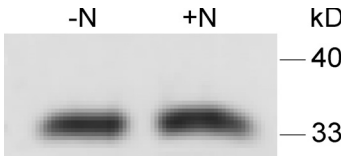

Fig. 4. Nitrogenase Fe-protein modification on arginine 102 is abolished in point mutation strain BHnifH_R142A. No band of lower electrophoretic mobility was observed 20 min after addition of 2 mM NH_4Cl (+N) as in the control negative without NH_4Cl. A positive control of the wild-type cell extracts is shown elsewhere (Fig. 1, Oetjen & Reinhold-Hurek, 2009).

Strategies for Fe-protein enrichment and purification. The success of mass spectrometric analyses is always dependent on the concentration and purity of the applied sample. Especially, determinations of covalent modifications on proteins require high quality samples, since a high coverage is mandatory in most cases. In order to increase the yield of the Fe-protein and to facilitate the subsequent analysis, strategies for its enrichment and purification have been elaborated. This has been particularly valuable for the mass determination of the undigested protein by MALDI-TOF MS and might be helpful for other approaches.

As displayed in Figure 5, proteins corresponding to the modified and unmodified form of the nitrogenase Fe-protein could be enriched by an approach consisting of cell fractionation by ultracentrifugation, polyethylene glycol (PEG)-6000 precipitation and DEAE-cellulose chromatography. However, many other proteins were still present in the elution fractions after DEAE-cellulose chromatography. Hence, after this step proteins were separated by 2D gel electrophoresis for purification purposes (Fig. 6).

After 2D gel electrophoresis the Fe-protein could be separated from other protein spots and excised for further processing. However, as can be depicted from Figure 6, a high extent of the ADP-ribosylation was apparently hydrolyzed during the enrichment procedure or did not enter the isoelectric focusing gel properly, as the relation of modified to unmodified form was not 1:1. A modification level of nearly 50% of NifH monomers was expected, as the protein was derived from nitrogen-fixing cells of *Azoarcus* sp. BH72 shocked by addition of 2 mM ammonium chloride for 15 min.

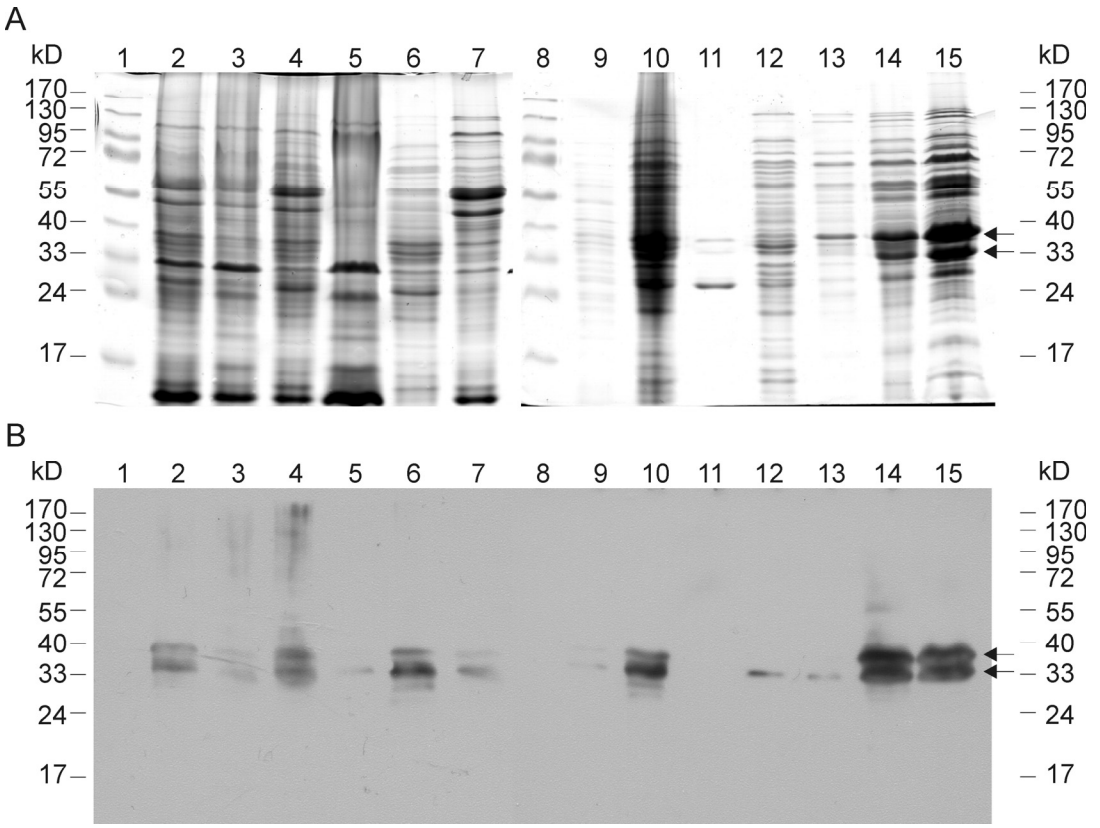

Fig. 5. Fe-protein enrichment. Coomassie brilliant blue R-250 stained SDS-PAGE gel (A) and Western Blot using antiserum against NifH (B) with different fractions from the enrichment procedure (7.5 µl or 5 µl have been loaded for SDS-PAGE or Western blotting, respectively): lane 1,8: Marker; lane 2: supernatant after cell lysis; lane 3: supernatant after ultrasonication of the resuspended pellet from the first cell lysis step; lane 4: cytoplasmic fraction; lane 5: membrane fraction; lane 6: supernatant after 10% PEG-6000 precipitation; lane 7: pellet after 10% PEG-6000 precipitation; lane 9: supernatant after 30% PEG-6000 precipitation; lane 10: pellet after 30% PEG-6000 precipitation; lane 11: flow through DEAE-cellulose chromatography; lane 12: 100 mM NaCl wash fraction DEAE-cellulose chromatography; lane 13: 200 mM NaCl wash fraction DEAE-cellulose chromatography; lane 14: 400 mM NaCl elution fraction DEAE-cellulose chromatography; lane 15: 400 mM NaCl concentrated elution fraction after DEAE-cellulose chromatography. Arrows indicate bands corresponding to the modified (upper band) and unmodified (lower band) dinitrogenase reductase.

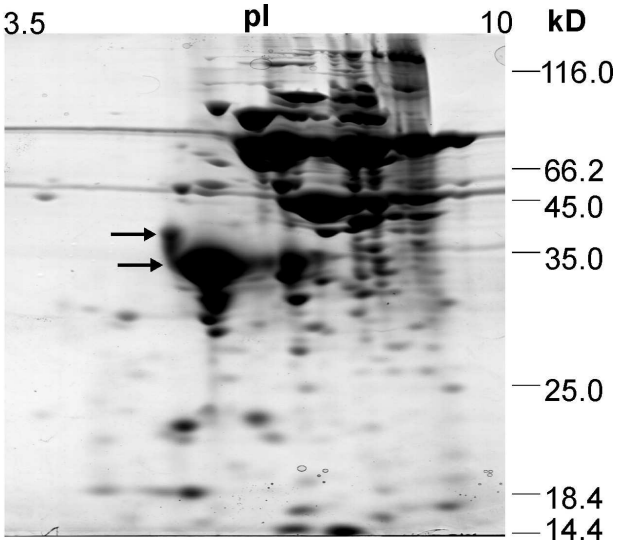

Fig. 6. Coomassie brilliant blue R-250 stained 2D gel from a 400 mM NaCl elution fraction after DEAE-cellulose chromatography. Arrows indicate the spots corresponding to the modified and unmodified nitrogenase Fe-protein.

MALDI-TOF analysis of the undigested Fe-protein. During the optimization of the protocol for detection of the modification on *Azoarcus* sp. BH72 Fe-protein, the undigested protein has been analyzed by MALDI-TOF MS as well. Approximately four Fe-protein containing spots from 2D gels of enrichment fractions were used for this purpose. After electroelution of the protein from acylamide gel pieces and precipitation, average Fe-protein mass has been determined by MALDI-TOF MS. The theoretical average mass of the protein is 32276 m/z, assuming that all cysteine residues were acrylamide modified. The determined average mass of the unmodified *Azoarcus* sp. BH72 Fe-protein was 32131 m/z producing an error of 0.4%. Unfortunately, the mass of the modified Fe-protein could not be measured. This would have been a possibility to exclude other potential kinds of modifications on this protein. However, this result clearly showed that mass determination of undigested proteins derived from acrylamide gels is possible with MALDI-TOF MS. The purified modified and unmodified Fe-protein subunits have been also subjected to an In-Solution digestion with trypsin and peptide analysis by Tris-tricine gel electrophoresis as well as MALDI-TOF analysis. However, although peptides were retrieved, no difference in the peptide band pattern during gel electrophoresis could be observed and no difference was detected in the compared spectra by MALDI-TOF (data not shown), confirming the previous observation that trypsin digestion alone is not sufficient to gain an analyzable peptide for the detection the ADP-ribose moiety.

Conclusions

The partial membrane association of the presumably active, unmodified nitrogenase Fe-protein in *Azoarcus* sp. BH72 suggests interaction of dinitrogenase reductase with a membrane protein under standard nitrogen fixation conditions. It is reasonable to speculate that this protein might be important for the electron transport to nitrogenase. Further evidence eventually achievable by protein interaction studies is necessary to confirm this. In *A. vinelandii* nitrogenase was found to be located in vascular membranous systems as well. However, these structures were suggested to play a role in protecting nitrogenase from oxygen (Oppenheim *et al.*, 1970). It could be verified here that arginine residue 142 – the homologous residue to *A. vinelandii* Fe-protein residue 140 - plays a crucial role in the docking of dinitrogenase reductase to dinitrogenase. Furthermore, the function of this residue was found to be essential for the Fe-protein in serving as a substrate of DraT.

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CHAPTER
2A

Characterization of the DraT/ DraG-System for Posttranslational Regulation of Nitrogenase in the Endophytic β-Proteobacterium Azoarcus sp. BH72

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Characterization of the DraT/DraG System for Posttranslational Regulation of Nitrogenase in the Endophytic Betaproteobacterium *Azoarcus* sp. Strain BH72▽†

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DraT/DraG-mediated posttranslational regulation of the nitrogenase Fe protein by ADP-ribosylation has been described for a few diazotrophic bacteria belonging to the class *Alphaproteobacteria*. Here we present for the first time the DraT/DraG system of a betaproteobacterium, *Azoarcus* sp. strain BH72, a diazotrophic grass endophyte. Its genome harbors one *draT* ortholog and two physically unlinked genes coding for ADP-ribosylhydrolases. Northern blot analysis revealed cotranscription of *draT* with two genes encoding hypothetical proteins. Furthermore, *draT* and *draG2* were expressed under all studied conditions, whereas *draG1* expression was nitrogen regulated. By using Western blot analysis of deletion mutants and nitrogenase assays in vivo, we demonstrated that DraT is required for the nitrogenase Fe protein modification but not for the physiological inactivation of nitrogenase activity. A second mechanism responsible for nitrogenase inactivation must operate in this bacterium, which is independent of DraT. Fe protein demodification was dependent mainly on DraG1, corroborating the assumption from phylogenetic analysis that DraG2 might be mostly involved in processes other than the posttranslational regulation of nitrogenase. Nitrogenase in vivo reactivation was impaired in a *draG1* mutant and a mutant lacking both *draG* alleles after anaerobiosis shifts and subsequent adjustment to microaerobic conditions, suggesting that modified dinitrogenase reductase was inactive. Our results demonstrate that the DraT/DraG system, despite some differences, is functionally conserved in diazotrophic proteobacteria.

Biological nitrogen fixation is one of the major biogeochemical contributions carried out by diazotrophic microorganisms. Nitrogenase, the enzyme catalyzing the reduction of nitrogen to ammonia, consists of the Fe protein (dinitrogenase reductase) and the MoFe protein (dinitrogenase) (2).

Since nitrogen fixation is an energy-demanding process for the cell, it is highly regulated at the transcriptional level and at the posttranslational level (11, 36). For the photosynthetic purple nonsulfur bacterium *Rhodospirillum rubrum* and other members of the *Alphaproteobacteria*, like *Azospirillum brasilense*, *Azospirillum lipoferum*, and *Rhodobacter capsulatus*, it has been shown that nitrogenase activity is rapidly switched off as a consequence of either the addition of ammonium or energy depletion (10, 26, 32, 35, 54). One subunit of the nitrogenase Fe protein is modified under switch-off conditions, with the modification leading to the inactivation of the enzyme. In the case of *R. rubrum* and *R. capsulatus*, it has been proven that the modifying group is an ADP-ribose moiety on the amino acid residue arginine101 or arginine102, respectively (25, 39). The enzyme catalyzing this ADP-ribosylation was identified as DraT (dinitrogenase reductase ADP-ribosyltransferase) in *R. rubrum* (33). Inactivated Fe protein can be activated in a reaction catalyzed by DraG (dinitrogenase reductase activating glycohydrolase) by removal of the ADP-ribosylation (43, 44). In *A. brasilense*, *A. lipoferum*, and *R. capsulatus*, *draT* and *draG* orthologs were discovered as well (10, 35). Moreover, in various genome projects (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi/), *draG*-like sequences and few *draT* orthologs can be identified. However, there is no report on their function in strains outside of the class *Alphaproteobacteria*.

Here we present the occurrence of a Dra-dependent modification system in a bacterium that does not belong to the class *Alphaproteobacteria*, the betaproteobacterium *Azoarcus* sp. strain BH72. Previously, the appearance of an Fe protein with lower electrophoretic mobility was revealed by Western blot analysis (22, 34), suggesting the presence of a posttranslational modification system in strain BH72. This endophyte, originally isolated from Kallar grass [*Leptochloa fusca* (L.) Kunth] (40, 42), possesses the ability to infect rice roots systemically (21). Reporter gene fusion experiments revealed that the *nifHDK* structural genes are expressed in the rice apoplast (5, 19). Another interesting feature of *Azoarcus* sp. strain BH72 is its ability to shift into a so-called hyperinduced state of nitrogen fixation under extremely low oxygen concentrations (20). This state is characterized by a very high rate of respiration and nitrogen fixation and the formation of diazosomes, intracytoplasmic membrane stacks which are also efficiently induced in coculture with an endophytic fungal strain, 2003, isolated from Kallar grass (22). Nitrogenase was shown to be present to a large extent in these membrane stacks. Interestingly, in the case of hyperinduction, an Fe protein with lower electrophoretic mobility was observed, which was not detectable by ^{32}P autoradiography (22). Mechanisms other than ADP-ribosylation were therefore suggested for these specialized conditions.

For a long time, there was no plausible explanation for the

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regulation of DraT and DraG activities. Recently, it could be shown for *A. brasilense* and *R. rubrum* that DraG membrane sequestration is involved in the regulatory mechanism. It is dependent on interaction with small signaling P_{II}-like proteins (such as GlnB, GlnK, GlnZ, and GlnJ), which can form complexes with the ammonium transporter protein AmtB (17, 49, 51). After an ammonium shock, DraG was found to be membrane associated in a P_{II}/AmtB-dependent manner. The current model for the regulation of the DraT/DraG system suggests that the steric separation of DraG from its substrate prevents nitrogenase activation. Ammonium-induced membrane sequestration of GlnK in an AmtB-dependent manner has also been shown for *R. capsulatus* (48). Interestingly, this observation was independent from the uridylylation status of the P_{II} protein. Additionally, some transport-incompetent AmtB variants have been described for *R. capsulatus*, which form ammonia-induced complexes with GlnK but fail to properly regulate nitrogenase (49). *Azoarcus* sp. strain BH72 harbors three paralogous P_{II}-like proteins—namely, GlnB, GlnK, and GlnY. The *glnK* gene as well as the *glnY* gene is located in an operon with a gene coding for an ammonium transporter (*amtB* or *amtY*, respectively). Surprisingly, in *Azoarcus* sp. strain BH72, GlnK membrane association was not dependent on AmtB (34).

In strain BH72, covalent Fe protein modification was abolished in mutants of the P_{II} signal transmitter proteins GlnB and GlnK and the ammonium transporter AmtB after ammonium addition to nitrogen-fixing cultures, whereas the switch-off response was affected only in a *glnK* or *amtB* mutant or in a *glnBK* double mutant (34). Thus, in the *glnB* mutant, the switch-off response was still operating without modification of the nitrogenase (termed the physiological switch-off mechanism) (34).

In *A. brasilense*, DraT is able to interact with the deuridylylated form of the P_{II} protein GlnB in vivo and in vitro, suggesting that this interaction leads to the stimulation of DraT activity and subsequently ADP-ribosylation of dinitrogenase reductase (15, 16). In yeast two-hybrid experiments, interaction of DraT and GlnB has been also demonstrated in *R. capsulatus* and *R. rubrum* (37, 57).

In addition, it has been demonstrated that the Fe protein redox status is important for the interaction with DraT or DraG, respectively (12). DraT exhibited higher activity with the Fe protein in its oxidized form, whereas DraG had no activity with oxidized Fe protein. However, nitrogenase inactivation that is independent from ADP-ribosylation was described for *A. brasilense* and *R. capsulatus* (38, 55). An ammonium switch-off was also found in *Herbaspirillum seropedicae* (9) and *Azospirillum amazonense* (46), both of which lack a DraT/DraG system. Inhibition of nitrogenase activity was rapid, but only partially, and did not occur in vitro in *A. amazonense* (9, 14).

In this study, we report the effect of deletion mutations in *draT* and in two *draG* copies on the switch-off response and nitrogenase modification in *Azoarcus* sp. strain BH72, which indicate that the DraT/DraG system is functionally conserved among members of the *Proteobacteria* despite some differences to the alphaproteobacterial system with respect to regulation. Our results demonstrate that switch-off is independent of DraT, indicating that in *Azoarcus* sp. strain BH72, Fe protein modification and switch-off response should be considered two discrete processes.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. *Escherichia coli* was grown in LB medium, following standard protocols (1). For mutant selection, *Azoarcus* sp. isolates were grown at 37°C in VM medium supplemented with ethanol (41). To obtain N_2-fixing cells of *Azoarcus* sp., cells were grown in 50-ml-batch cultures in N-free synthetic malate (SM) medium (40) essentially as described by Karg and Reinhold-Hurek (27); however, the incubation conditions were 37°C at 150 rpm, with 1% acetylene in the headspace. For *draG* expression analysis, strains BHGGTG1 (*draG1*::*gus* fusion), BHGGTG2 (*draG2*::*gus* fusion in wild-type strain BH72), and BHΔG1GGTG2 (*draG2*::*gus* fusion in strain BHΔdraG1) were grown under nitrogen-fixing conditions or microaerobically under nitrogen excess in SM plus N medium containing 10 mM NH_4Cl and 0.1 g/liter yeast extract.

Anaerobiosis and NH_4Cl treatments. For nitrogenase repression (switch-off) analysis, N_2-fixing cells were freshly diluted in N-free SM medium to an optical density at 578 nm (OD_{578}) of 0.1 to 30 ml. Cells were grown for 2 hours at 37°C under microaerobic conditions (1% O_2 and 10% acetylene in the headspace of 1-liter rubber stopper-sealed Erlenmeyer flasks). For nitrogenase repression, either 1 mM or 0.5 mM NH_4Cl was added to the cells. To analyze the effects of the NH_4Cl addition in a *draG* mutant strain, cells were treated as described above, except that nitrogenase activity was repressed by the addition of 200 μM NH_4Cl. Anaerobiosis experiments were carried out with cells grown in a 2-liter bioreactor (B. Braun, Melsungen, Germany) in order to control the oxygen supply (6). To determine the nitrogen fixation rate, which cannot be measured in the bioreactor directly, 20 ml of cells grown in SM medium at 1% oxygen were transferred to microaerobic rubber stopper-sealed 250-ml Erlenmeyer flasks with 10% acetylene. Anaerobic conditions were achieved in the bioreactor by flushing with nitrogen gas and were controlled with an oxygen sensor. After 30 min of anaerobic incubation, 20 ml of cells were transferred to anaerobic flasks as described above. Cells were further incubated at 37°C for 15 min, and the acetylene reduction rate was determined. Thereafter, microaerobic conditions were adjusted in the flasks and the acetylene reduction was assessed. For experiments with chloramphenicol to avoid protein de novo synthesis, N_2-fixing cells were grown in 1-liter Erlenmeyer flasks as described above and treated with 37.5 μg/ml chloramphenicol 45 min before incubation under anaerobic conditions for 15 min.

DNA analysis. Chromosomal DNA was isolated as described previously (18). Additional DNA techniques were carried out by following standard protocols (1). Genomic clone characterization was performed by using restriction mapping and Southern blot analysis with digoxigenin-labeled gene probes or by using PCR analysis.

Northern blot analysis. Total RNA was extracted using the peqGOLD TriFast extraction kit according to the manufacturer's instructions (Peqlab Biotechnologie GmbH, Erlangen, Germany) from cells grown with N_2 or aerobically under conditions of ammonium excess. Twelve micrograms of RNA per sample in a buffer containing 10% formamide, 1.3% formaldehyde, and 8 μg/ml ethidium bromide was separated on a 1.0% agarose gel containing 5.4% formaldehyde, blotted onto a nylon membrane (Hybond-N; GE Healthcare, Piscataway, NJ), and hybridized according to stringent standard conditions (1). Hybridization was carried out with a digoxigenin-labeled *draT* probe or 16S rRNA probe at 65°C overnight.

Construction of mutants and transcriptional fusion strains. For construction of an in-frame deletion mutant of *draT*, a 786-bp fragment containing the first part and the upstream region of *draT* and an 858-bp fragment containing the 3′ end and the downstream region of *draT* were amplified with *Pfu* DNA polymerase (Qiagen, Hilden, Germany). Primer sequences included recognition sites for restriction enzymes. CGG was introduced behind the HindIII restriction site of primer draTvornRev in order to perpetuate the *Azoarcus* codon usage. PCR fragments were cloned separately into the pPCR-Script Amp SK cloning vector (Stratagene Europe, Amsterdam, The Netherlands), resulting in pPCRdraTup and pPCRdraTdown. In a second cloning step, the downstream fragment was cloned into pPCRdraTup by HindIII digestion, resulting in pPCRΔdraT containing a 708-bp deletion. Thus, the amino acids phenylalanine, arginine, serine, and asparagine were inserted between leucine10 and alanine248 of DraT. The deletion fragment was subcloned into pUC18 (53) by SacI/EcoRI digestion, yielding pUC18ΔdraT, and further subcloned into pK18*mobsacB* (45) by XbaI/EcoRI digestion, resulting in pK18ΔdraT.B. The *draG1* deletion mutant was obtained by using a similar strategy by cloning the 3′ end and the downstream

TABLE 1. Bacterial strains, plasmids, and primer sequences

Strain, plasmid, or primer	Description or sequence[a]	Source or reference
Strains		
Escherichia coli		
DH5αF′	F′ *recA1 endA1 hsdR17*(r_K^-, m_K^+) *supE44* (λ^- *thi-1 relA1* ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169*	13
XL10-Gold Kan ultracompetent cells	Tet^r Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [*proAB lac*I^{q}ZΔM15 Tn*10* (Tet^r) Tn*5* (Km^r) Amy]	Stratagene
One Shot TOP10	F′ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 deoR araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
Azoarcus sp.		
BH72	Wild type	40
BHΔdraT	BH72 Δ*draT*, in-frame deletion at bp 33–741	This study
BHΔdraG1	BH72 Δ*draG1*, in-frame deletion at bp 217–654 (see text for details)	This study
BHΔdraG2	BH72 Δ*draG2*, in-frame deletion at bp 318–764 (see text for details)	This study
BHΔdraG1ΔdraG2	Δ*draG1* Δ*draG2* deletion (see above)	This study
BHGGTG1	Km^r, BH72::pK18GGTG1, chromosomally integrated transcriptional *draG1*::(*gfp gusA*) fusion in wild-type strain BH72	This study
BHGGTG2	Km^r, BH72::pK18GGTG2, chromosomally integrated transcriptional *draG2*::(*gfp gusA*) fusion in wild-type strain BH72	This study
BHΔG1GGTG2	Km^r, BHΔdraG1::pK18GGTG2, chromosomally integrated transcriptional *draG2*::(*gfp gusA*) fusion in *draG* deletion mutant BHΔdraG1	This study
Plasmids		
pUC18	Ap^r, ColE1 replicon	53
pPCR-Script Amp SK(+)	Ap^r, ColE1 origin, f1(+) origin, *lacZ′*, P_{lac}	Stratagene
pCR2.1-TOPO	Ap^r Km^r, ColE1 origin, f1 origin	Invitrogen
pRK2013	Km^r, RK2 *tra* gene cloned in ColE1 replicon	7
pK18*mobsacB*	Km^r, RP4 *mob* region, containing *sacB* gene	45
pK18GGST	Km^r, ColE1 replicon, RP4 *mob* region, containing promoterless *gfp* and *gusA* gene	Krause et al., unpublished data
pPCRdraTup	Ap^r, 0.88-kb PCR fragment containing first part of *draT* in SrfI site of pPCR-Script Amp SK	This study
pPCRdraTdown	Ap^r, 0.86-kb PCR fragment containing last part of *draT* in SrfI site of pPCR-Script Amp SK	This study
pPCRΔdraT	Ap^r, 0.86-kb HindIII fragment from pPCRdraTdown in pPCRdraTup, containing 708-bp in-frame deletion of *draT*	This study
pUC18ΔdraT	Ap^r, 1.74-kb SacI/EcoRI deletion fragment from pPCRΔdraT in pUC18	This study
pK18ΔdraT.B	Km^r, 1.74-kb XbaI/EcoRI deletion fragment from pUC18ΔdraT in pK18*mobsacB*	This study
pPCRdraG1down	Ap^r, 0.76-kb PCR fragment containing last part of *draG1* in pPCR-Script Amp SK	This study
pCRdraG1up	Ap^r, 0.75-kb PCR fragment containing first part of *draG1* in pCR2.1-TOPO	This study
pPCRΔdraG1	Ap^r, 0.75-kb HindIII/XhoI fragment from pCRdraG1up in pPCRdraG1down, containing 437-bp deletion of *draG1* (see text for details)	This study
pK18ΔdraG1.B	Km^r, 1.51-kb BamHI/PstI deletion fragment from pPCRΔdraG1 in pK18*mobsacB*	This study
pPCRdraG2up	Ap^r, 0.73-kb PCR fragment containing first part of *draG2* in pPCR-Script Amp SK	This study
pPCRdraG2down	Ap^r, 0.75-kb PCR fragment containing last part of *draG2* in pPCR-Script Amp SK	This study
pPCRΔdraG2	Ap^r, 0.73-kb HindIII/SalI fragment from pPCRdraG2down in pPCRdraG2, containing 447-bp deletion of *draG2* (see text for details)	This study
pK18ΔdraG2.B	Km^r, 1.48-kb BamHI/SalI deletion fragment from pPCRΔdraG2 in pK18*mobsacBA*	This study
pK18GGTG1	Km^r, 0.75-kb HindIII/XbaI fragment from pCRdraG1up in pK18GGST	This study
pK18GGTG2	Km^r, 0.73-kb HindIII/XbaI fragment from pPCRdraG2up in pK18GGST	This study
Primers		
draTvornFor2	5′-GCTCTAGATCACGATGCCCTTGGCGATG-3′[b]	This study
draTvornRev	5′-CGAAGCTTGCCAACAGCGCGGGGTCGATGTC-3′[b]	This study
draThintenFor	5′-GCAAGCTTCGCCAAGGTGCTGGCGTTCT-3′[b]	This study
draThintenRev	5′-GCCTCGAGCGGACGCGATCCAGTTGCCT-3′[b]	This study
draGvorneFor	5′-GCGGATCCGGAAATGACGCTGAACGATT-3′[b]	This study
draGvorneRev	5′-GCAAGCTTCGGCTTCATCGTCGACAC-3′[b]	This study
draGhintenFor	5′-GCAAGCTTTCAAAGGTCATCGTGGTGTCA-3′[b]	This study
draGhintenRev	5′-GCCTGCAGAATTCATCGCCCACCTGAT-3′[b]	This study
draG2vorneFor	5′-GCGGATCCCCGAGTTCGATGAGTTCCTG-3′[b]	This study
draG2vorneRev	5′-GCAAGCTTCATGAAGCCAGCGTTGATAGG-3′[b]	This study
draG2hintenFor	5′-GCAAGCTTCAAACCTGGCCACAACAGAAG-3′[b]	This study
draG2hintenRev	5′-GCGTCGACGGCGATGAGTTCGCTGTAGT-3′[b]	This study
draTfor	5′-CATGACCGCCCATTTCCTGC-3′[c]	This study
draTrev	5′-CACGGTCGCCGAATTCATCC-3′[c]	This study
draG1for3	5′-CACCGATGACACCACGATGACCTT-3′[c]	This study
draG1rev3	5′-GACCGGCGCGAGTTCGAGCAGACC-3′[c]	This study
draG2for	5′-ATCGGATTGTTGCCAGGTA-3′[c]	This study
draG2rev	5′-AGTCAGCGCGCAATAAAGTC-3′[c]	This study

[a] Abbreviations for drug resistance phenotypes are as follows: Ap^r, ampicillin; Tet^r, tetracycline; Str^r, streptomycin; Km^r, kanamycin.
[b] Primers designed for construction of mutant strains. Recognition sites for restriction enzymes are underlined as follows: TCTAGA, XbaI; AAGCTT, HindIII; CTCGAG, XhoI; GGATCC, BamHI; CTGCAG, PstI; GTCGAC, SalI.
[c] Primers used for generation of the *draT* probe and for validation of *draG* mutants by PCR analysis.

region of *draG1* into pPCR-Script Amp SK, resulting in pPCRdraG1down. The first part and the upstream region of *draG1* were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA), yielding pCRdraG1up. The upstream fragment was cloned into pPCRdraG1down by HindIII/XhoI digestion, resulting in pPCRΔdraG1 containing a 437-bp deletion. A thymidine was introduced behind the HindIII restriction site of primer draGhintenFor in order to get an in-frame deletion. The deletion fragment obtained was subcloned into pK18*mobsacB* by PstI/BamHI digestion, yielding pK18ΔdraG1.B. For the *draG2* deletion mutant,

analogously the *draG2* upstream region and the downstream region were cloned into pPCR-Script Amp SK, resulting in pPCRdraG2up and pPCRdraG2down, respectively. A cytosine was introduced behind the HindIII restriction site of primer draG2vorneRev in order to obtain an in-frame deletion. The downstream fragment was cloned into pPCRdraG2up, yielding pPCRΔdraG2 by HindIII/SalI digestion. The resulting fragment containing a 447-bp deletion was subcloned into pK18*mobsacB* by BamHI/SalI digestion, resulting in pK18ΔdraG2.B. *Azoarcus* deletion mutants were obtained by sucrose selection according to the method of Schäfer et al. (45) after conjugation and triparental mating with *E. coli* bearing pK18ΔdraT.B, pK18ΔdraG1.B, or pK18ΔdraG2.B, respectively, and an *E. coli* helper strain (pRK2013) (7).

To analyze the expression of *draG1* and *draG2*, transcriptional fusions with *gfp* and *gusA* were constructed. In the case of *draG1*, a fragment containing the *draG1* 5′ end and the upstream region was cloned from plasmid pCRdraG1up into the mobilizable plasmid pK18GGST (Krause et al., unpublished data) upstream of the promoterless reporter genes *gfp* and *gusA*, resulting in pK18GGTG1. In the case of *draG2*, the 0.73-kb BamHI fragment from plasmid pPCRdraG2up was first cloned into the BamHI site of pUC18. The fragment was then subcloned through HindIII/XbaI digestion into pK18GGST, resulting in pK18GGTG2. Recombinant strains were obtained as depicted above. Selection of recombinant strains was performed by inspecting for kanamycin resistance, and integration was proven by Southern blot analysis (data not shown).

Protein extraction. For nitrogenase modification analysis from cells out of in vivo assays, 1 ml of cell suspension was removed from cultures with a syringe and immediately processed for sodium dodecyl sulfate (SDS) protein extraction as described previously (21).

SDS-PAGE and Western blotting. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Laemmli (31). The modified and the unmodified forms of nitrogenase Fe protein were separated in a 12% low-cross-linker polyacrylamide gel with a ratio of acrylamide to bisacrylamide of 172:1 (26).

Western blotting was performed as described previously (22). The Fe protein of *Azoarcus* strains was detected by using antiserum against the *Rhodospirillum rubrum* Fe protein. Chemiluminescence detection was carried out with SuperSignal West Pico substrate (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. Unless stated otherwise, chemiluminescence detection and quantification were performed with an image analyzer using charge-coupled-device technology (LAS-3000; Fujifilm, Japan) and ImageQuant 5.1.

Enzyme assays. Nitrogenase activity was measured by the acetylene reduction method described previously elsewhere (5). Activity of β-glucuronidase (Gus) was measured quantitatively using the method described by Jefferson et al. (24) and according to Egener et al. (5) and expressed in Miller units, defined as $E_{420} \times 1{,}000/t$ (min) $\times$ OD_{600}. A 2-ml culture with an OD_{578} of approximately 0.3 was centrifuged, and cells were resuspended in 2 ml of GusA buffer (0.6 M Na_2HPO_4, 0.4 M NaH_2PO_4, 1 mM EDTA, 14 mM β-mercaptoethanol, pH 7). Cells were perforated by the addition of 0.004% SDS and 0.04× Tris-EDTA incubated at 37°C for 10 min. The reaction with the chromogenic substrate 2-chloro-4-nitrophenyl-β-D-glucoside at 37°C was stopped by the addition of 0.6 M 2-amino-2-methyl-propandiol, and cell debris was removed by centrifugation. The increase in A_{420} was monitored spectrophotometrically against a control from wild-type cells not expressing *gusA*.

Phylogenetic analysis. A phylogenetic tree of DraG protein sequences was constructed. Protein sequences were identified by a BLASTP similarity search using DraG1 or DraG2 as a query. The respective first 100 best hits were redirected for an all-against-all BLASTP search using the BioEdit program (Ibis Biosciences, Carlsbad, CA). Sequences that occurred twice were deleted, as well as sequences that produced alignments with an E value of $>10^{-5}$ (52). The final alignment was done with the online version of MAFFT v6 (28, 29) using the G-INS-i strategy with a BLOSUM62 scoring matrix, a gap-opening penalty of 1.5, and an offset value of 0.14. Phylogeny reconstruction was performed by using the minimum evolution algorithm of MEGA 4.0 and pairwise deletion (47) with a Jones-Taylor-Thornton correction distance matrix and consisted of 500 bootstrap replicates.

RESULTS

Occurrence of *draT* and *draG* in the *Azoarcus* sp. strain BH72 genome. One *draT* copy and two *draG* paralogs were identified in the genome of *Azoarcus* sp. strain BH72. The amino acid sequence of DraT had 36% or 35% identity over the entire length to *R. rubrum* or to *A. brasilense* DraT, respectively. Like in *R. rubrum*, the *nifHDK* operon was located upstream of *draT* (see Fig. S1 in the supplemental material) and transcribed in the opposite direction (8). In contrast to *R. rubrum*, where *draT* is cotranscribed with *draG* and an open reading frame (ORF) of unknown function (32), *draT* was not found to be located in an operon with *draG*. Two ORFs followed the 3′ end of *draT* in the same transcriptional orientation, azo0536 and *dcrH1* (azo0535). The gene product of azo0536 had similarity to a hypothetical protein (Daro_1418) of "*Dechloromonas aromatica*" RCB (55% identity), *Pseudomonas stutzeri* (PST_1325; 54% identity) and *Azotobacter vinelandii* (AvinDRAFT_4208; 52% identity), which showed a similar genomic organization adjacent to the 5′ region of the *nifHDK* operon (see Fig. S1 in the supplemental material).

One *draG* copy, designated *draG1*, was localized in the neighborhood of the *nif* structural genes 6.7 kb downstream of *draT*. The peptide sequence of this copy had 45% identity to *R. rubrum* DraG and 43% identity to *A. brasilense* DraG. A gene encoding a putative NifQ protein was located in the same transcriptional orientation upstream of *draG1*. As a transcriptional terminator was found upstream of the putative *nifQ* and another operon was located at the 3′ end of *draG1* in the opposite transcriptional direction, only *nifQ* and *draG1* are potentially cotranscribed in *Azoarcus* strains. In addition, a putative sigma 54-dependent promoter sequence (GT**GG**CGT CTCGCCT**G**CC [highly conserved positions are in bold]; 122 bp in front of the putative *nifQ* start codon) and a putative NifA-specific upstream activator sequence (**TGT**CGGGCAG CGA**ACA**; 603 bp upstream of the putative *nifQ* start codon) could be identified in front of *nifQ*. However, so far there is no experimental evidence for cotranscription of *nifQ* and *draG1*.

A second putative *draG* copy, designated *draG2*, was distant from the *nifHDK* genes, and the gene product had only 24% or 23% identity to *R. rubrum* or *A. brasilense* DraG, respectively. Two genes orientated in the same transcriptional direction and coding for a hypothetical protein or a conserved hypothetical protein, respectively, were located at the 3′ end of *draG2*. Neither the putative sigma 54-binding site nor putative terminator sequences were predicted in this region.

Phylogenetic analysis of DraG in *Bacteria* and *Archaea*. In order to gain a better understanding of the potential function of the two DraG paralogs, a phylogenetic tree was constructed based on amino acid sequences. According to the minimum evolution method, four phylogenetically distinct groups of ADP-ribosylhydrolases could be assigned (see Fig. S2 in the supplemental material). Group I contained sequences of alpha-, beta-, delta-, and gammaproteobacteria, including the DraG sequences of *R. rubrum*, *A. brasilense*, *A. lipoferum*, and *R. capsulatus*, where the function of DraG as dinitrogenase reductase ADP-ribosylglycohydrolase is confirmed. All the strains present in this group had *nifH* and *draT* orthologs in their genomes. This suggests that group I DraG sequences might be NifH-specific ADP-ribosylglycohydrolases. *Azoarcus* sp. DraG2 clustered in group IV together with sequences of diverse species, where so far nothing is known about the potential function of these ADP-ribosylhydrolases. Group III comprises a small number of sequences from members of the *Chlorobea* and from a cyanobacterium. Some *Rhodopseudomonas* species also possess two genes coding for ADP-ribosylhy-

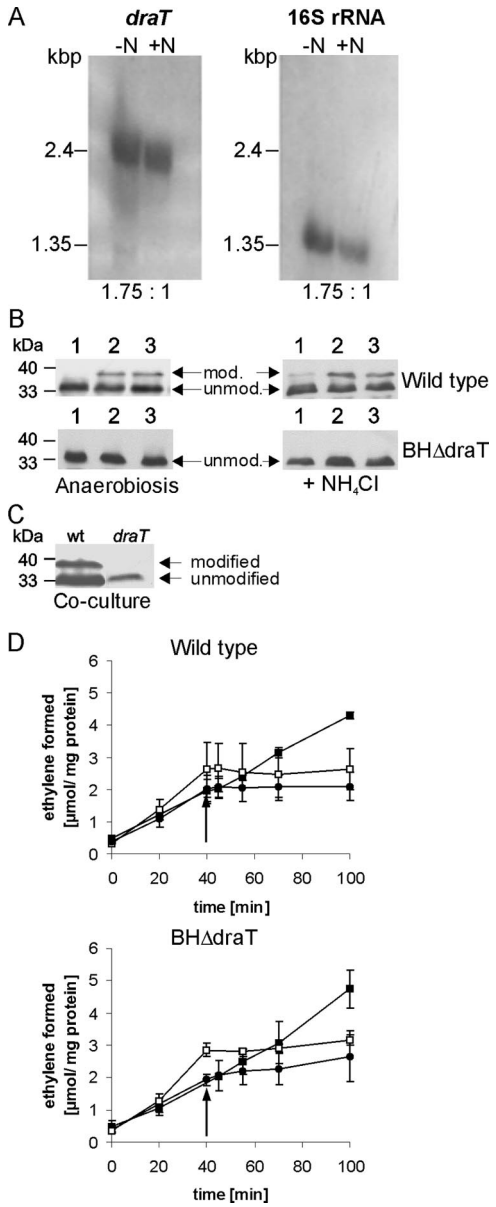

FIG. 1. Expression of *draT* and role of DraT in posttranslational modification of nitrogenase Fe protein and switch-off of nitrogenase activity. (A) Northern blot hybridized with a *draT* probe and with a 16S rRNA probe as indicated. Band intensities were quantified using the ImageQuant 5.1 program to estimate loading amounts as described below. RNA was extracted from cells grown in SM medium under nitrogen-fixing conditions (−N) or grown aerobically under conditions of nitrogen excess (+N). (B) Western blot analysis of nitrogenase Fe protein of wild-type *Azoarcus* sp. strain BH72 and BHΔdraT, indicating that DraT is responsible for posttranslational modification of nitrogenase Fe protein of *Azoarcus* sp. strain BH72. Left panels exhibit the effect of anaerobiosis on nitrogenase Fe protein modification. Cells were taken before (lane 1) and 5 min (lane 2) or 20 min (lane 3) after transfer to anaerobic conditions. Right panels show the effect of addition of 1 mM ammonium chloride to nitrogen-fixing cells. Cells were taken before (lane 1) and 5 min (lane 2) or 15 min (lane 3) after addition of 1 mM ammonium chloride. (C) Western blot analysis of cell extracts from N_2-fixing coculture of *Azoarcus* sp. strain with fungal strain 2003 7 days after inoculation. wt, wild type. (D) Acetylene reduction assays demonstrating that the switch-off response is not abolished in the *draT* mutant strain. Diagrams show the ethylene production of wild-type *Azoarcus* sp. strain BH72 and *draT* mutant BHΔdraT as indicated without ammonium chloride (filled squares) or with the addition of 1 mM (open squares) or 0.5 mM (filled circles) ammonium chloride at the time indicated by arrows. Error bars were derived from two repetitions.

drolases in their genomes, one belonging to group I and the other belonging to group II. ADP-ribosylhydrolase sequences with unknown specificity of taxonomically diverse bacteria fall into group II. Though it is not clear which specificities the ADP-ribosylhydrolases of this group have, it could be shown that the *Anabaena variabilis* Fe protein is not modified by ADP-ribosylation (3).

Expression analysis of *draT*, *draG1*, and *draG2*. *draT* expression was analyzed by Northern blot analysis using a *draT*-specific probe from cells grown microaerobically under conditions of nitrogen fixation or of nitrogen excess under atmospheric O_2 concentrations. The analysis revealed that expression of *draT* was not upregulated under conditions of N_2 fixation (Fig. 1A). In 16S rRNA controls, the proportion of band intensities under both tested conditions was 1.75:1 (conditions of nitrogen fixation/nitrogen excess), as it was in the case of the *draT* hybridization. Since the size of the detected band did not correspond to a monocistronic transcript of *draT*, it is likely that *draT* is cotranscribed with the following two ORFs of unknown functions, azo0536 and *dcrH1*, with a predicted transcript size of 2,423 bp; this is also indicated by a putative sigma 70 promoter in front of *draT* and a predicted terminator sequence at the 3′ end of *dcrH1* (data not shown).

To analyze the expression of *draG1* and *draG2*, which could not be detected by Northern blot analysis, transcriptional reporter gene fusions were used. *gusA* encoding Gus was transcriptionally fused to either *draG1* or *draG2*. In Gus assays, a 19-fold-higher expression level of *draG1* was detected in cells grown under nitrogen-fixing conditions than in cells grown microaerobically with ammonium chloride as the nitrogen source (582 ± 86 or 31 ± 12 Miller units, respectively). This is contrary to the observation of *R. rubrum*, where DraG was detected under all studied growth conditions (50). Under the conditions described above, expression levels of *draG2* were comparably low (45 ± 9 or 89 ± 9 Miller units, respectively). To test whether *draG2* expression was higher in the absence of *draG1*, the expression level of this gene was determined in a *draG1* mutant background under conditions of N limitation or excess, and expression levels remained equally low (41 ± 20 or 77 ± 19 Miller units, respectively). The low expression of *draG2* in comparison to the examined expression level of *draG1* in this bacterium (13-fold higher) and slightly increased expression on ammonium chloride suggest that DraG2 might not play a key role in nitrogenase regulation.

Effect of DraT on nitrogenase posttranslational modification and switch-off. To elucidate the effect of DraT on the posttranslational modification and on the physiological switch-off of nitrogenase, an in-frame deletion mutant of *draT* (BHΔ draT) was constructed to avoid polar effects on the cotranscribed genes azo0536 and *dcrH1*. Western blot analysis with *R. rubrum* antiserum against the nitrogenase Fe protein confirmed that DraT was required for the posttranslational modification of the Fe protein in *Azoarcus* sp. strain BH72 after

anaerobiosis shifts or the addition of ammonium (Fig. 1B). Under nitrogen fixation conditions, the Fe protein was nearly unmodified in the wild-type strain BH72. By changing the conditions to an environment unfavorable for nitrogen fixation, achieved by supplementation with ammonium as a nitrogen source or by oxygen deprivation, a second protein of lower electrophoretic mobility appeared in wild-type extracts (Fig. 1B, top). This suggested the occurrence of a modified NifH protein (22, 34), and the modification could recently be identified as ADP-ribosylation on arginine residue 102 in *Azoarcus* sp. strain BH72 (Oetjen et al., unpublished data). In contrast to that in wild-type strain BH72, no covalently modified NifH appeared in extracts of the *draT* mutant (Fig. 1B, bottom).

Earlier results have shown that upon diazosome formation—a characteristic physiological state reproducibly achieved by coculturing *Azoarcus* sp. with fungal strain 2003—a NifH protein of lower electrophoretic mobility was formed which was not detected by ^{32}P autoradiography (22). In order to investigate whether this modified NifH protein was dependent on DraT, cell extracts from strain BHΔdraT cocultured with the fungal isolate 2003 were analyzed (Fig. 1C). While wild-type *Azoarcus* isolates showed a modified Fe protein, this was not detected in cell extracts from strain BHΔdraT. Therefore, Fe protein modification in *Azoarcus* sp. strain BH72 under these conditions was also dependent on DraT.

To test the role of DraT for a physiological switch-off of nitrogenase activity, acetylene reduction assays were performed. In the wild-type strain BH72 as well as in the mutant strain BHΔdraT, a prompt inhibition of nitrogenase activity was observed after the addition of 1 mM or 0.5 mM ammonium chloride, respectively (Fig. 1D). Thus, a second mechanism for nitrogenase switch-off after the ammonium addition operated in strain BH72 independently of DraT. Nitrogenase inactivation as a consequence of the anaerobiosis shift has also been observed for the *Azoarcus* wild-type strain and the *draT* mutant (data not shown). As stated previously (34), this inactivation might be a result of energy limitation in this strictly respiratory bacterium.

Effect of DraG1 and DraG2 on removal of the posttranslational modification and recovery of nitrogenase activity. To assess whether NifH protein modification is a reversible mechanism mediated by DraG in *Azoarcus* sp. strain BH72, deletion mutants *draG1* and *draG2* and a *draG1 draG2* double mutant were constructed.

The effect of anaerobiosis shifts and the subsequent adjustment of microaerobic conditions was analyzed in wild-type strain BH72 and in the *draG* mutant strains (Fig. 2). For this, cells were grown in an oxygen-controllable bioreactor to an OD_{578} of approximately 0.2 and flushed with nitrogen gas to achieve anaerobic conditions. Nitrogenase activity reactivation after adjustment to microaerobiosis was assessed (Fig. 2A) in parallel to the Fe protein modification status at different time points during the experiment (Fig. 2B). The wild-type strain BH72 and the *draG2* mutant showed similar reactivation curves (Fig. 2A). These strains could recover their initial nitrogenase activities (147 ± 26 for the wild-type strain BH72 or 121 ± 44 for strain BHΔdraG2) within 5 to 10 min after adjusting to microaerobiosis. This was accompanied by a rapid, complete Fe protein demodification (Fig. 2B). Nitrogenase activity restoration and the Fe protein demodification reaction

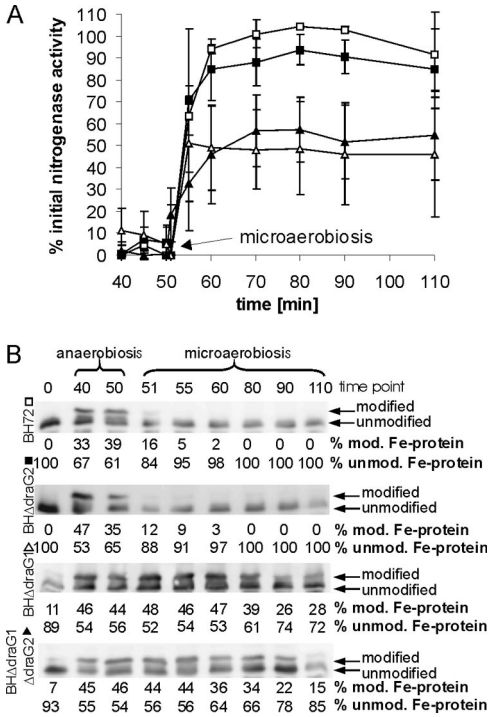

FIG. 2. Effect of anaerobiosis shifts on nitrogenase activity and Fe protein modification of nitrogen-fixing cultures of wild-type and *draG* mutant *Azoarcus* strains. (A) Acetylene reduction assay representing the percentage of initial nitrogenase activity (nanomoles ethylene/milligrams protein × minute) of nitrogen-fixing cultures of wild-type *Azoarcus* sp. strain BH72 (open squares), BHΔdraG1 (open triangles), BHΔdraG2 (filled squares), and BHΔdraG1ΔdraG2 (filled triangles) after shifts to anaerobic conditions followed by adjustment to microaerobiosis. Initial activities were for wild-type strain BH72 (147 ± 26), BHΔdraG2 (121 ± 44), BHΔdraG1 (122 ± 12) or BHΔdraG1ΔdraG2 (135 ± 51) (ethylene/milligrams protein × minute), respectively. (B) Western blot analysis with antiserum against nitrogenase Fe protein. Samples were taken at the time points indicated in panel A. The relative amounts of modified Fe protein were determined with a luminescent image analyzer with charge-coupled-device technology and quantified with ImageQuant 5.1. Error bars were derived from two independent experiments with two replicates per strain. mod., modified; unmod., unmodified.

were impaired in the *draG1* mutant and the *draG* double mutant strain (Fig. 2). This result indicated that indeed Fe protein modification leads to nitrogenase inhibition in *Azoarcus* sp. strain BH72. However, after adjustment to microaerobiosis, recovery of nitrogenase activity to levels of about 50% was possible in both strains within 5 to 10 min (initial activities were 122 ± 12 for strain BHΔdraG1 and 135 ± 51 for strain BHΔdraG1ΔdraG2). Surprisingly, at the same time, the Fe protein has still been modified to a large extent (for an example, see strain BHΔdraG1 with 46% modified Fe protein monomers at time point 55 [Fig. 2B]). In addition, Fig. 2B displays that Fe protein modification diminished over the determined time period even in the *draG1* mutant and in the *draG* double mutant.

The relative decrease of modified Fe protein even in the *draG* mutant strains might be due to protein de novo synthesis or might indicate that removal of the covalent modification was dependent not only on DraG proteins. Therefore, the experi-

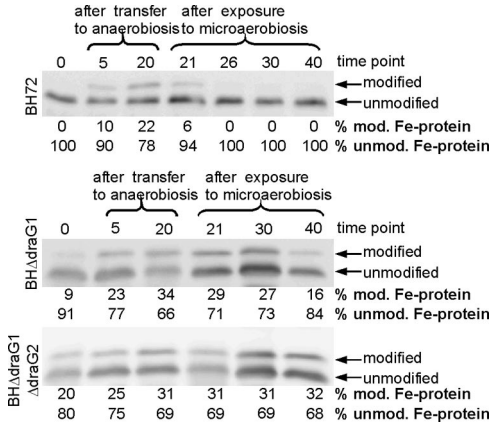

FIG. 3. Posttranslational modification of nitrogenase under anaerobic conditions in the presence of chloramphenicol (37.5 μg/ml) for inhibition of protein de novo synthesis. Western blot analysis with antiserum against NifH is shown. Strains, as indicated, were transferred to anaerobiosis for 15 min. Then conditions were adjusted to 1% oxygen in the headspace. Samples were taken at the time points indicated. Quantification of the relative amounts of modified Fe protein was as described in legend to Fig. 2. A representative result out of two independent experiments is shown. mod., modified; unmod., unmodified.

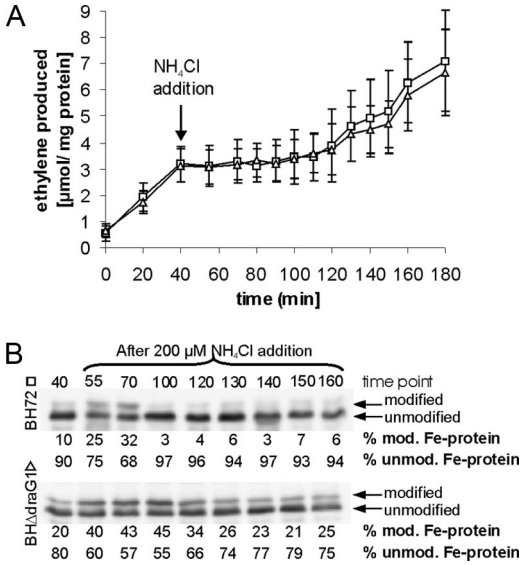

FIG. 4. Effect of ammonium chloride addition (200 μM) on nitrogenase activity and Fe protein modification of nitrogen-fixing cultures of *Azoarcus* sp. strain BH72 and the *draG1* mutant strain. (A) Acetylene reduction (nitrogenase activity in micromoles ethylene/milligram protein) of wild-type *Azoarcus* strain BH72 (open squares) and *draG1* mutant strain BHΔdraG1 (open triangles). Error bars derive from three independent experiments with two replicates each. (B) Western blot analysis with antiserum against nitrogenase Fe protein. Samples were taken at the indicated time points according to the method described for panel A. Quantification of the relative amounts of modified Fe protein as described in legend to Fig. 2. One representative result out of three independent experiments with two replications is shown. mod., modified; unmod., unmodified.

ment was repeated with chloramphenicol-treated cells to inhibit protein synthesis. Under these conditions, the Fe protein was fully demodified only in wild-type *Azoarcus* sp. strain BH72, whereas the Fe protein of the double mutant remained at about 30% in the modified form, as it was during anaerobiosis (Fig. 3). In strain BHΔdraG1, demodification of the Fe protein took place; however, the response was not complete and proceeded more slowly than in the wild type. In this case, DraG2 might have partially complemented DraG1. Thus, nitrogenase de novo synthesis was likely to be responsible for the observed increase in cellular unmodified Fe protein content in experiments without chloramphenicol treatments.

Wild-type and *draG1* mutant strains were also analyzed for their abilities to remove the posttranslational modification of the nitrogenase Fe protein and to recover nitrogenase activity after the addition of low concentrations of ammonium chloride (Fig. 4). The addition of 200 μM ammonium chloride to nitrogen-fixing cultures of wild-type strain BH72 and of the *draG1* mutant strain led to a time-limited inhibition of nitrogenase activity and a subsequent reactivation after consumption of the external nitrogen source (Fig. 4A). Surprisingly, strain BHΔdraG1 restored nitrogen fixation as equally well as the wild type. A discrepancy was observed between the nitrogenase activity and the Fe protein modification status. Considering the high percentage of modified Fe protein in the mutant strain BHΔdraG1 (Fig. 4B), the similar acetylene reduction rates might have been achieved if the wild-type *Azoarcus* sp. strain did not exploit the maximal nitrogen fixation capacity. Less-unmodified dinitrogenase reductase dimers in strain BHΔ draG1 achieved the same nitrogenase activity rates as those of the wild type, indicating that in the wild type a limitation different from Fe protein availability might have occurred. The complete shutdown of nitrogenase activity after the addition of ammonium chloride (Fig. 4A), which was not reflected by the Fe protein modification status (Fig. 4B), was probably due to the ammonia-mediated physiological switch-off in this bacterium. Western blot analysis of the wild-type *Azoarcus* sp. revealed that a nearly complete demodification occurred within 60 min after the ammonium chloride addition (time point 100 in Fig. 4B). Demodification in strain BHΔdraG1 was delayed and not complete. However, the ratio of unmodified-to-modified Fe protein still increased with the time, correlating well with results of anaerobiosis experiments. Again, this effect might be due to de novo protein synthesis.

DISCUSSION

Here we presented for the first time evidence for the occurrence of a functional DraT/DraG system outside of the alpha subgroup of the *Proteobacteria.* In the betaproteobacterium *Azoarcus* sp. strain BH72, we have analyzed expression of the genes involved in the posttranslational regulation of nitrogenase and studied the effect of deletion mutations on the modification and physiological switch-off response. As we demonstrate here, the posttranslational regulation is similar to the DraT/DraG system of members of the *Alphaproteobacteria*.

The operon structure of the *dra* genes and expression regulation differed in *Azoarcus* sp. strain BH72 from the situation in members of the *Alphaproteobacteria.* In *R. rubrum*, *A. brasilense*, and *A. lipoferum*, *draT* and *draG* are linked to the *nifHDK* structural genes and they are probably cotranscribed with an ORF with unknown function (8, 23, 32, 56). In *R. capsulatus*, the *draTG* operon is located widely separated from

the nitrogenase structural genes. A *draTG* downstream ORF could not be found (35). *dra* gene expression in members of the *Alphaproteobacteria* is most probably not under the control of a *nif* promoter. *R. rubrum* DraG could be detected under all studied growth conditions, indicating a constitutive gene expression (50). In *A. lipoferum*, expression of the *dra* operon was shown to be regulated in response to oxygen but not to NH_4^+ ions (23). The genomic organization clearly shows that *Azoarcus* sp. strain BH72 *draT* is not located in an operon with *draG* as it is in the *Alphaproteobacteria*. Therefore, expression levels of and regulatory circuits for *draT* and *draG* might be different in *Azoarcus* spp. Expression of *draT* was found to be constitutive in contrast to expression of *draG1*, which was increased under conditions of N_2 fixation as opposed to the alphaproteobacterial system. Expression regulation of *draG1* in response to NH_4^+ ions is in agreement with its putative role under conditions of N_2 fixation. In *Azoarcus* spp., *draT* is likely to be cotranscribed with two ORFs of unknown function, of which one is widely distributed in this genomic region in diazotrophic betaproteobacteria and gammaproteobacteria.

We have shown that DraT is required for nitrogenase Fe protein modification in *Azoarcus* sp. strain BH72 in pure culture or in coculture with an endophytic fungus strain, 2003. Recently, the Fe protein ADP-ribosylation of arginine residue 102 could be demonstrated in *Azoarcus* sp. strain BH72 by applying a mass spectrometric approach (Oetjen et al., unpublished data). Previously it has been shown that Fe protein modification in *Azoarcus* sp. strain BH72 is dependent on the P_{II}-like proteins GlnK and GlnB and the ammonium channel protein AmtB after the addition of ammonium (34). *Azoarcus* sp. strain BH72 possesses three genes coding for P_{II}-like proteins. Of those, *glnK* and *glnY* are physically linked with an *amt* gene (34). Interestingly, the regulatory system in *Azoarcus* spp. appears to deviate from alphaproteobacterial mechanisms with respect to membrane sequestration of GlnK or other P_{II}-like proteins, which is dependent on the ammonium transporter AmtB (17, 49, 51); binding of DraG to this complex leads to separation of DraG from its substrate, preventing nitrogenase activation (17, 51). In contrast, in *Azoarcus* sp. strain BH72, membrane sequestration of GlnK was still possible in an *amtB* mutant (34). Whether membrane sequestration of DraG1 in *Azoarcus* accounts for a regulatory mechanism still needs to be elucidated. If so, then probably a different membrane protein might be the target.

Previous studies have revealed that in *Azoarcus* spp., physiological switch-off was affected only in an *amtB* or *glnK* mutant but not in a *glnB* mutant (34). Simultaneously, Fe protein modification was abolished in the *glnB* mutant. Together with our data presented here, showing that in contrast to the mechanism in alphaproteobacteria harboring *draT*, DraT was not obligatory for the physiological switch-off response in strain BH72, it is apparent that unmodified Fe protein in *Azoarcus* spp. can undergo a switch-off response. This verifies that nitrogenase Fe protein modification and the physiological switch-off response should be considered two discrete phenomena. A second mechanism for regulation of nitrogenase activity in response to ammonium has been described for *R. capsulatus* and *A. brasilense* (38, 55). NifH mutant proteins, deficient in their abilities to be a substrate for ADP-ribosylation, still allowed a switch-off response. However, DraT was required for the regulation as *draT* mutants did not show a switch-off response (35, 54, 56). A DraT-independent ammonium switch-off was described for *H. seropedicae* and *A. amazonense* (9, 46). Inhibition of nitrogenase activity was rapid without a transition phase, as seen in *A. brasilense*, but only partially (9, 14). Switch-off in *Azoarcus* sp. strain BH72 is completely independent of DraT, as it is in *A. amazonense* or *H. seropedicae*. However, with 1 mM ammonium chloride, the switch-off response appeared to be rapid and complete in *Azoarcus* strain BHΔdraT compared to that in *H. seropedicae*, for example (9). *Azoarcus* spp. seem to share both nitrogenase regulatory mechanisms, the DraT/DraG-mediated and a so-far-unexplained DraT-independent way of inactivation.

The mechanism of the DraT-independent physiological switch-off yet needs to be elucidated. It was previously assumed for phototrophs (12, 38) that the electron transport might play a role in the regulation of nitrogenase activity. In *Azoarcus* sp. strain BH72, a ferredoxin (FdxN) encoded in the *nif* operon was found to be the main but not the essential electron donor for nitrogenase (6). During *Azoarcus* sp. strain BH72 genome annotation, flavodoxin-encoding genes (*nifF1*, *nifF2*) and several genes coding for FdxN-like proteins have been identified which could play a role in electron transport to nitrogenase (30). However, for the switch-off response of nitrogenase, FdxN was essential (6). Therefore, blocking the electron transport to nitrogenase might be the crucial factor in the regulation of nitrogenase activity in this bacterium, different from *R. rubrum*, where switch-off was unaffected in *fdxN1* and *fdxN2* mutants (4).

Our physiological analysis of *draG* mutant strains has revealed that DraG1 is the major protein responsible for Fe protein demodification but that DraG2 probably has an additive effect. The role of DraG1 as dinitrogenase reductase ADP-ribosylglycohydrolase was also suggested by phylogenetic analysis (see Fig. S2 in the supplemental material). It is therefore assumed that DraG1 in *Azoarcus* sp. strain BH72 plays a similar role as that in the well-studied alphaproteobacterial systems. However, it was surprising to us that *draG* mutant cells could recover up to 50% of their initial nitrogenase activity in anaerobiosis shock experiments (Fig. 2) and that they showed similar acetylene reduction curves after the addition of low ammonium chloride concentrations (Fig. 4), although nitrogenase Fe protein was largely ADP-ribosylated. Based on the available amount of unmodified NifH protein, this unusual observation indicates that the initial nitrogenase activity was not used to its full extent. Therefore, under certain culture conditions, it is not the cellular pool of available unmodified dinitrogenase reductase that seems to be crucial to exhibit a maximum nitrogenase activity in *Azoarcus* but rather other factors, like possibly the electron supply to nitrogenase.

The physiological, DraT-independent switch-off reaction appeared to be highly efficient in strain BH72 while the modification of the Fe protein was not complete (less than 50% of modified monomers); after the addition of low ammonium chloride concentrations, a complete inactivation of nitrogenase activity was achieved.

Interestingly, in all analyzed strains, including the *draG* mutants, the relative amount of modified Fe protein diminished over the determined time period, although the decline in modified Fe protein content occurred more slowly in the *draG1*

mutant and the *draG* double mutant. In order to assess whether rapid de novo synthesis of nitrogenase might contribute to this effect, we studied the demodification ability of *draG* deletion strains after chloramphenicol addition and anaerobiosis treatments. Since demodification was abolished in the *draG* double mutant, we assume that a fast nitrogenase de novo synthesis was responsible for the response in the *draG* mutants without the addition of chloramphenicol. As demodification in the presence of chloramphenicol occurred in strain BHΔdraG1, although the demodification reaction did not reach wild-type levels, DraG2 might have accounted for the observed demodification.

In summary, the phylogenetic and functional analyses suggest that the DraT/DraG system might be operating in a wider range of proteobacterial diazotrophs than previously suspected, including members of the *Deltaproteobacteria* and *Betaproteobacteria*, albeit with some functional distinctions.

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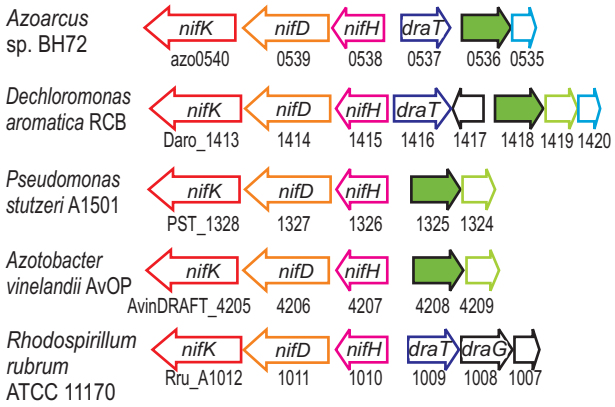

Fig. S1. Genome organization of the *draT*- azo0536 region in *Azoarcus* sp. strain BH72 compared to strains bearing a putative azo0536 ortholog. For comparative purpose the *Rhodospirillum rubrum* gene organization around *draT* is shown. Numbers below the arrows represent the locus-tags for each strain.

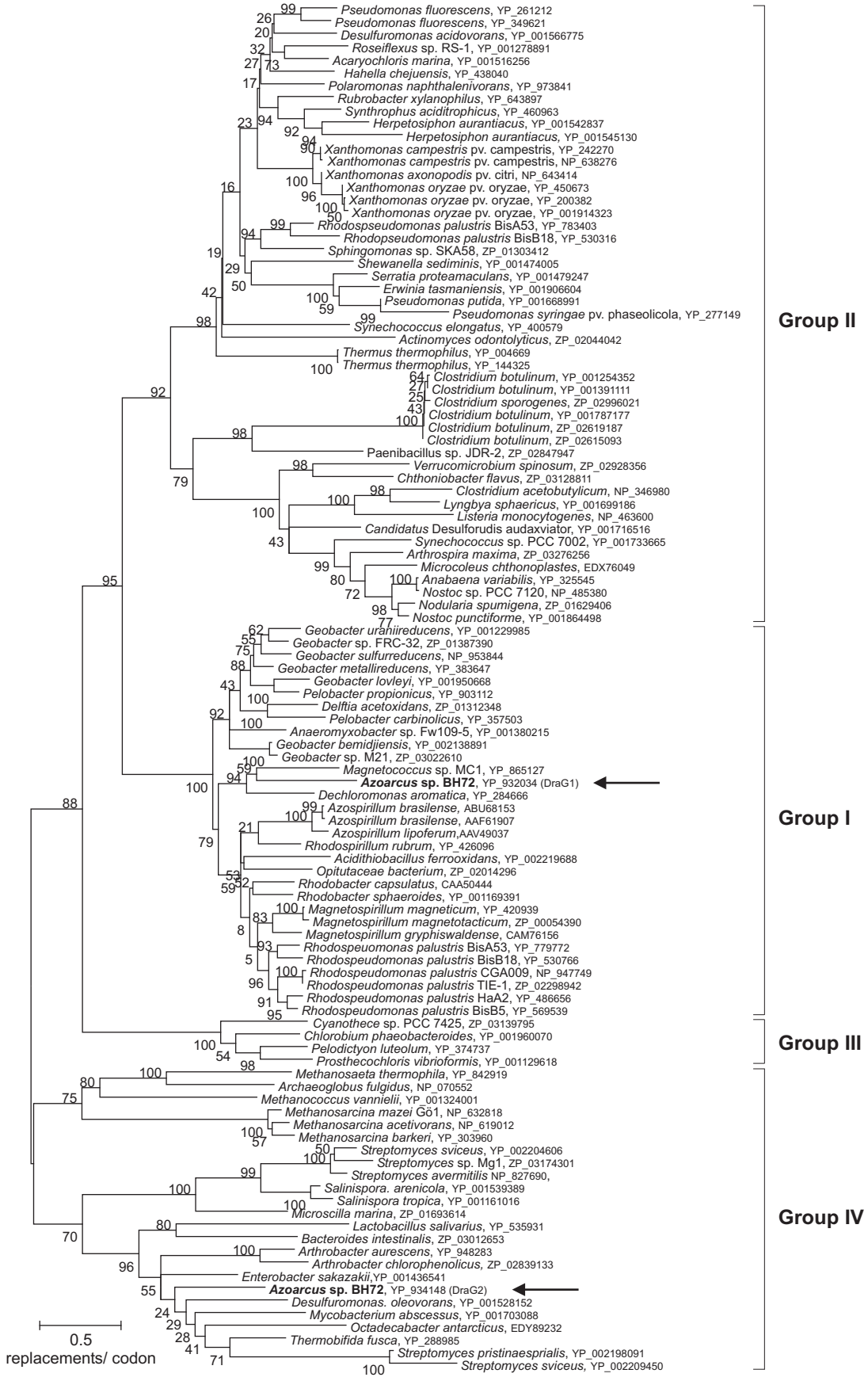

Fig. S2. Protein tree of ADP-ribosylhydrolases (DraG) and homologues of unknown function showing four phylogenetically distinct groups. Bootstrap values (500 replicates) are given as the percentage at each node.

CHAPTER
2B

Functional analysis of azo0536 and azo0535– the genes co-transcribed with draT

Functional analysis of azo0536 and azo0535 – the genes co-transcribed with *draT*

Abstract

Reversible covalent ADP-ribosylation of nitrogenase Fe-protein by the DraT/DraG-system is a rapid way to regulate the activity of this enzyme posttranslationally. This process is functionally characterized for a few diazotrophic alphaproteobacteria and in the betaproteobacterium *Azoarcus* sp. BH72, but it is probably more widespread in the diazotrophic *Proteobacteria*. In contrast to the alphaproteobacterial system, in the grass endophyte *Azoarcus* sp. BH72 *draT* was shown to be co-transcribed with two genes – namely azo0536 and *dcrH1* - coding for a hypothetical protein or a protein with sequence similarity to a hemerythrin, respectively. Here it was demonstrated that a polar knock-out mutation in azo0536, had an effect on the diazotrophic growth of this endophytic bacterium, when cells were grown microaerobically in cultures with shifting oxygen concentrations. Interestingly, cells grew normal in a bioreactor with controlled oxygen concentrations of 0.6%, leading to speculations about an oxygen scavenging function of DcrH1. The knock-out mutation did not alter the ability of this endophyte to modify the nitrogenase Fe-protein. Results from nitrogenase *in-vitro* assays verified that Fe-protein ADP-ribosylation in *Azoarcus* sp. BH72 leads to nitrogenase inactivation.

INTRODUCTION

The DraTG-system is known to regulate nitrogenase activity posttranslationally by reversible ADP-ribosylation of a specific arginine residue of one subunit of nitrogenase Fe-protein in response to the nitrogen status or energy deprivation. Dinitrogenase reductase ADP-ribosyltransferase (DraT) catalyzes the ADP-ribosylation, while dinitrogenase activating glycohydrolase (DraG) functions antagonistically and catalyzes removal of the covalent modification (Ludden, 1994). The posttranslational regulation system has been studied in molecular detail in diazotrophic alphaproteobacteria (Liang *et al.*, 1992, Zhang *et al.*, 1992, Masepohl *et al.*, 1993, Fitzmaurice *et al.*, 1989) and recently as well in the endophytic betaproteobacterium *Azoarcus* sp. BH72 (Oetjen & Reinhold-Hurek, 2009), where nitrogenase Fe-protein ADP-ribosylation has been identified to occur on arginine residue 102 (Oetjen *et al.*, 2009). Different to the situation in the alphaproteobacteria, in *Azoarcus* sp.

BH72 *draT* is co-transcribed with two ORFs of unknown function, termed azo0536 and *dcrH1* (Oetjen & Reinhold-Hurek, 2009). In addition, some features particularly regarding the regulation of the switch-off response differed in strain BH72 to the mechanism discovered in members of the *Alphaproteobacteria*. Ammonium-induced nitrogenase inactivation – called switch-off response – was independent of DraT. This suggests the presence of a second mechanism responsible for nitrogenase inactivation in response to NH_4^+-ions. Even nitrogenase reactivation after addition of low ammonium chloride concentrations or after anaerobiosis shifts and subsequent adjustment to microaerobiosis was unusual in this bacterium, which harbors two genes coding for ADP-ribosylhydrolases. *draG*-mutant strains could recover nitrogenase activity equally well after addition of low ammonium chloride concentrations as the wild-type strain BH72, though it's Fe-protein was partially modified in contrast to the wild-type. Anaerobiosis shift experiments suggested that as expected Fe-protein modification leads to nitrogenase inactivation, as nitrogenase recovery was impaired in a *draG1*-mutant or a *draG1draG2* double mutant. In addition, this experiment indicated that the cellular pool of nitrogenase was not used to full capacity in *Azoarcus*.

This chapter describes preliminary, experimental attempts aiming to extend the understanding of the unusual features with respect to nitrogenase activity regulation in *Azoarcus* sp. BH72. As the genes azo0536 and *dcrH1* are co-transcribed with *draT*, the proteins might contribute to sensing or modulation of the posttranslational regulation. Therefore, a azo0536 knock-out mutant has been constructed and analyzed physiologically in order to investigate whether azo0536 might be involved in any of the observed differences or in a putative other kind of Fe-protein modification, which was suggested to occur in diazosome containing cells of this bacterium. In addition, nitrogenase *in-vitro* assays have been performed to figure out whether nitrogen fixation might be possible with a modified nitrogenase in this bacterium.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Table 1: Bacterial strains and plasmids.

Strain or plasmid	Description[1]	Reference
Strains		
Escherichia coli		
DH5αF`	F`*recA1 endA1 hsdR17*(rK^-, mK^+) *supE44* (λ-thi-1 relA1 ϕ80d*lacZΔM15* Δ(*lacZYA-argF*)U169	Hanahan, 1983
Azoarcus		
BH72	wild-type	Reinhold et al., 1986
BHOrf12Ω	Sm^r/Spc^r , azo0536::Ω chromosomal insertion	this study
BHΔdraG1ΔdraG2	BH72, Δ*draG1*, in-frame deletion bp 217-654, ΔdraG2, in-frame deletion bp 318-764	Oetjen & Reinhold-Hurek, 2009
BHΔdraT	BH72, Δ*draT*, in-frame deletion bp 33-741	Oetjen & Reinhold-Hurek, 2009
Plasmids		
pUC19	Ap^r, ColE1 replicon	Yannish-Perron, 1985
pK18mob2	Km^r, pMB1-Replikon, RP4 *mob*-region	Tauch et al., 1998
pBGVN13	Ap^r, 9.0 kb Sau3A1 fragment containing the *nif*-region in pUC19	Egener et al., 2001
pHP45Ω	Ap^r, containing the Ω-fragment harboring a Sm^r /Spc^r-restistence cassette	Prentki & Krisch, 1984
pRK2013	Km^r **,** RK2 *tra* gene cloned in ColE1 replicon	Figurski & Helinski, 1979
pMob2.1593	Km^r, 1.59 bp Eco47III fragment from pBGVN13 in SmaI site of pK18mob2	this study
pMob2.1593Ω	Km^r, 2.0 kb Ω-fragment from pHP45Ω in XmnI site of pMob2.1593	this study

[1] Ap^r, Km^r, Sm^r, Spc^r: ampicillin, kanamycin, streptomycin, spectinomycin resistance, respectively.

Construction of an azo0536::Ω mutant. For insertional mutagenesis of azo0536 (Figure 1), a 1593 bp Eco47III-fragment containing the azo0536 region from plasmid pBGNV13 (Egener *et al.*, 2001) was cloned into pK18mob2 bearing a kanamycin (Km) resistance gene (Tauch *et al.*, 1998) resulting in pMob2.1593. A 2.0 kb SmaI cassette containing the Ω-fragment consistent of a streptomycin/spectinomycin (Sm/Spc) antibiotic resistance gene derived from plasmid pHP45Ω (Prentki & Krisch, 1984) was cloned into the PdmI site of azo0536 in pMob2.1593 to yield plasmid pMob2.1593Ω. An *E. coli* strain bearing this plasmid together with an *E. coli* helper strain pRK2013 and *Azoarcus* sp. BH72 was used for conjugation and triparental mating. Double crossover strains characterized by a Sm/Spc resistance concomitant with a failure to grow on kanamycin containing agar plates were selected. Positive clones were validated by Southern blotting using a digoxygenin labeled probe derived from the Ω-fragment by SmaI-digestion of pHP45Ω using the DIG High Prime-Kit according to manufacturers instruction. As expected, an EcoR1 digest of genomic DNA of double recombinant

clones generated a fragment of 4.6 kb (data not shown). Hybridization was carried out under stringent standard conditions (Ausubel *et al.*, 1987).

Media and growth conditions. Nitrogen fixing batch-cultures of *Azoarcus* sp. BH72 wild type and the azo0536::Ω mutant strain BHOrf12Ω were grown in Erlenmeyer flasks or in a bioreactor. Cells were precultured in SM+N medium as described (Oetjen & Reinhold-Hurek, 2009). For the cell growth with shifting oxygen concentrations, cell were grown over night in nitrogen-free SM medium in rubber stopper sealed Erlenmeyer flasks as described (Oetjen & Reinhold-Hurek, 2009). For the growth experiments, cells were freshly diluted in Erlenmeyer flasks with a starting oxygen concentration of 1.4% (Oetjen & Reinhold-Hurek, 2009). Due to respiration, the oxygen concentration in the flasks decreased to approximately 0.8% during the growth. For *in vitro* assays and growth experiments of wild-type and azo0536 mutant, cells were grown in a 2-litre bioreactor (Biostat B; B. Braun Melsungen AG, Melsungen, Germany) in N-free SM medium with controlled oxygen concentration set to 0.6% O_2 to an OD_{578} of approximately 0.8.

Nitrogenase assays. *In vivo* nitrogenase assays have been performed as previously described (Egener *et al.*, 1999). Time limited nitrogenase inactivation was induced by addition of 200 µM ammonium chloride to nitrogen fixing cells as explained before (Oetjen & Reinhold-Hurek, 2009). *In vitro* assays were carried out essentially as described (Ludden & Burris, 1976).

Protein extraction, SDS-PAGE and Western blotting. For *in vitro* assays cells were harvested anaerobically in the presence of 1 mM dithionite at an OD_{578} of 0.8, and all following steps were done anaerobically. Crude extracts were obtained from approximately 2 g (fresh weight) of cells resuspended in 18 ml of 0.1 M Tris·Cl, pH 7.8 by ultrasonication (4x 20 s at 50 watts output with 20s breaks) under a stream of nitrogen gas. Phenylmethanesulphonylfluoride (PMSF) was added to a concentration of 0.5 mM. Unbroken cells were removed by centrifugation at 24,000 × g for 10 min at 4°C. The supernatant was used for ultracentrifugation (Optima^{TM} LE-80K, Beckman Coulter, Fullerton, USA) at 115,000 × g for 90 min at 4°C. The supernatant (cytoplasmic fraction) was stored in liquid nitrogen until use. The pellet (referred to as membrane fraction) was washed with 0.1 M Tris·Cl, pH 7.8 buffer containing 0.5 M NaCl and ultracentrifuged for 30 min at 115,000 × g at 4°C. The membrane pellet and the salt wash fraction frozen in liquid nitrogen were stored at -20°C under atmospheric conditions until use. For nitrogenase modification analysis from cells out of *in vivo* assays, 1 ml of cell suspension was removed from cultures with a syringe and immediately processed for SDS protein extraction as described (Hurek *et al.*, 1994).

Analysis of nitrogenase Fe-protein modification by MALDI-TOF MS. Mass spectrometric analysis of the nitrogenase Fe-protein modification in the azo0536::Ω mutant was carried out as recently described (Oetjen et al., 2009). Both forms of nitrogenase Fe-protein were separated by two-dimensional gel electrophoresis, NifH containing spots were excised and further processed by trypsin and endoproteinase Asp-N in-gel double digestion (Oetjen et al., 2009). After extraction peptides were analyzed by MALDI-TOF MS as described (Oetjen et al., 2009).

RESULTS

Diazotrophic growth rate of strain BHOrf12Ω. To study the possible function of the genes co-transcribed with *draT*, a knock-out mutant was constructed by inserting a streptomycin-spectinomycin (Sm/Spc) resistance cassette into azo0536 which probably had a polar effect *dcrH1* (Figure 1). The diazotrophic growth of *Azoarcus* was not impaired by this mutation under controlled microaerobic conditions of 0.6% oxygen. Strain BHOrf12Ω had a similar doubling time of 2.1 ±0.4 hours (two repetitions) as the wild-type (2 hours; Martin *et al.*, 2000) when cells were cultured in a bioreactor on malic acid. In order to verify this result, wild-type strain BH72 and the mutant strain BHOrf12Ω were grown together in a bioreactor to enable exactly the same growth conditions. The determined doubling time was 2.0 ±0.1 hours (two repetitions) under nitrogen fixation conditions as already elsewhere determined for the wild-type in pure culture (Martin et al., 2000). Cell suspension aliquots have been taken at different time points during the experiment and checked for Sm/Spc resistance. At time point 0, when cells had been inoculated to an OD_{578} of 0.004, 40% (representing BHOrf12Ω) could grow on Sm/Spc containing agar plates. At an OD_{578} of 0.35 nearly the same amount of colonies (41.5%) were identified as BHOrf12Ω, while at an OD_{578} of 1.3 with the beginning of the stationary phase 50% of the colonies were detected on Sm/Spc containing agar, clearly showing that under these conditions growth rate of strain BHOrf12Ω was normal or even slightly elevated.

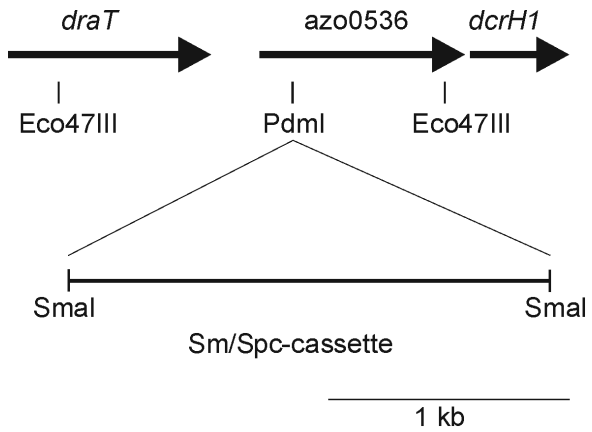

Fig. 1. Gene organization of the azo0536 region. Restriction sites for enzymes used are indicated. A 2 kb SmaI fragment carrying a resistance gene for streptomycin and spectinomycin (Sm/Spc-cassette) was integrated into the PdmI site of azo0536. The same fragment was used for construction of a probe that was taken for Southern hybridization to verify correct insertion (not shown).

Interestingly, when cells were grown in cultures in rubber-stopper sealed Erlenmeyer flasks under nitrogen fixation conditions with a starting oxygen concentration of 1.4%, diazotrophic

growth of strain BHOrf12Ω was impaired (Fig. 2). In comparison to wild-type strain BH72 with an doubling time of 2.9 ±0.2 hours in the Erlenmeyer approach, the determined doubling time of *Azoarcus* mutant strain BHOrf12Ω was 3.8 ±0.6 hours (the experiment was performed to two times with three parallels each). This suggests that the higher starting oxygen concentration under these conditions compared to the bioreactor approach was inhibitory for the nitrogen fixation reaction in strain BHOrf12Ω.

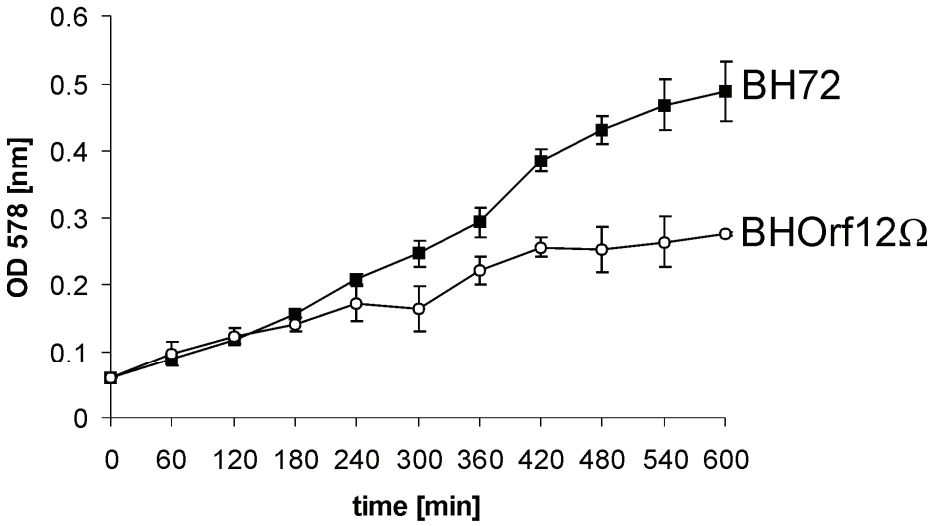

Fig. 2. Growth of *Azoarcus* sp. BH72 wild-type and azo0536 mutant BHOrf12Ω as indicated under nitrogen fixing conditions in Erlenmeyer flasks. Error bars derive from two independent experiments with three parallels each.

Nitrogenase activity and "switch-off" after addition of low ammonium chloride concentrations. Since strain BHOrf12Ω showed a diazotrophic growth deficiency under the conditions of inconstant oxygen concentrations, it is interesting to study the ability of this strain to fix nitrogen. Therefore the *in-vivo* acetylene reduction rate was determined in two independent experiments with two repetitions each. Strain BHOrf12Ω was able to fix nitrogen, but nitrogenase activity was relatively low in comparison to strain BH72 (45 ±34 nmol ethylene/ mg protein × min for strain BHOrf12Ω or 106 ±21 nmol ethylene/ mg protein × min for strain BH72, respectively). An ammonium-induced switch-off was possible in strain BHOrf12Ω, and nitrogenase could be reactivated after addition of 200 µM ammonium chloride (Fig. 3). However, it appeared that nitrogenase reactivation was delayed in strain BHOrf12Ω in comparison to the wild type which might be a side effect of the anyway restricted growth capability of this strain.

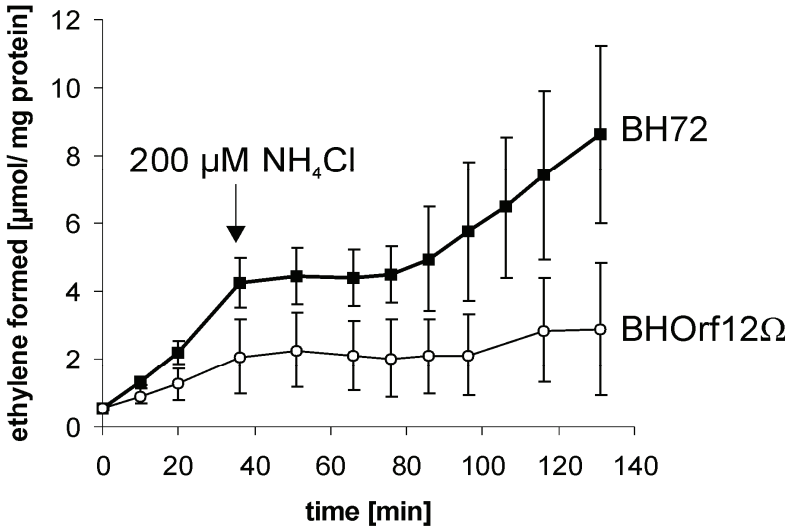

Fig. 3. Comparison of acetylene reduction activity of the wild-type strain BH72 in comparison to the azo0536 knock-out strain BHOrf12Ω before and after "switch-off" that was induced by addition of 200 µM NH_4Cl, as indicated by an arrow. Error bars result from two individual experiments with two parallels each.

Formation of a higher molecular mass form of the Fe-protein during SDS-PAGE occurred in the azo0536-mutant after 200 µM ammonium chloride addition to nitrogen fixing bacteria (Figure 4). A second protein of lower electrophoretic mobility was observed in Western blots with antiserum against the NifH-protein and it disappeared after assimilation of the external nitrogen source. Therefore the decrease in the nitrogen fixation rate was likely to be not due to a higher degree of modified dinitrogenase reductase dimers in strain BHOrf12Ω. It even appeared that the relative amount of modified Fe-protein was reduced in comparison to the wild type, as the bands corresponding to the higher molecular mass form of this protein were only faintly visible. However, the phenotypical differences were not pronounced and therefore not significant.

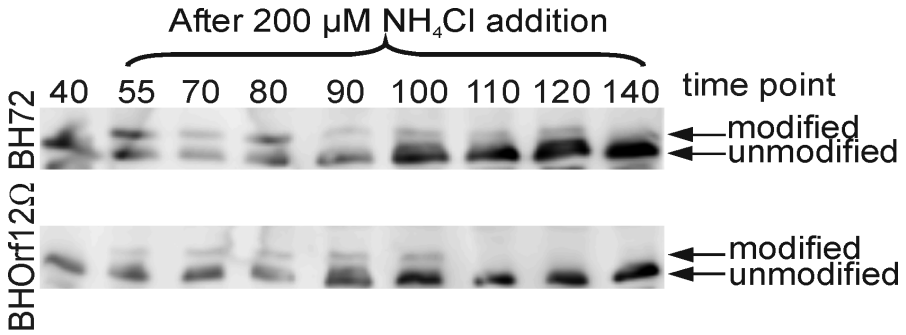

Fig. 4. Western blot with antiserum against NifH of protein extracts from representative cultures of 200 µM ammonium shock experiments shown in Figure 2. Like wild-type strain BH72, strain BHOrf12Ω could modify and demodify its nitrogenase Fe-protein.

ADP-ribosylation of the Fe-protein in the azo0536 mutant. To investigate weather the gene products of azo0536 or azo0535 (*dcrH1*) had any detectable effect on the covalent modification of the Fe-protein itself, the modified Fe-protein was analyzed by MALDI-TOF MS. However, a peak of 3765 m/z representing the ADP-ribosylated peptide 87-117 was identified in strain BHOrf12Ω, and no difference was observed to the spectra of the wild-type (Fig. 5, and compare to (Oetjen et al., 2009) Fig. 3). Thus the gene products of azo0536 or azo0535 did not have any detectable influence on the nature of the Fe-protein modification.

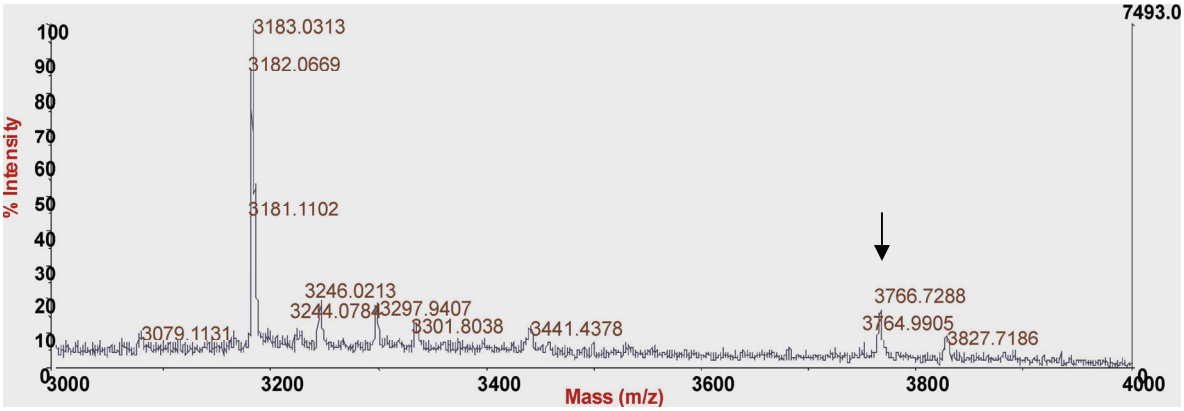

Fig. 5. MALDI-TOF analysis of modified Fe-protein from *Azoarcus* strain BHOrf12Ω. A peak specific for the ADP-ribosylated peptide 87-117 was observed (arrow) .

Nitrogenase *in vitro* activity of modified and unmodified Fe-protein. An unusual partial nitrogenase reactivation was observed in *Azoarcus* mutants impaired in Fe-protein demodification in mutants of *draG1* and in a *draG1draG2* double mutant (Oetjen & Reinhold-Hurek, 2009). Hence, the question arose if nitrogen fixation in this bacterium might be possible with the Fe-protein in its ADP-ribosylated form. In order to address this issue, nitrogenase *in vitro* assays have been performed in cooperation with A. Norén (Department of Biochemistry and Biophysics, Stockholm University, Stockholm). Crude extracts have been prepared from a *draG* double mutant treated with 2 mM ammonium chloride or incubated for 15 min under conditions of anaerobiosis to induce Fe-protein modification, and were compared to the *draT* mutant serving as the positive control with unmodified Fe-protein. Surprisingly, in case of modified Fe-protein obtained from strain BHΔdraG1ΔdraG2 which has been treated with ammonium chloride, a weak increase in acetylene reduction could be determined (Fig. 6A), although NifH was nearly completely modified (Fig. 6C). However, as a full modification is hardly achievable and the unmodified and modified forms of the

Fe-protein could not be separated from each other, remaining activity might have been accomplished by residual unmodified protein. In addition, a higher increase in ethylene production took place in assays with the unmodified Fe-protein. No activity of modified Fe-protein was detectable *in vitro* when cells had been shocked by anaerobiosis in comparison to unmodified Fe-protein with low but detectable activity (Fig. 6B). Results from the respective Western blot demonstrated that nitrogenase Fe-protein was modified before and after the assay when protein derived from strain BHΔdraG1ΔdraG2 (Fig. 6D). This suggests that Fe-protein ADP-ribosylation in *Azoarcus* sp. BH72 led to nitrogenase inactivation *in vitro*.

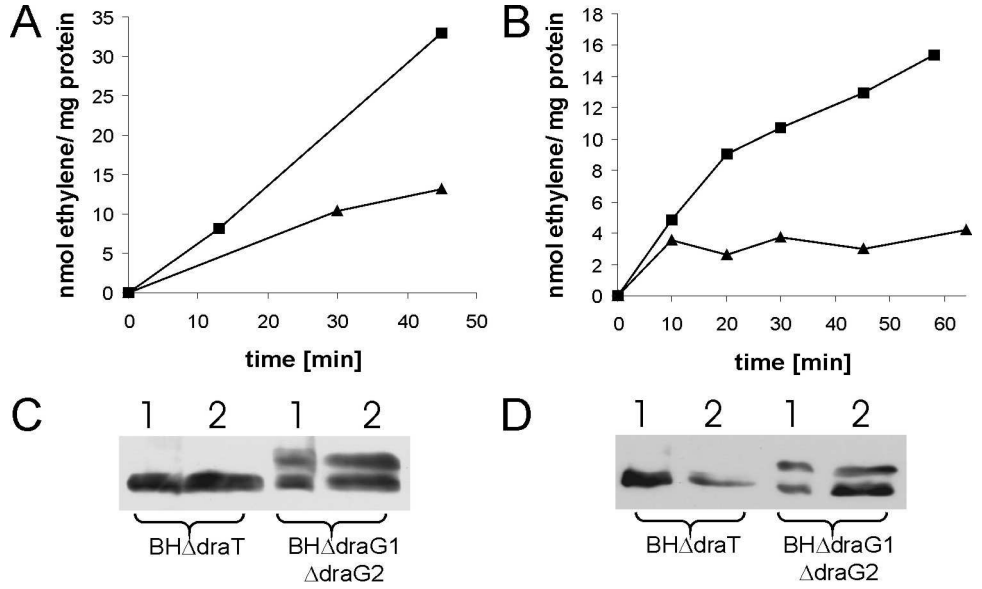

Fig. 6. *In vitro* assay of nitrogenase activity of extracts of a *draT* mutant (—■—) and a *draG1draG2* double mutant (—▲—), shocked by 2 mM ammonium chloride (A) or by anaerobiosis (B). (C, D): Western blots from cytoplasmic fractions (lane 1) and cytoplasmic fractions after *in vitro* assays (lane 2) from the *draT* mutant and *draG* double mutant as indicated after ammonium chloride treatment (C) or anaerobiosis (D).

DISCUSSION

In this work the putative physiological role of the gene products of azo0536 and *dcrH1* in the posttranslational regulation of nitrogenase and on the diazotrophic growth potential was investigated in the endophytic bacterium *Azoarcus* sp. BH72. The knock out mutation in azo0536, which was most probably polar on *dcrH1*, did not affect the posttranslational control system of this betaproteobacterium *per se*. The azo0536 knock-out mutant strain BHOrf12Ω was able to perform an ammonium-induced switch-off and the immunological detection of a modified Fe-protein suggested that the ADP-ribosylation reaction took place after induction with the respective stimulus. MALDI-TOF analysis verified that the covalent modification of

nitrogenase Fe-protein in strain BHOrf12Ω indeed occurred *via* ADP-ribosylation. However, a phenotype was detected, as the diazotrophic growth of strain BHOrf12Ω was reduced in Erlenmeyer flasks with shifting oxygen concentrations while it was normal when cells were grown under controlled conditions of 0.6% oxygen in a bioreactor. Since a polar mutation was constructed, it is not possible to specify weather one of the two gene products or even both were responsible for the observed phenotype.

Unfortunately, assigning a putative function to azo0536 with the help of bioinformatic tools failed, because its gene product does not show any reliable sequence similarity to protein domains of known function in the Pfam-database. However, *dcrH1* was found to encode a protein with sequence identity to a single-domain hemerythrin. These proteins can bind oxygen through a nonheme di-iron center and they occur in invertebrates as single-domain hemerythrins and in a quite high number of bacteria as single- or multi-domain hemeryrhins (reviewed in French *et al.*, 2008). The putative molecular functions of hemerythrins involve (i) oxygen binding as a storage mechanism or for delivery to oxygen-requiring enzymes (Karlsen *et al.*, 2005); (ii) binding of oxygen as a sensory mechanism (often multi-domain hemerythrins) (Isaza *et al.*, 2006); (iii) binding of oxygen as a detoxification mechanism (French et al., 2008); (iv) binding of iron as a storage mechanism (Baert *et al.*, 1992) and (v) binding of iron or other metals as a detoxification mechanism (Demuynck *et al.*, 1993). With this knowledge, it is tempting to speculate that DcrH1 in *Azoarcus* sp. BH72 may act as an oxygen-binding protein that protects nitrogenase from oxygen damage under conditions of slightly elevated oxygen concentrations, as 1.4%. This would explain, why a diazotrophic growth deficiency of *Azoarcus* sp. strain BHOrf12Ω has been only observed in cultures with higher starting oxygen concentrations and not in a bioreactor, where oxygen levels were kept low at 0.6%. An oxygen scavenging protein might be important for an adaptation to changing oxygen concentrations in the natural environment of the endophytic bacterium *Azoarcus* sp. BH72. However, this will have to be corroborated by analysis of a deletion mutation of the *dcrH1* gene. The growth experiments would need to be repeated with simultaneous measurement of the acetylene reduction and the oxygen concentration in Erlenmeyer flasks. Furthermore, spectroscopic evidence for the oxygen-binding capacity of DcrH1 will need to be provided.

The unusual features with respect to nitrogenase posttranslational regulation like the partial nitrogenase reactivation after anaerobiosis shifts and subsequent adjustment to microaerobiosis which have been observed in a *draG1*-mutant in *Azoarcus* (Oetjen & Reinhold-Hurek, 2009) could not be explained by the analysis of the azo0536 knock-out

mutant. It might be possible that the gene product of azo0536 plays a role in addition to DraG1 in the nitrogenase "switch-on" mechanism. However, this would need to be clarified by an in-frame or deletion mutation of this gene. In addition, physiological analysis of a *draG1*-azo0536 double mutant might assist in clarifying this question. A normal Fe-protein ADP-ribosylation could be detected in the knock out mutant BHOrf12Ω after ammonium addition to nitrogen-fixing cells, but it seemed that lesser dinitrogenase reductase dimers were modified. Unfortunately, up to now this does not allow for reliable conclusions as more Western blot experiments would need to be performed. Moreover, it would be interesting to study the ADP-ribosylation capability of strain BHOrf12Ω in response to anaerobiosis treatment.

In addition to the putative role of DcrH1, it was verified here that nitrogenase *in vitro* activity was diminished when Fe-protein has been ADP-ribosylated, implying that modified Fe-protein is inactive.

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CHAPTER 3

Identification of proteins accompanying diazosome-related N_2-fixation by the grass endophyte *Azoarcus* sp. strain BH72 by proteomics

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Status of the manuscript: In preparation for submission to the Journal of Bacteriology.

Own contribution:

- Pure and co-culture cell growth in collaboration with F. Battistoni
- Extraction and fractionation of bacterial proteins in collaboration with F. Battistoni
- Two dimensional gel electrophoresis in collaboration with F. Battistoni
- Rewriting of the manuscript

Contributions F. Battistoni:

As depicted above and:

- Image Master analysis
- Alcohol dehydrogenase assays
- Writing of the first version of the manuscript
- Construction of Table 1

Contributions C. Scharf:

- Protein identification by MALDI-TOF analysis

Contributions T. Karg:

- HPLC analysis

Note: The manuscript style has been adapted for better reading purpose.

Identification of proteins accompanying diazosome-related N_2-fixation by the grass endophyte *Azoarcus* sp. strain BH72 by proteomics

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Running title: Proteomic analysis of the N_2-fixing *Azoarcus* sp. BH72.

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Abstract

The betaproteobacterium *Azoarcus* sp. BH72 can supply nitrogen to its natural host Kallar grass under gnotobiotic conditions and is, as an endophyte of grasses, an interesting model organism for investigations of the nitrogen fixation process. In co-culture with the fungal strain 2003, an endophytic ascomycete of Kallar grass, *Azoarcus* sp. BH72 can develop intracytoplasmatic membrane stacks, termed diazosomes that are associated with an efficient and economic nitrogen fixation reaction. Diazosomes are absent under standard N_2-fixing conditions. With the aim to identify diazosome related proteins, a differential display proteomic approach was conducted on strain BH72 grown under both N_2-fixation conditions. Membrane and cytoplasmic proteins were isolated from *Azoarcus* sp. BH72 and from a non-diazosome forming mutant strain BH1599. Different two-dimensional polyacrylamide gel electrophoresis profiles were compared and proteins of interest were identified by MALDI-TOF mass spectrometry. Results suggested that a differentiation process occurred in strain BH72 cells during co-culture growth. We identified proteins specifically up-regulated or induced in co-culture cells of the wild type, which therefore might be important for the diazosome specific nitrogen fixation process, the formation of the internal membranes or the bacterium-fungus interaction. Our results indicated that electron transport processes play an important function for the metabolism of diazosome containing cells of *Azoarcus* sp. BH72. The detection of several membrane proteins, conserved hypothetical or hypothetical secreted proteins may be the basis for further studies.

INTRODUCTION

Oxygen plays an important role in biological nitrogen fixation. In their natural aerobic environment diazotrophs often have to deal with very low O_2 concentrations which allow energy generation and N_2-fixation simultaneously, as the key enzyme for nitrogen fixation - nitrogenase - is oxygen-sensitive. N_2-fixation takes place in grass root-associated bacteria such as *Azospirillum* spp. and *Azoarcus* spp. at dissolved oxygen concentration (DOC) around 1 µM (24, 44). The model endophyte *Azoarcus* sp. BH72 was isolated from the endorhizosphere of Kallar grass, a C4 plant grown in the Punjab of Pakistan (44, 46) and was shown to supply nitrogen to its host plant in an "unculturable" state (21). Moreover *in situ* hybridization demonstrated that *Azoarcus* sp. nitrogenase genes (*nif*) are expressed in the aerenchyma of uninoculated field-grown Kallar grass plants (20). In gnotobiotic culture strain BH72 shows a similar colonization pattern of rice roots in comparison to its original host (23) and the *nifH* gene was shown to be expressed in the aerenchyma (14). An unusual feature of *Azoarcus* sp. BH72 is the formation of intracytoplasmic membrane stacks, diazosomes (25). Cells fixing nitrogen under standard conditions (e.g. around 2 µM of dissolved oxygen) do not harbor these membranes. They become apparent (25) in the case of hyperinduction, a physiological state of augmented activity and efficiency of nitrogen fixation in optimized batch cultures at extremely low O_2-concentrations (25). Mutational analysis of structural genes and immunohistochemical localization of the dinitrogenase reductase suggested that these highly organized structures are involved in the efficient nitrogen fixation process (25). A reproducible induction of these intracytoplasmic membrane stacks occurred in co-cultures of strain BH72 with an ascomycete strain 2003 (related to *Acremonium alternatum*), which was isolated from surface-sterilized roots of Kallar grass (22). Bacteria adhered to the actively respiring fungus may encounter extremely low DOCs which are maintained over a long period of time. These conditions might trigger the induction of diazosomes (25). Global changes in protein patterns of total cellular and membrane proteins were detected in diazosome-containing bacteria in comparison with cells fixing N_2 under standard conditions, however only few proteins could be identified by N-terminal amino acid sequence analysis (27).

Recently the genome of strain BH72 has been sequenced and annotated (30). This was the basis for a comprehensive proteomic analysis, which allows for the detection of constitutive expressed proteins as well as proteins specifically induced in diazosome containing bacteria. Membrane and cytoplasmic fractions were compared from cells grown under pure or co-

culture conditions. Different 2D-PAGE gels were evaluated and spots of interest were analyzed by MALDI-TOF for their identification. Membrane and cytoplasmic 2D-PAGE proteomic patterns of N_2-fixing strain BH72 grown in pure- and co-culture had significant differences reflecting adaptations in the bacterial metabolism to cope with the different environmental conditions studied. Metabolic pathways were reconstructed from both conditions and new proteins were identified and correlated with previous and current data acquired by using additional functional approaches. The results obtained here contribute to a better understanding of the *Azoarcus* sp. BH72 ecology as well as the role of the diazosomes in the strain BH72 metabolism. Condition-specific proteins identified with this work will provide new targets for genome-wide mutagenesis.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions. Strain BH1599 is a mutant of wild-type *Azoarcus* sp. BH72 (46) which carries a transposon insertion in the *pilAB* genes, is unable to form Type IV pili and shows decreased attachment to the fungal mycelium and diazosome formation in co-culture. Fungus strain 2003 has been isolated from the rhizosphere of Kallar grass (25). *Azoarcus* sp. or the fungal strain 2003 were grown at 37° or 30°C, respectively, on VM-ethanol agar plates (44). For standard nitrogen fixation in pure culture, *Azoarcus* sp. BH72 was grown microaerobically in nitrogen-free SM medium essentially as described previously (45) in an oxygen controlled bioreactor (Biostat B; B. Braun Melsungen AG, Melsungen, Germany). Precultures were grown in 20 ml of SM medium with overnight shaking, and cells were washed two times in nitrogen-free SM medium. Temperature and stirring were set to 37°C and 600 rpm, respectively and the oxygen concentration to 0.6%. Cultured cells were harvested at OD_{578} = 0.65. For co-cultures of *Azoarcus* strains and the fungal isolate 2003, bacterial and fungal cells were precultured separately on VM-ethanol agar and then cultivated together in sealed 1-liter serum flasks as previously described (25). Cells were harvested when the O_2 concentration in the headspace had decreased to 4.0%. Quantification of the oxygen- and ethylene concentration was done by gaschromatography as previously described (27).

Protein extraction and solubilization. Prior to protein extraction bacterial cells from co-cultures were separated from the fungal spores and hyphae by treatment with detergents and differential filtration as previously described (25). Cells were resuspended at 4°C in 50 mM phosphate buffer (pH 7) and disrupted by four passages through a French press cell at 1500 lb/in^2. Cells debris was removed by centrifugation (20 min at 20000 × g at 4°C) and the remaining supernatant was used for membrane sedimentation by ultracentrifugation (2 h 200,000 × g at 4°C). The supernatant was referred to as the soluble cytoplasmic fraction. Proteins out of this fraction were precipitated by trichloroacetic acid (TCA) as described (33) and washed two times with ice cold acetone. The pellet after ultracentrifugation (referred to as the membrane fraction) was washed twice with 50 mM phosphate buffer (pH 7.0) containing 600 mM NaCl to remove nonspecifically or loosely bound proteins. The membrane pellet and the cytoplasmic fraction were dissolved in extraction buffer (0.7 M sucrose, 0.5 M Tris, 30 mM HCl, 0.1 M KCl, 50 mM EDTA; 2% (vol/vol) 2-mercaptoethanol), sonicated until they have been resuspended and purified with Tris·Cl-buffered phenol (pH 8.0) essentially as described (35). Proteins were precipitated with 0.1 M ammonium acetate in methanol overnight at -20°C. Final pellets were resuspended in 50-80 µl of sample buffer (5 M urea, 2 M thiourea, 2% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2% (w/v) 3-[N,N-dimethyl(3-myristoylaminopropyl)ammonio]propanesulfonate amidosulfobetaine-14 (ASB-14), 10 mM DTT and 2% (v/v) ampholines pH 3-10 (BioRad, Hercules, California, USA). Protein concentration was determined using a RC DC Protein Assay kit (BioRad).

Two dimensional gel electrophoresis. Isoelectric focusing (IEF) in glass tubes as a first dimension and SDS-PAGE as a second dimension were carried out as previously described (25) except that approximately 200 µg of protein in a sample volume of 50 µl to 80 µl was loaded on the top of the IEF tube gels. Before performing the second dimension extruded IEF gels were incubated 20 min in equilibration buffer (60 mM Tris·Cl, pH 6.8, 1% SDS, 20% glycerol, 50 mM dithiothreitol). Vertical gel electrophoresis in 13×16 cm SDS-PAGE gels was carried out with a 12 % (wt/vol) polyacrylamide gel as described by Laemmli (31).

Staining procedures and image analysis. Coomassie Brilliant Blue staining and scanning of the gels was carried out as described (35). Spot detection, quantification, background subtraction, gel-to-gel matching and

differential display analysis were performed on three independent biological repetitions. Intensities of individual spots were normalized based upon the total density of all spots detected in each gel. Apparent masses of each protein spot were calculated by the use of the software, according to the molecular weight ladder used (Unstained Protein Molecular Weight Marker, Fermentas International Inc., Burlington, Canada).

Preparation of peptide mixtures for MALDI-MS. Proteins were manually excised from Coomassie Brilliant Blue stained 2D gels and transferred into 96 well microplates loaded with 100 µL LiChrosolv® water (Merck, Darmstadt, Germany) per well. Digestion with trypsin and subsequent spotting of peptide solutions onto the MALDI-targets were performed automatically in an Ettan Spot Handling Workstation (Amersham-Biosciences, Uppsala, Sweden) using a modified standard protocol. Briefly, gel pieces were washed twice with 100 µL 50 mM ammoniumbicarbonate/ 50% (v/v) methanol for 30 min and once with 100 µL 75% (v/v) acetonitrile (ACN) for 10 min. After 17 min drying 10 µL trypsin solution containing 20 ng/µL trypsin (Promega, Madison, WI, USA) in 20 mM ammoniumbicarbonate was added and incubated at 37°C for 120 min. For peptide extraction gel pieces were covered with 60 µL 50% (v/v) ACN/ 0.1% (w/v) trifluoroacetic acic (TFA) and incubated for 30 min at 37°C. The peptide containing supernatant was transferred into a new microplate and the extraction was repeated with 40 µL of the same solution. The supernatants were dried at 40°C for 220 min completely. Peptides were dissolved in 2.2 µL of 0.5% (w/v) TFA/ 50% (v/v) ACN and 0.7 µL of this solution were directly spotted on a MALDI-target. Then, 0.4 µL of matrix solution (50% (v/v) ACN/ 0.5% (w/v) TFA) saturated with alpha-cyano-4-hydroxycinnamic acid (CHCA) was added and mixed with the sample solution by aspirating the mixture five times. Prior to the measurement in the MALDI-TOF instrument the samples were allowed to dry on the target for 10 to 15 min.

MALDI-TOF MS analysis. The MALDI-TOF measurement of spotted peptide solutions was carried out on a Proteome-Analyzer 4700 (Applied Biosystems, Foster City, CA, USA). The spectra were recorded in reflector mode in a mass range from 800 to 3700 Da with a focus mass of 2000 Da. For one main spectrum 25 sub-spectra with 100 shots per sub-spectrum were accumulated using a random search pattern. If the autolytic fragments of trypsin with the mono-isotopic $(M+H)^+$ m/z at 1045.5 and/or at 2211.1 reached a signal to noise ratio (S/N) of at least 10, an internal calibration was automatically performed using at least one peak for one- or two peaks for two-point-calibration. Calibration was performed manually for the less than 1% samples for which the automatic calibration failed.

Additionally, MALDI-TOF-TOF analyses were performed for the five strongest peaks of the TOF-spectrum after subtraction of peaks corresponding to background, keratin and trypsin fragments. For one main spectrum 20 sub-spectra with 125 shots per sub-spectrum were accumulated using a random search pattern. The internal calibration was automatically performed as one-point-calibration if the mono-isotopic arginine (M+H)+ m/z at 175.119 or lysine (M+H)+ m/z at 147.107 reached a signal to noise ratio (S/N) of at least 5.

After calibration a combined database search of MS and MS/MS measurements was performed using the GPS Explorer software (Applied Biosystems, Foster City, CA, USA) with the following settings: (i) MS peak filtering: mass range from 800 to 3700 Da; minimum S/N filter of 10; peak density of 50 peaks per range of 200 Da and maximal 200 peaks per protein spot; mass exclusion list contained background peaks and trypsin fragments with an exclusion tolerance of 100 ppm (ii) MS/MS peak filtering: mass range from 60 Da to a mass that was 20 Da lower than the precursor mass; peak density of 50 peaks per 200 Da and maximal 65 peaks per MS/MS; minimum S/N filter of 10 (iii) database search: precursor tolerance 35 ppm and MS/MS fragment tolerance 0.65 Da.

The peptide search tolerance was 35 ppm but the actual RMS value was between 5 and 15 ppm. Peak lists were compared with an *Azoarcus* sp BH72 specific database using the Mascot search engine (Matrix Science Ltd, London, UK). Peptide mixtures that yielded a mowse score of at least 49 were regarded as positive identifications ($P < 0.05$).

Analysis of fungal exudates by HPLC. To analyse the exudates produced by the fungus strain 2003 a HPLC system (Sykam, Gilching, Germany) with a S1000 pump and a RI-detector ERC 7512 (Erma, Tokyo, Japan) was used. Peaks were evaluated with the help of an integration programme (Sykam). 100 µl of sterile-filtered supernatant from co-cultured cells as well as from fungus strain 2003 grown without bacteria was loaded onto a stainless steel column with a DVB-styrene-copolymer as stationary phase. As negative control 100 µl of FU-medium was applied. Material was eluted from the column with 1 mM sulphuric acid at 0.8 ml/min. A standard including lactate, formiate, acetate, propionate, buryrate, isopropanol, valerate, n-butanole and capronoate was used, where each compound was adjusted to 100 µM. As additional standards 1 mM solutions of glucose, formiate, acetaldehyde, formaldehyde, succinate, methanol and ethanol were applied.

Ethanol production by the fungus in the growth medium. Ethanol production by strain 2003 grown in pure and co-culture with *Azoarcus* sp. BH72, was determined by using an enzyme assay (alcohol dehydrogenase EC: 1.1.1.1) (26). 500 µl of cell-free culture supernatant obtained by centrifugation at 4°C (10 min 14000 × g) was applied as a substrate for the enzymatic assay. Two independent biological experiments were done with four different cultures per condition.

Siderophore detection in the SM and FU culture media. Detection of hydroxamate and catechole-type siderophores in culture supernatant was carried out as previously described (4, 5). For siderophore detection independent of their structure a chrome azurol-S (CAS) assay was used as described (50).

RESULTS AND DISCUSSION

Comparison of two-dimensional membrane and cytoplasmic proteome patterns of *Azoarcus* sp. strain BH72 grown under pure and co-culture N_2-fixation conditions. Proteome patterns of membrane and cytoplasmic fractions from *Azoarcus* sp. strain BH72 grown under different N_2-fixing condition were compared with the aim to detect proteins specifically expressed in co-culture with the ascomycete strain 2003. ImageMaster® software was used to process three different gels originating from independent experiments out of twelve biological repetitions per condition. This analysis software allows for the quantitative comparison of protein spots while adjusting for experimental variations caused by problems such as irregular protein loading or staining variations. For gel alignments, the spot matching alignment algorithm was used. In the comparative analyses, the proteome patterns from N_2-fixing cells grown under pure culture condition were taken as references, while the mutant strain BH1599 was used as a negative control. This pilus mutant strain shows decreased attachment to the fungal mycelium and no diazosome formation in co-culture (13).

Pure-culture membrane and cytoplasmic proteome patterns of strain BH72 revealed significant differences to dual-culture patterns. Approximately 150 spots per condition were evaluated on the Coomassie-stained 2D gels within the pH 3-10 and size range 10-122 kDa (Figure 1). A cut-off of 2 fold was used in the ImageMaster® software to specify that a particular spot was up-regulated or down-regulated. In membrane fractions 53% of the spots were absent or down-regulated while 18% were novel or up-regulated under co-culture conditions (Figure 1E and F). From the last group, ten novel spots were detected in the wild-type proteome pattern (Figure 1E, spot numbers 19; 33; 52; 92; 95; 101; 132; 141; 147; 174). These proteins might be related to the presence of internal membranes formed under co-culture conditions or might be important for the bacterium-fungus interaction. Within the cytoplasmic fraction, 46% and 10% of the spots were down- or up-regulated, respectively, compared to the reference gel pattern (Figure 1A and B). In this case four novel induced spots were found in the wild-type proteome pattern (Figure 1B, spot numbers 15; 28; 55; 65). The observed down regulation of proteins from both fractions in co-culture indicated that under these conditions, cells may have a more specialized metabolism to cover their metabolic requirements with fewer enzymes. Consistent with this observation, a decrease of proteins under co-culture growth compared to standard nitrogen fixation conditions was already earlier observed (27).

The most abundant proteins present on Coomassie-stained 2D gels were picked and analyzed by mass spectrometry (MALDI-TOF). In total, approximately 800 protein spots were evaluated from several gels in the pH range of 3-10. The gene products of 180 different genes were identified by Peptide Mass Fingerprints (PMF), which represent 4.5% of the genome. 140 gene products out of this group contained EC number. These numbers were used to search the Kyoto Encyclopedia of Genes and Genomes (KEGG) database for possible metabolic pathways.

As shown in Table 1, nineteen membrane proteins, including outer membrane proteins, were identified. Using the SMART (49) and TMHMM (51) blast analysis, transmembrane domains were detected in three of the amino acid sequences. Moreover, seven hypothetical secreted or conserved hypothetical secreted proteins have been also identified; in this case two of the protein sequences possessed a transmembrane domain (Table 1, spot numbers 157 and 159). This confirmed that the methodology for membrane protein extraction and separation has been successfully applied. Nevertheless, a degree of under-representation of membrane proteins is expected. For example out of the five ABC-transporter systems identified, only the ATP-binding protein or the periplasmic binding protein subunits were detected (Table1). This was also observed in proteomic studies of membrane fractions in *Sinorhizobium meliloti* and *Alcanivorax borkumensis* (12, 48).

Several proteins in *Azoarcus* sp. BH72 produced multiple spots probably due to post-translational modifications. In most cases the shift in position was horizontal, suggesting that the modification influenced only the isoelectric point and left the molecular mass substantially unchanged (Figure 1 and Table 1). Exceptions to that were NifH, NifD and NuoG, from which two isoforms with different molecular weight have been identified (Figures 1A, E, F, spot numbers 1; 2; 97). The modifying group of NifH represents an ADP-ribosylation (33, 39, 40).

Metabolic activity profiles in *Azoarcus* sp. BH72

Nitrogen metabolism. As expected the pathways involved in the N_2-fixing process and subsequent assimilation of the ammonia produced were active under all conditions tested. The three structural subunits of the nitrogenase complex NifHDK were identified under the conditions studied, as well as enzymes related to its synthesis (NifS, NifU, NifN and NifE) and activation (NifM). Previous work in strain BH72 demonstrated that in pure-culture the NifH protein is uniformly disperse in the cytoplasm, while in co-culture conditions it was mainly located on internal membrane stacks formed (diazosomes) (25). In agreement, here the NifH protein was down-regulated in co-culture cytoplasmic fractions, while it was always present in membrane fractions. Moreover, the NifM protein, which is required for activation and stabilization of NifH (47), was down-regulated in the cytoplasm and up-regulated in the membrane fraction.

Other proteins involved in nitrogen fixation or assimilation like for example a ferredoxin (FdxD), the global regulator NtrC, the glutamine synthetase type I (GlnA) and a protein probably involved in glutamine transport (GlnQ1) were identified, confirming that an active nitrogen fixation and assimilation process was taking place under all studied conditions. In addition the large subunit of the uptake hydrogenase complex (HupL) was detected to be constitutively expressed in strain BH72 N_2-fixing cells. Hydrogenases are responsible for the recycling of the hydrogen produced by nitrogenase (see reference (52) for a review).

Interestingly, CarA and CarB the two subunits of a carbamoyl phosphate synthetase (glutamine-hydrolyzing) protein were detected to be constitutively expressed in the cytoplasm and up-regulated in the membrane, respectively. In particular, the large subunit (CarB) was unique in the wild-type strain. The carbamoyl phosphate synthetase is involved in the glutamine assimilation by forming glutamate and carbamoyl-phosphate as a part of the glutamate and pyrimidine metabolism (1, 19). Since no GOGAT protein was detected under the conditions studied, the glutamine assimilation to glutamate might occur in *Azoarcus* sp. BH72 by using this pathway. However the GS-GOGAT system is present in the *Azoarcus* sp. BH72 genome (30).

Another enzyme related to nitrogen metabolism is the UreC protein, which was induced in the wild type. The urease alpha subunit (UreC) is part of the urease complex, which catalyzes the hydrolysis of urea to form ammonia and CO_2. Since a complete urea cycle is not encoded in the *Azoarus* sp. BH72 genome (30) no urea should be produced by this strain. Therefore

urea could have been formed by the fungus strain 2003, serving as an alternative nitrogen source for strain BH72.

Carbon metabolism. In order to meet the fungus growth demands, the co-culture medium was supplied with the osmoprotectants betaine hydrochloride and choline chloride, L-proline, vitamins and glucose. It should be noted, that *Azoarcus* sp. BH72 is unable to grow on glucose (44). Differences with respect to carbon metabolism are therefore expected when strain BH72 was grown in pure or in co-culture with the ascomycete strain 2003. The SM medium, which was used under pure culture conditions, contained L-malate as sole carbon source (45). The added concentration of L-malate in the FU medium was chosen to be 1% from those taken under pure culture conditions with the aim to promote the growth of strain BH72 in the beginning and to bypass the lag-phase (25). Therefore after consumption of the malate, *Azoarcus* sp. BH72 was dependent on fungal exudates as C-sources. HPLC analysis demonstrated that under pure culture growth conditions strain 2003 excreted acetate, lactate, formiate and an unidentified compound (final concentration in mM: 1.13, 0.89 and 1.0 respectively; 100% for the unidentified). Using the same approach it was also demonstrated that under co-culture conditions, the amount of acetate and lactate but not formiate decreased drastically (final concentration in mM: 0, 0, and 0.83 respectively; 60% for the unidentified). These results are in correlation with experiments showing that *Azoarcus* sp. BH72 can grow on acetate and lactate but not on formiate as a sole carbon source (44). We could detect here an induction of a probable acetyl-coenzyme A synthetase (AcsB) in cells grown under co-culture conditions (Table 1, spot number 40 and Figure 2). AcsB catalyze the formation of acetyl-coA from acetate. The produced acetyl-coA could be channeled into several metabolic pathways including the tricarbon acid cycle (TCA), glyoxylate cycle, propanoate metabolism and fatty acids biosynthesis from where enzymes have been identified (Table1). Alternatively, acetyl-coA can be converted by the ThlA enzyme, which was detected constitutively expressed, into acetoacetyl-CoA. This compound could be directed into the poly-β-hydroxybutyrate (PHB) biosynthetic pathway, from where two enzymes involved in their synthesis and regulation were up-regulated (Table 1, spot numbers 64 and 65).

Almost all the enzymes involved in the TCA-cycle were identified in the different conditions studied (Table 1 and Figure 2), confirming the key role of this pathway in the *Azoarcus* sp. BH72 metabolism for energy production, as well as a source of different metabolic intermediates. This is reflected by the finding that numerous enzymes involved in the amino acid, fatty acid or cofactor biosynthetic pathways as propanoate or porphyrins, which use TCA intermediate compounds as precursors, have been also detected (Table 1).

The glyoxylate cycle, through which organism can convert acetate into oxaloacetate, was active under the conditions studied. A key enzyme from the glyoxylate cycle, the isocitrate lyase AceA protein, which catalyzes the conversion of isocitrate into succinate and glyoxylate, was up-regulated in co-cultured cells, supporting that strain BH72 can grow on the acetate formed by the fungus. The produced succinate could maintain the levels needed for a continuous flow of the TCA cycle or it could be directed to other key biosynthetic pathways, e.g. porphyrine and heme synthesis. The glyoxylate formed in this reaction could be transformed to malate, which can be used in the TCA cycle.

Constitutive expressed and membrane associated a protein with homology to a $NADP^+$-dependent malic enzyme (MaeB2), which catalyzes the formation of pyruvate from malate, was detected. Pyruvate formed could be channeled into the amino acid biosynthesis pathways or - converted to acetyl-coA - into the TCA-cycle. In correlation with the decreased malate concentration in the FU medium one component of a C4-dicarboxylate transporter system DctP2, was detected to be down-regulated in co-culture cells (Table 1 spot number 151 and Figure 2).

In *Azoarcus* sp. BH72, the TCA and glyoxylate cycles are linked to the gluconeogenesis pathways by the phosphoenolpyruvate carboxykinase (PckG) enzyme, which was up-regulated in co-culture (Table 1 spot number 33, Figure 2). *pck* mutants in *S. meliloti* showed a reduced level of nitrogen fixation, while in *Rhizobium leguminosarum* no apparent symbiotic phenotype was detected (42). However, this enzyme was found only in proteins extracted from *S. meliloti* cultured cells (11). Under N_2-fixing conditions the role of this protein as a link between these two key pathways in the *Azoarcus* sp. BH72 metabolism especially in co-culture growth seems to be evident. Other enzymes out of the gluconeogenesis pathways, like a probable enolase, a probable phosphoglycerate mutase and a probable phophoglycerate kinase were also active under all studied conditions (Table 1). Fructose-bisphosphate aldolase was down-regulated in cells from co-culture experiments.

Strongly up-regulated in the cytoplasm, two copies of the alcohol dehydrogenase ExaA2 and ExaA3 enzymes, as well as the quinoprotein ExaB1, were detected (Table1). The ExaB1 protein was only present in strain BH1599, while four isoforms of the ExaA2 protein were present in all co-cultured cells (Figure 1B, spot number 48). Spots with the same coordinates in 2D-gels have been previously identified as up-regulated proteins in gels of total protein extract from diazosome-containing cells (27). Alcohol dehydrogenases catalyze the oxidation of ethanol to acetaldehyde. The acetaldehyde formed is most probably converted to acetate by the NAD^+-aldehyde dehydrogenase protein (AldA), which was also up-regulated in co-culture

cells, and then to acetyl-coA by the mentioned AscB enzyme. Therefore enzymes necessary for the assimilation of ethanol were active, when strain BH72 was grown in co-culture with fungus strain 2003. This is in agreement with the fact that ethanol can be used as a sole carbon source (44). In order to determine the ethanol concentration during one week of growth in medium from co-cultures with strain BH72 or strain BH1599 an alcohol dehydrogenase enzyme assay was applied. As a control fungus isolate strain 2003 was grown in pure culture. Results show that the ethanol concentration was high during the first 3 days and decrease to no activity after 5 days. The same result was observed in both pure- and co-culture growth conditions, with the only difference that in co-culture the activity decreased faster (data not shown). Therefore we could show here that the ascomycete strain 2003 produced and alternatively also metabolized ethanol, while strain BH72 was able to utilize this compound as a carbon source in agreement with the up-regulation of enzymes involved in the ethanol assimilation.

Amino acid metabolism. Most of the proteins involved in the amino acid metabolism detected in cells grown under co-culture conditions were down-regulated or constitutively expressed (Table 1). This is in agreement with the previous observation that diazosome-containing cells were arrested in growth (25) and therefore reduce their anabolic demands. Two proteins from the glycine cleavage multienzyme complex (GCV) were identified under all studied conditions. The GCV complex is involved in one carbon transfer reactions and it catalyzes the cleavage of glycine into CO_2, NH_3 and a C1 unit. Like in *E. coli,* the C1 unit could be used as substrates in other metabolic pathways like the biosynthesis of purines, thymidine, histidine, coenzyme A and methionine in *Azoarcus* sp. BH72 (18), from where proteins were also identified (Table 1). However, the glycine cleavage reaction is reversible; glycine can be also synthesized by the GCV complex.

Energy production. The process of nitrogen fixation is highly energy demanding as nitrogenase requires at least 16 molecules of ATP for the reduction of 1 molecule of N_2 to ammonium. During this process, which occurs under extremely low oxygen concentrations, a respiratory chain with high affinity to oxygen closely coupled to ATP production is required. During this work we could detect in the *Azoarcus* sp. BH72 proteome several membrane associated subunits of the NADH-ubiquinone oxidoreductase complex (Nuo), involved in the respiratory chain (Table 1). PetA1, a probable ubiquinol-cytochrome c reductase iron sulfur protein was constitutively expressed. Interestingly CycH, a conserved hypothetical cytochrome c-type biogenesis protein was exclusively detected in co-culture wild-type cells. The *cycHJKL* genes of *S. meliloti* were shown to be required for symbiotic nitrogen fixation

(28). Consistent with the induction of CycH, the gene product of *ccmA* (also termed *helA*) was induced under co-culture conditions. The *ccmABCD* genes encode an ABC transporter specific for heme (16). This complex is a component of the system I for cytochrome c biogenesis that is usually found in alpha- and gammaproteobacteria (17). Therefore a role of cytochrome c in a specialized electron transport in diazosome-containing cells of *Azoarcus* sp. BH72 might be possible. In the cytoplasmic fractions three electron transfer flavoproteins EtfA1, Etf1 and IsiB were identified (Table 1). In particular, the Etf1 protein was up-regulated in correlation with an intensive, high energy demanding N_2-fixation process under co-culture conditions. Finally, several subunits of the ATP synthase were detected membrane-associated under co-culture growth conditions (Table1). In summary, these results are in agreement with the hypothesis that diazosomes formed in strain BH72 under co-culture conditions are not only involved in the N_2-fixation, but also in the respiration process (25).

Protein synthesis and folding. As expected proteins involved in the synthesis, folding and reparation of proteins have been identified under all studied conditions.

A large number of proteins involved in chaperoning were identified including the constitutive expressed GroES1, DnaK, Tig, PpiB, Hsp18 and ClpX proteins (Table 1). The first three appeared to be the major spot in the gels and their role in protein folding, degradation and in the assembly of large protein complexes are well known in bacteria (7). Particularly DnaK (Table 1, spot number 118) was down-regulated in membrane fractions. Different expressed chaperones might reflect that a distinctive set of proteins was produced under the varied growth conditions. A change in the chaperone set might also be due to the different temperatures used during cultivation (Table 1). Some enzymes related to protein processing were up-regulated in cells grown under co-culture conditions. This included PrlC, CtpA and a probable Zn dependent peptidase (azo0765, Table1). The Pcm and the PrlC proteins were detected in the membrane fraction and PrlC, an oligopeptidase, was even identified as a novel protein spot in wild-type cells. Interestingly the gene product of *pnp,* a polyribonucleotide nucleotidyltransferase (PNPase), was induced in wild-type cells. PNPase was reported to participate in RNA degradation (6) and was shown to be involved in the cold shock adaptation in *E. coli* (54). Furthermore PNPase negatively controls the expression of *spv* virulence genes in *Salmonella enterica* (56) and it was shown to control the expression of outer membrane proteins via small noncoding RNAs (2). Interestingly, in *E. coli* PNPase has been also shown to be responsible for residual *in vivo* polyadenylation of RNA in the absence of Poly(A) polymerase and for the incorporation of non-A residues into poly(A) tails (36). A polyadenylated *nifH* mRNA could be isolated from uninoculated Kallar grass roots whose

cDNA was identical to the *Azoarcus* sp. BH72 *nifH* gene(21). The observed tail was heteropolymeric, suggesting other enzymes than Poly(A) polymerase for its synthesis. It might be reasonable to speculate that under specific conditions PNPase accounts for such mRNA processing in *Azoarcus* sp. BH72. Therefore this protein might be also important for *Azoarcus* sp. BH72 during diazosome formation.

Iron uptake and metabolism related proteins. Five putative outer membrane (OM) TonB-dependent receptors for iron compounds, a substrate-binding component (FbpA1) of an ABC iron-transporter system and a copy of the bacterioferritin protein Bfr2, were identified (Table 1). In Gram-negative bacteria, these types of receptors, transporters and storage proteins (Bfr2) are responsible for the specific uptake of ferric-siderophore complexes under iron-limited conditions, as well as in the maintenance of the intracellular iron homeostasis (3). Interestingly, the OM receptor azo3556 as well as the FbpA1 and Bfr2 proteins were up-regulated in co-culture. In particular, the FbpA1 protein (Table 1, spot number 154) was up-regulated only in wild-type co-culture cells, suggesting a specific role of this protein in diazosome formation. In *S. meliloti* FbpA is specific for nodule bacteria (11). The mentioned receptor, azo3556, has high sequence identity to OM receptors of the TonB-dependent receptor family for vitamin B12 uptake. In strain BH72, this gene is located in a genomic region which contains genes related to vitamin B12 uptake and synthesis. Vitamin B12 was supplied in the FU medium used for co-culture growth. This receptor might be involved in the vitamin B12 uptake and the gene be regulated by the presence of vitamin B12.

Blast analysis of the additional OM receptor sequences identified showed a high degree of similarity with putative OM TonB-dependent receptors for ferric-citrate, hydroxamate and catecholate siderophore-types. Siderophore compounds are synthesized and secreted by bacteria under iron limitation (3). In order to study whether *Azoarcus* or isolate 2003 are able to produce siderophores, different tests were applied to the supernatant of the fungus medium. Chemical assays for specific or general siderophore detection did not show positive results during one week of growth when strain BH72 or BH1599 have been grown in pure or co-culture. Moreover, no siderophore production could be detected in pure culture of fungus strain 2003. These results are in agreement with the absence of siderophore biosynthetic pathways in the genome of strain BH72 (30). However, in this study both media used were iron rich, as this element was supplied in the form of Fe^{3+}-EDTA. In *S. meliloti* and *R. leguminosarum* TonB-dependent receptors were iron-regulated (32, 55), but not involved in nitrogen fixation. Therefore they were not necessary during symbiosis. On the other hand, it was reported that OM TonB-dependent receptors are important for the perception of

environmental signals and that they are associated with pathogenicity of plant pathogens (29). Results obtained in strain BH72 suggested an iron-independent regulation of these OM receptors under both N_2-fixing conditions studied and moreover, that these receptors are involved in other process than iron uptake.

Cellular protection and detoxification. Several proteins involved in the detoxification of reactive oxygen species (ROSs) were identified under all conditions. From these some proteins were up-regulated under co-culture growth conditions, such as AhpC, SodC, Ohr, Tpx (only in the strain BH1599), and a conserved hypothetical peroxiredoxin (Table1). The antioxidant AhpC appeared in the wild-type co-culture membrane fractions as a multiple spots with slightly different molecular weights and pIs (Table 1 and Figure 1E, spot number 111). The same observation was made in *Desulfovibrio vulgaris,* suggesting stress-dependent posttranslational modifications (9). SodB and KatA have been demonstrated to be important for the detoxification of ROSs during symbiotic growth of *S. meliloti* (11, 12), underlining the importance of these proteins during a highly effective nitrogen fixation process.

Structural elements: membrane proteins. A set of three major constitutive spots in the membrane fraction were identified as outer membrane porins (Table 1, spot numbers 135, 136, 137). These proteins belong to the general bacterial porin (GBP) family (T.C.:1.B.1). Porins serve as “molecular sives” for several compounds (e.g., sugars, nucleotides, phosphate, pyrophosphate) (38). In *S. meliloti* a set of outer membrane proteins with unknown function were also identified in cultured and nodule bacteria (11). In many host-microbe interactions porins act as virulence factors (34). Interestingly isoforms of the porins mentioned in strain BH72 were up-regulated under co-culture conditions (Figure 1E and 1F, spot numbers 135, 136 and 137).

Proteins related to the cell envelope biogenesis, integrity and shape were detected in both conditions studied. Interestingly, Pal - a putative peptidoglycan-associated lipoprotein and member of the OmpA family - was down-regulated in the wild-type strain and constitutive in strain BH1599 (Table 1, spot number 143). The mayor role of Pal in the outer membrane integrity in association with peptidoglycan was reported in *E. coli* as well as in *Haemophilus influenzae* (8, 43). Additionally, a rod shape-determining protein MreB (Table 1, spot number 140) was down-regulated in co-culture cells. Previous microscopic observations from co-cultured cells of strain BH72 revealed frequently dumbbell-shaped bacteria (25). Therefore it might be possible that MreB and Pal are involved in the observed cell morphology changes in *Azoarcus* sp. BH72 grown under co-culture conditions.

Several membrane and membrane associated proteins were up-regulated in the wild-type strain (Table1). Two (Table 1, spot number 146 and 147) were annotated as conserved hypothetical membrane proteins. In both protein sequences, a transmembrane motif was detected. In particular, the protein azo3667 was also present in the mutant strain BH1599, but significantly less (wt/mut: 0.59± 0.29/0.2±0.17), suggesting that these proteins could be involved in diazosome formation or function.

Under the growth conditions studied two proteins related to the transport and/or efflux of compounds were identified. An AggA (TolC-like) protein, member of the outer membrane efflux protein (OEP) family, was detected constitutively expressed (Table 1, spot number 138). Additionally, a membrane fusion protein (azo0244) member of the HlyD family was down-regulated in the wild-type strain and constitutive expressed in strain BH1599. Moreover, several isoforms of this protein have been also detected to be up-regulated in the mutant strain (Figure 1F, spot number 139). Both proteins are part of a type I secretion system involved in the secretion of a number of proteins and molecules, and require the help of proteins belonging to the ABC transporter family (38). The differential expression of these two proteins in the two analyzed strains suggested different efflux processes during co-culture conditions.

Finally, two different outer membrane proteins (OprM1 and OmpW), probably involved in drug or antibiotic resistance were up-regulated (Table 1, spot number 141, 142). The OprM1 protein, which is a probable outer membrane efflux protein, member of the resistance-nodulation-cell division (RND) superfamily (T.C.: 2.A.6.-) was detected exclusevely in co-culture wild-type cells. Additionally, the outer membrane protein precursor, member of the bacterial porin OmpW family (T.C.:1.B.39), was up-regulated in both strains. OmpW was reported to be related to the ampicillin and tetracycline resistance in *E. coli* (53), while in *Vibrio cholerae* the expression was found to be dependent on *in vitro* culture conditions such as temperature, salinity, and availability of nutrients or oxygen (37). However, the induction of OprM1 in strain BH72 under co-culture conditions is interesting, since only this strain is able to attach to its fungal partner. This outer membrane protein could therefore be important for the interaction.

Conserved hypothetical and hypothetical secreted proteins. Several conserved hypothetical or hypothetical secreted proteins were differentially expressed under the studied conditions (Table 1). For example the gene of a conserved hypothetical secreted protein (azo3738, Table 1, spot number 160) was up-regulated under co-culture conditions. azo3738 is located upstream of the *glnKamtB*-operon and it was shown by RT-PCR that these genes

are cotranscribed (A. Sarkar, unpublished results). The gene product of azo2696 (Table 1, spot number 174) was induced only in wild-type strain BH72 under co-culture growth. Via a pfam search we could identify a BNR/ Asp box repeat (E-value of 0.011) within the amino acid sequence of this protein. BNR/ Asp box repeats occur in glycosyl hydrolases or extracellular proteins and are believed to bind polysaccharides (10). Therefore this protein could be an interesting candidate for further research, since it might be involved in the bacterium-fungus interaction.

Miscellaneous. A protein (azo1228) with 77% similarity to a choline dehydrogenase (EC 1.1.99.1) was strongly up-regulated in the cytoplasmic fraction. Choline dehydrogenase catalyzes the four-electron oxidation of choline to glycine-betaine a potent osmoprotectant as described for *Haemophilus elongata* and *S. meliloti* (15, 41). The expression of this protein in co-culture is correlated to the presence of cholinechloride as well as betainhydrochloride in the FU medium used as an osmoprotectant.

Interestingly proteins involved in the synthesis and transport of exopolysacharides (Wza2, Table 1, spot number 51) were up-regulated or induced (WbnF, Table 1, spot number 52) under co-culture conditions. These proteins might be important for the bacterium-fungus interaction.

Conclusions. Internal membrane stacks (diazosomes) are formed in *Azoarcus* sp. BH72 during co-culture with the ascomycete strain 2003 and at low O_2 concentration (25). The approach used in this work allowed for the detection of specific proteins which are expressed only in diazosome containing cells and which could most probably be involved in diazosome formation. We could verify here that the NifH protein was partially membrane associated in *Azoarcus* sp. BH72 under co-culture growth. Proteins playing a role in nitrogen as well as carbon metabolism have been identified under all studied conditions. The up-regulation of specific proteins from the acetate and ethanol assimilation demonstrated that the *Azoarcus* carbon metabolism was adapted to the utilization of fungal exudates. The expression of chaperones and enzymes involved in protein synthesis and processing changed under co-culture growth, indicating an adaptation to the different environmental conditions. Of particular interest has been the identification of proteins up-regulated in the wild-type strain BH72 co-cultured with the fungus isolate 2003 and not in strain BH1599, which is unable to attach to the fungal mycelium and to produce internal membrane stacks. To this group belonged for example WbnF, a protein probably involved in exopolysaccharide synthesis and therefore likely to be important for the bacterium-fungus interaction. Additionally a protein probably located in these specialized structures might be the CycH protein, which is possibly

involved in the respiration process. Interesting has been as well the induction of Pnp, a polyribonucleotide nucleotidyltransferase probably playing a role in RNA processing and messenger RNA degradation and PrlC, an oligopeptidase. We could identify several membrane proteins, conserved hypothetical and hypothetical secreted proteins specifically induced in strain BH72 under co-culture growth. Therefore our approach may give rise to further studies and subsequent characterization of proteins important for interaction, diazosome structure and function.

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Table 1. Differentially expressed proteins derived from both membrane and cytoplasmic protein fractions of N_2-fixing *Azoarcus* sp. BH72 cells grown in pure- or co-culture with fungus strain 2003.

Spot n°.[a] Description	Mol wt (kDa)	pI	Locus tag[b]	Protein name[b]	Cov. (%)	Differential abundance[c]
Nitrogen metabolism						
1:C, M Iso.	31,2	4,7	azo0538	NifH: nitrogenase iron protein probable	30	↓ (2,62± 0,51); CON
2:C, M Iso.	55,3	6,3	azo0539	NifD: nitrogenase molybdenum-iron protein alpha chain	59	CON, iso. novel wt
3:C Iso., M	59,1	5,68	azo0540	NifK: nitrogenase molybdenum-iron protein beta chain	45	CON
4:C	25,2	4,78	azo0543	NifY1: NifY protein	56	↓
5:C, M	32,0	5,1	azo0546	NifM: probable peptidylprolyl isomerase	49	↓; ↑
6:C	43,7	6,43	azo0552	NifS**:** cysteine desulfurase	34	CON
7:C	31,6	4,95	azo0553	NifU:Probable nitrogen fixation protein NifU	42	↓ (2.7 ± 0,69)
8:M	54,3	5,6	azo0562	NifE: nitrogenase iron-molybdenum cofactor biosynthesis protein	34	CON
9:M	48,8	5,83	azo0561	NifN: probable nitrogenase iron-molybdenum cofactor biosynthesis protein	42	↓
10:C	13,0	6,7	azo0528	FdxD: Ferredoxin V (FdV) (Ferredoxin plant-type)	43	CON
11:M	66,3	6,19	azo3787	HupL: probable ferredoxin hydrogenase, large chain	27	wt/mut.: ↓/CON
12:C, M	53,1	5,87	azo0735	NtrC: nitrogen regulation protein NR(I)	52	CON; ↓
13:C	51,9	5,29	azo0738	GlnA: Glutamine synthetase I	39	CON
14:M	26,7	7,08	azo0439	GlnQ1: probable glutamine transport ATP-binding protein	34	wt/mut.: ↓/CON
15:C	60,3	5,3	azo3504	UreC : urease alpha subunit	33	↑ novel wt
18:C	40,4	6,4	azo1381	CarA: Carbamoyl-phosphate synthase small chain	43	CON
19:M	116,8	5,2	azo1382	CarB: carbamoyl-phosphate synthase	31	↑ novel wt
TCA, glyoxylate shunt, gluconeogenesis and pyruvate metabolism						
20:M	64,8	6,05	azo1551	SdhA: succinate dehydrogenase, flavoprotein subunit	55	CON
21:M, M Iso.	27,1	6,19	azo1552	SdhB: succinate dehydrogenase, iron-sulfur subunit	45	CON; iso. novel co-culture
22:C, M	41,2	5,24	azo3332	SucC: probable succinyl-CoA synthetase, beta chain	56	CON
23:C, M	31,0	6,54	azo3333	SucD: Probable succinyl-CoA synthetase, alpha chain	44	CON; novel wt
24:C, M Iso.	48,3	6,15	azo1554	GltA: probable citrate synthase	37	CON; iso.1: wt/mut.: CON/↓ (4,76± 1,355); iso.2: CON
25: M	41,7	5,74	azo1556	OdhB: dihydrolipoamide S-succinyltransferase	47	↑
26:C	49,7	6,83	azo1557	OdhL: Probable dihydrolipoamide dehydrogenase	32	CON
27:M	82,0	5,58	azo3211	Maeb2:Probable malic enzyme	23	CON
28:C	98,3	5,7	azo1533	AcnA: aconitate hydratase	27	↑ novel wt
29:C, M	92,7	5,49	azo1534	AcnB: aconitate hydratase	38	CON; wt/mut: CON/↓

30:C, C Iso.	35,5	5,78	azo1547	Mdh: malate dehydrogenase	52	CON, iso. novel co-culture
31:C, M	47,9	5,6	azo1117	AceA: isocitrate lyase	46	↑,↑ novel wt
32: C	40,0	5,63	azo3860	YeaU: tartrate dehydrogenase	34	↓
33:M	68,3	5,5	azo0820	PckG: probable phosphoenolpyruvate carboxykinase	21	↑ novel wt
34:C	45,9	4,79	azo2144	Eno: enolase probable	16	CON
37:C	27,9	5,89	azo2808	GpmA: phosphoglycerate mutase, probable	46	CON
38:M,C	41,5	5,75	azo2839	PgK: phosphoglycerate kinase, probable	41	CON ; ↑
39:C	38,2	5,7	azo2843	FbaA: fructose-bisphosphate aldolase	38	↓
40:M, C	71,5	5,77	azo2414	AcsB: probable acetyl-coenzyme A synthetase	37	CON; novel wt
41:M	62,7	5,8	azo1371	LpdA: dihydrolipoamide dehydrogenase	38	wt/mut.: CON/↓ (3,2 ± 1)
42:C Iso.	40,6	6,55	azo2172	ThlA: probable acetoacetyl-CoA thiolase	28	↑
43:M	50,4	5,79	azo3638	Pta: conserved hypothetical phosphate acetyltransferase	30	↓
44:C	13,6	5,0	azo3228	GloA: lactoylglutathione lyase	57	↑ novel mut.
47:C	55,1	6,4	azo2939	AldA: aldehyde dehydrogenase (NAD+)	33	↑
48:C Iso.	77,3	7,72	azo2972	ExaA2 :quinoprotein ethanol dehydrogenase, probable	24	↑
49:C	41,7	8,23	azo2975	ExaA3: quinoprotein ethanol dehydrogenase, probable	25	↑
50:C	15,6	6,9	azo2933	ExaB1: probable cytochrome c550	53	↑ novel mut.
Propanoate metabolism						
53:M	73,3	5,76	azo0688	PccA: probable propionyl-CoA carboxylase alpha chain	39	CON
Amino acids metabolism						
16:C	13,0	4,0	azo1286	GcvH: glycine cleavage system H protein	65	CON
17:C	36,2	6,5	azo1287	GcvT:glycine cleavage system T protein	45	↑
35:C	30,9	5,63	azo1096	DapA:dihydrodipicolinate synthase	42	↓
36:C	28,2	5,7	azo2576	DapB: dihydrodipicolinate reductase	28	↓
54:M	36,9	6,42	azo3156	IlvC: ketol-acid reducto isomerase	54	↓
55:C	65,7	5,7	azo0632	IlvD: dihydroxy-acid dehydratase	33	↑ novel wt
56:C	26,3	4,86	azo3345	HisA: phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	47	CON
57:M	47,0	5,51	azo0818	HisD: Histidinol dehydrogenase	33	↑
58:C	45,2	6,6	azo2801	GlyA: serine hydroxymethyltransferase	31	CON
59:C	39,2	5,7	azo1042	Asd: aspartate-semialdehyde dehydrogenase	39	CON
60:C	34,3	6,26	azo2399	CysK: cysteine synthase A	61	↓
61:C	55,4	5,3	azo3162	LeuA1: 2-isopropylmalate synthase	51	↓
Fatty acids metabolism						
45:C	63,7	5,72	azo1702	Acyl-CoA dehydrogenase	34	↑
46:C	27,5	5,1	azo0790	PaaF1: putative enoyl-CoA hydratase	37	↓
62:C	27,2	5,9	azo2130	FabI: probable enoyl-[acyl-carrier-protein] reductase	55	CON
67:C	89,3	4,14	azo1626	AcpP: Acyl carrier protein	49	↓
69:M	49,6	6,2	azo0862	AccC1: probable biotin carboxylase	45	↓ (6,6 ± 4,3)
Butanoate metabolism						
63:C, M	26,1	6,32	azo1023	PhbB2: acetoacetyl-CoA reductases	17	CON, ↑ novel wt

64:M	23,6	4,11	azo1024	PhbF: polyhydroxyalkanoate synthesis repressor	45	↑
65:C	35,9	6,1	azo3867	AcoA2: probable acetoin dehydrogenase, alpha subunit	32	↑ novel wt
Cofactors biosynthesis						
66:C	39,1	5,37	azo0319	RibAB: GTP cyclohydrolase II	46	↑
68:M	42,5	5,25	azo0995	HemX: putative uroporphyrin-III C-methyltransferase	44	CON
70:M	34,5	5,42	azo3537	CbiH :Precorrin-3B C17- methyltransferase		CON
Pyrimidine and purine biosynthesis						
71:C	13,7	4,0	azo3700	TrxC2: probable thioredoxin-disulfide reductase	70	↑ novel mut.
72:C	44,7	5,8	azo2895	PurD: phosphoribosylamine-glycine ligase	24	↑
73:C	60,1	5,76	azo2146	PyrG: CTP synthetase	36	CON
74:C	11,9	6,52	azo2658	TrxA2: putative thioredoxin-disulfide reductase	58	CON
75:C	11,9	4,8	azo1030	TrxA1: thioredoxin-disulfide reductase	58	↓
76:C	33,9	5,44	azo1363	TrxB2: thioredoxin-disulfide reductase	25	↓
77:C	23,6	5,98	azo1473	Adk: adenylate kinase	49	↓
78:C	15,4	5,6	azo0923	Ndk: nucleosidediphosphate kinase	39	CON
Protein synthesis, folding and reparation						
79:M, C	35,7	5,41	azo3391	RpoA: DNA-directed RNA polymerase	63	CON
80:C	20,3	6,34	azo3429	NusG: transcription antitermination protein	42	↑ novel mut.
81:C, C Iso., M	62,9	5,19	azo1072	RpsA: 30S ribosomal protein S1	25	wt/mut.: CON/↓ (4,85± 1,98); iso. novel co-culture; wt/mut.: (2,23 ± 0,89)
82:M	28,1	7,0	azo1909	RpsB: 30S ribosomal protein S2	59	↑ novel mut.
83:M	14,5	7,07	azo0718	RpsF: 30S ribosomal protein S6	40	↑ novel mut.
84:C	16,3	5,51	azo0721	Rpl1: 50S ribosomal protein L9	63	↓
85:C	21,5	5,97	azo0754	RplY: 50S ribosomal protein L25	67	CON
86:C	20,7	4,84	azo0086	Efp: putative elongation factor P	56	CON
87:C, M	43,1	5,42	azo3419	TufA: elongation factor Tu (EF-TU)	33	CON
88:C, M	43,1	5,42	azo3431	TufB: elongation factor Tu (EF-TU)	33	CON
89:C, M	77,4	5,09	azo3420	FusA2: Elongation factor EF-G	47	CON ; ↓
90:C	51,9	5,29	azo1908	Tsf: elongation factor EF-Ts	23	↓
91:C	41,7	4,94	azo1497	PrfB: peptide chain release factor	24	CON
92:M	75.3	5.2	azo2103	Pnp: polyribonucleotide nucleotidyltransferase	29	↑ novel wt
93:M	23,9	5,0	azo0848	Pcm: putative protein-L-isoaspartate O-methyltransferase	32	CON
94:C	49,3	7,2	azo2806	CtpA: carboxy-terminal processing protease precursor	41	↑
95:M	77.2	5.3	azo2877	PrlC: oligopeptidase A	70	↑ novel wt
96:C	54,1	8,0	azo0765	Probable Zn dependent peptidase	39	↑

Respiratory chain and energy production						
97:M	22,9	4,73	azo1398	NuoC: respiratory-chain NADH dehydrogenase, chain C	41	↓ (2,63 ± 0,54)
98:M, M Iso.	81,9	6,13	azo1402	NuoG: NADH-ubiquinone oxidoreductase chain G	35	CON, iso. novel wt
99:M	18,5	6,89	azo1404	NuoL: NADH-ubiquinone oxidoreductase, chain I	70	CON
100:M	21,0	6,43	azo0960	PetA1: probable ubiquinol-cytochrome c reductase iron-sulfur protein. TM	54	CON
101:M	43,2	4,96	azo3927	CycH: conserved hypothetical cytochrome c-type biogenesis protein. TM	39	↑ novel wt
102:C	31,0	5,36	azo1700	EtfA1: probable electron transfer flavoprotein, alpha subunit	70	CON
103:C	59,7	6,42	azo2151	Etf1: probable electron transfer flavoprotein-ubiquinone oxidoreductase	29	↑
104:C	19,7	4,13	azo0954	IsiB: probable flavodoxin	51	↓
105:M	55,3	5,61	azo0157	AtpA: ATP synthase alpha chain	38	CON
106:C, M	50,6	5,23	azo0159	AtpD: ATP synthase beta chain	65	CON
107:M	18,8	4,5	azo0156	AtpH: putative ATP synthase delta chain	26	↑
108:C	21,5	5,97	azo1358	Ppa: probable inorganic pyrophosphatase	37	CON
Detoxification related proteins						
109:C	21,5	5,84	azo1466	SodB: superoxide dismutase probable	62	CON
110:C	17,5	7,4	azo0522	SodC: superoxide dismutase [Cu-Zn] precursor	34	↑
111:C, M Iso.	20,6	5,74	azo0769	AhpC: alkyl hydroperoxide reductase subunit C	68	CON, novel wt
112:C	55,6	5,4	azo0770	AhpF: alkyl hydroperoxide reductase subunit F	37	↓
113:C	15,0	6,2	azo2405	Ohr: probable organic hydroperoxide resistance protein	28	↑
114:M	24,5	6,4	azo2663	Conserved hypothetical peroxiredoxin	31	↑
115:C	17.2	5.2	azo0105	Tpx: probable thiol peroxidase	42	↑ novel mut.
116:C	31,4	4,8	azo2548	Dyp: Dye-decolorizing peroxidase	37	↓
117:M	18,3	4,62	azo0616	Bfr3: Bacterioferritin	25	↑
Chaperones						
118:C, M	68,7	4,69	azo1063	DnaK: chaperone protein. (Heat shock protein 70)	64	CON ; ↓ (3,4 ± 1,2)
119:C	48,0	4,93	azo2072	Tig: trigger factor	35	CON
120:C	10,4	5,43	azo0973	GroES1: chaperonin	80	CON
121:C	57,9	4,9	azo0974	GroEL1: 60 kDa chaperonin	31	↑
122:C	18,2	5,49	azo1056	PpiB: peptidyl-prolyl cis-trans isomerase B	70	CON
123:C,M	15,9	5,67	azo3243	Hsp18: putative small heat shock protein (chaperonine)	71	CON
124:M	67,9	5,52	azo1568	PpiD: putative peptidyl-prolyl cis-trans isomerase.TM	46	wt/mut. : CON/↓ (3,2 ± 1,0)
125:M	95,8	5,64	azo1566	ClpB: probable ATP-dependent Clp protease, ATP-binding subunit	47	↓
126:C	71,8	5,17	azo1425	HtpG: probable chaperon protein HtpG	46	↓
127:C	46,5	5,52	azo2070	ClpX: ATP-dependent Clp protease ATP-binding subunit	39	CON
128:C, M	17,5	4,54	azo2062	Conserved hypothetical peptidyl-prolyl cis-trans isomerase	24	CON ; ↑
Membrane proteins						
129:M	76,4	5,9	azo3023	Probable TonB-dependent outer membrane receptor	41	CON

130:M	78,7	5,81	azo2396	Probable TonB-dependent outer membrane receptor	31	CON
131:M	50,0	4,76	azo2978	Probable TonB-dependent outer membrane receptor	15	CON
132:M	71,6	5,93	azo3556	Probable TonB-dependent outer membrane receptor	40	↑ novel wt
133:M	74,8	5,57	azo0378	Probable TonB-dependent outer membrane receptor	56	wt/mut. : CON/↓ (3,9 ± 1,2)
134:M	79,0	5,05	azo2887	OstA: organic solvent tolerance protein precursor	18	CON
135:M, M Iso.	39,4	8,43	azo3017	Outer membrane porin protein precursor. TM	45	CON, iso. novel co-culture
136:M, M Iso.	38,0	6,97	azo3290	Outer membrane porin protein precursor	70	CON, iso. novel co-culture
137:M, M Iso.	37,8	9,11	azo3291	Outer membrane porin protein precursor	16	CON, iso. novel co-culture
138:M	53,3	6,65	azo0654	AggA: putative outer membrane efflux protein	43	CON
139:M, M Iso.	41,4	7,71	azo0244	Membrane fusion protein	65	wt/mut.: ↓/CON; iso. novel mut.
140:M	36,7	4,98	azo0174	MreB : rod shape-determining protein	35	CON
141:M	49,9	5,8	azo0246	OprM3: probable outer membrane efflux protein	22	↑ novel wt
142:M	26,3	6,9	azo3741	OmpW: outer membrane protein W precursor	23	↑
143:M	18,1	6,59	azo0416	Pal: putative peptidoglycan-associated lipoprotein	69	wt/mut.: ↓ (3,6 ± 1,5)/CON
144:M	29,1	5,63	azo3212	ParA3: sporulation initiation inhibitor protein Soj	23	CON
145:M Iso.	30,2	7,1	azo1017	ComL: probable competence lipoprotein precursor	46	↑
146:M	11,0	6,28	azo3667	Conserved hypothetical membrane protein. TM	32	↑
147:M	31,1	5,3	azo2269	Conserved hypothetical membrane protein. TM	33	↑ novel wt
Motility and adhesion						
148:M	50,0	4,76	azo2693	FliC2: flagellin	47	CON
149:M	49,8	5,78	azo2704	FliC3: flagellin	40	CON
150:M	19,0	5,6	azo3647	PilP: putative type 4 fimbrial biogenesis protein	32	↑ novel mut.
Transporters						
151:C	37,2	8,79	azo0919	DctP2: probable C4-dicarboxylate-binding periplasmic protein	55	↓
152:M, M Iso.,C	41,7	8,23	azo2795	Putative periplasmic substrate binding protein	51	wt/ mut.: ↓/CON; iso. novel co-culture; novel wt
153:M	31,4	5,01	azo0677	UrtD: Conserved hypothetical ABC transporter, ATP binding protein	30	CON
154:C	37,0	8,33	azo2205	FbpA1: Iron transport system substrate-binding protein probable	45	↑ novel wt
155:M	61,5	5,74	azo1275	Yjjk: probable ABC transporter, ATP-binding protein	39	↓
156:C	22,0	7,4	azo3936	CcmA: probable heme exporter protein A	11	↑
Hypothetical secreted proteins						
157:M	51,4	4,86	azo3760	Hypothetical secreted protein. TM	50	↓

158:M	22,3	8,69	azo3886	Hypothetical secreted protein	58	↑ novel mut.
159:M	23,3	9,65	azo0869	Conserved hypothetical secreted protein. TM	40	↑ novel mut.
160:M	26,9	5,25	azo3738	Conserved hypothetical secreted protein	40	↑
161:C	13,7	7,77	azo2563	Conserved hypothetical secreted protein	56	↑
162:M	63,4	5,58	azo0758	Conserved hypothetical secreted protein	53	CON
177:C	22,3	7,1	azo3874	Conserved hypothetical secreted protein	25	↑
Miscellaneous						
51:M	22,4	5,28	azo3283	Wza2: conserved hypothetical polysaccharide export protein. TM.	56	↑ novel mut.
52:M	37,3	6,1	azo0852	WbnF: Nucleoside-diphosphate-sugar epimerase	28	↑ novel wt
163:C	56,9	8,01	azo1228	Choline dehydrogenase	29	↑
164:C	23,2	6,31	azo0963	SspA2: probable stringent starvation protein A	42	CON
165:C	35,2	7,23	azo0296	Probable metallo-beta-lactamase superfamily protein	60	CON
166:C	36,2	6,0	azo3496	AscD: probable CDP-6-deoxy-L-threo-D-glycero-4-hexulose-3-dehydrase reductase	31	↓
167:C	12,1	5,7	azo3319	Fkbp: peptidyl-prolyl cis-trans isomerase	41	↑ novel mut.
168:M	48,5	6,19	azo3637	GlmU: DP-N-acetylglucosamine pyrophosphorylase / diamine N-acetyltransferase	39	wt/mut.: CON/↓
Conserved hypothetical						
169:C	26,0	4,85	azo0574	YebC: Conserved hypothetical protein	29	wt/mut.: ↓/CON
170:C	22,31	4,81	azo1131	Conserved hypothetical protein	59	↓
171:C	15,6	4,3	azo0475	Conserved hypothetical protein	30	↑ novel mut.
172:M	24,0	6,6	azo1168	Conserved hypothetical protein	29	↑
173:C	40,2	5,6	azo2298	Conserved hypothetical protein	35	↑
174:M	30,0	5,7	azo2696	Conserved hypothetical protein	17	↑ novel wt
175:C	30,8	5,36	azo2934	Conserved hypothetical amino acid-binding protein	73	↑
176:C	33,2	6,25	azo2935	Conserved hypothetical protein	49	↑
178:M	75,7	7,8	azo3950	Conserved hypothetical protein	49	CON

[a] C: cytoplasmic fraction, M: membrane fraction, Iso: isoform. TM: transmembrane domains (based on TMHMM, a transmembrane helix prediction method based on a hidden Markov model). [b] Locus tag and protein name are presented according to the annotated genome (Krause *et al.,* 2006). [c] For differential abundance calculations pure culture N_2-fixing gels were used as reference. A cutoff of 2 fold was used to define a spot as up or down regulated. For these, relative spot intensities calculated from three experiments are given with standard deviation. ↑: up-regulated or novel in co-culture, ↓: down regulated or absent in co-culture, CON: constitutive in both growth conditions. wt: wild-type strain BH72, mut: mutant strain BH1599.

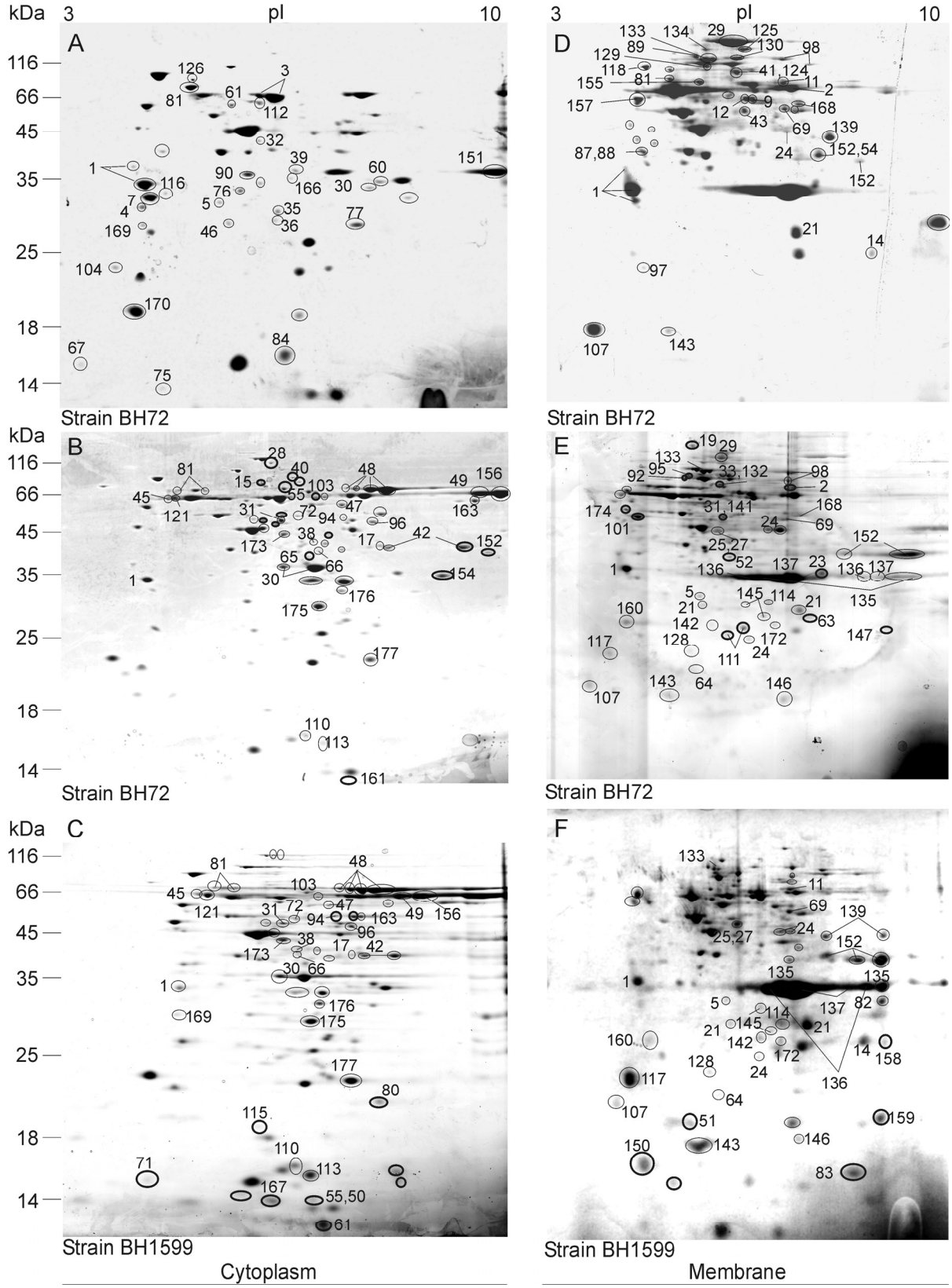

Figure 1. 2D-proteome pattern of cytoplasmic (A, B and C) and membrane (D, E and F) fractions of N_2-fixing *Azoarcus* sp. BH72 cells. In panels A and D strain BH72 was grown in pure culture, while in panels B, C, E and F cells were grown in co-culture with fungus strain

2003. C and F: proteome pattern of mutant *Azoarcus* sp. BH1599. Isoelectric focussing was performed using a capillary tube gel of pH 3-10. The focused proteins were separated on 12% SDS-PAGE gels and were stained with Coomassie brilliant blue R-250. Spots encircled in panels A and D were down-regulated in co-cultures cells, while spots in slim black circles in panels B, C, E and F were up-regulated in co-culture cells. Spots in bold black circles were unique in wild-type strain cells (panels B and E) or in mutant strain BH1599 cells (panel C and F). Numbers correlate with spot numbers and the corresponding proteins listed in Table 1.

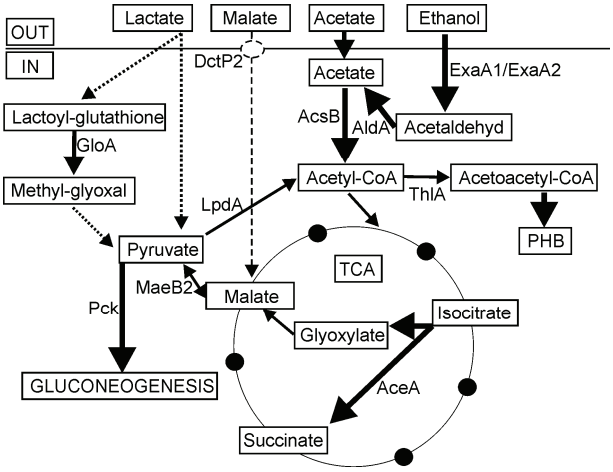

Figure 2. Schematic representation of major carbon metabolic pathways identified in the proteome of N_2-fixing *Azoarcus* sp. BH72 cells. Abbreviations refer to the following proteins identified and listed in Table 1: DctP2: C4-dicarboxylate-binding periplasmic protein, ExaA1 of ExaA2: quinoprotein ethanol dehydrogenase, AldA: aldehyde dehydrogenase (NAD^+), AcsB: acetyl-coenzyme A synthetase, ThlA: acetoacetyl-CoA thiolase, LpdA: dihydrolipoamide dehydrogenase, GloA: lactoylglutathione lyase, AceA: isocitrate lyase, MaeB2: Probable malic enzyme ($NADP^+$) and PckG: phosphoenolpyruvate carboxykinase.