Functional analysis of the GlnK$_1$ protein of *Methanosarcina mazei* strain Gö1: Aspects of nitrogen regulation

Dissertation

zur Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultäten

der Georg-August-Universität zu Göttingen

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Göttingen 2004

D7

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Tag der mündlichen Prüfung: 2. November 2004

Teile dieser Arbeit wurden veröffentlicht in:

Ehlers C, Grabbe R, Veit K, Schmitz RA (2002). Characterization of $GlnK_1$ from *Methanosarcina mazei* strain Gö1: Complementation of an *Escherichia coli glnK* mutant strain by *M. mazei* $GlnK_1$. *J. Bacteriol*. 184, 1028-1040.

Ehlers C, Veit K, Gottschalk G, Schmitz RA (2002). Functional organization of the *nif* operon in the mesophilic archaeon *Methanosarcina mazei* strain Gö1. *Archaea* 1, 143-150.

Ehlers C, Weidenbach K, Veit K, Deppenmeier U, Metcalf W, Schmitz RA (2004). Development of genetic methods and construction of a chromosomal *glnK₁* mutant in *Methanosarcina mazei* strain Gö1. Molecular Genetics and Genomics, *submitted.*

Ehlers C, Weidenbach K, Veit K, Forchhammer K, Schmitz RA (2004). Unique mechanistic features of post-translational regulation of glutamine synthetase activity in *Methanosarcina mazei* strain Gö1 in response to nitrogen availability. Molecular Microbiology*, submitted.*

Außerdem werden vorraussichtlich folgende Veröffentlichungen aus der Arbeit hervorgehen:

Veit K, Ehlers C, Ehrenreich A, Deppenmeier U, Schmitz RA (2004). Nitrogen regulation in *Methanosarcina mazei* strain Gö1: Global analysis of transcriptional regulation of genes involved in nitrogen metabolism. *In preparation.*

Veit K, Ehlers C, Schmitz RA (2004). Differential expression of soluble methyltransferases in response to different nitrogen and carbon sources in *Methanosarcina mazei* strain Gö1. *In preparation.*

ACKNOWLEDGMENTS

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SUMMARY

- In the genome of the methanogenic archaeon *Methanosarcina mazei* strain Gö1 a gene cluster encoding for a molybdenum-dependent nitrogenase was identified. Growth analysis confirmed that *M. mazei* is able to fix molecular nitrogen and use it as sole nitrogen source. Besides these *nif*-genes, four genes encoding for PII-like proteins were identified, which are known to be involved in nitrogen regulation of bacteria. Two of them are located within the *nif*-gene cluster and were designated *nifI$_1$* and *nifI$_2$*. The other two PII encoding genes are both organized in an operon together with a gene encoding for an ammonium transporter (*amtB*) and were designated *glnK$_1$* and *glnK$_2$*, respectively.

- The *glnK$_1$*-gene of *M. mazei* was cloned and sequenced. The deduced amino acid sequence of GlnK$_1$ showed 44 % identity to the *Escherichia coli* GlnK protein. In the T-loop region of the *M. mazei* GlnK$_1$ the conserved tyrosin residue (Tyr51) was identified, which is the site of covalent modification of most bacterial PII-like proteins. After heterologous expression in an *E. coli* strain carrying chromosomal deletions of the *glnB*- and *glnK*-genes, the *M. mazei* GlnK$_1$ protein was purified to an apparent homogeneity of 98 % by mercaptoethanol precipitation, anion exchange chromatography on Q-sepharose and gel filtration.

- The molecular mass of GlnK$_1$ monomers was determined to be 14 kDa, which corresponds to the predicted mass based on the DNA sequence. Gel filtration analysis and native gel electrophoresis of purified GlnK$_1$ revealed a molecular mass of 42 kDa, clearly demonstrating GlnK$_1$ being a trimer under native conditions. *In vitro* uridylylation assays using purified *M. mazei* GlnK$_1$ and *E. coli* uridylyltransferase GlnD' indicated that GlnK$_1$ is not modified by uridylylation. This was confirmed by *in vivo* ammonium-upshift experiments, in which a modification of GlnK$_1$ upon changes in nitrogen availability would have resulted in a different mobility in native gel electrophoresis.

- *In vitro* and *in vivo* analyses demonstrated that GlnK$_1$ does not form heterotrimers with bacterial GlnK proteins. Denaturating and renaturating purified *M. mazei* GlnK$_1$ in the presence of purified *E. coli* GlnK resulted exclusively in the reassembly of homotrimers indicating species-specific structural features of the respective GlnK monomers. Consistent with this finding, no heterotrimers were detected, when *M. mazei* GlnK$_1$ was expressed in *Klebsiella pneumoniae* under nitrogen limitation.

- Transcriptional analysis by Northern blotting and quantitative reverse transcriptional (RT) PCR revealed that the $glnK_1amtB_1$ operon is strongly regulated in response to nitrogen availability and is induced 170-fold under conditions of nitrogen limitation. This expression regulation was confirmed by Western blot analysis using cell extracts of *M. mazei* cells grown under nitrogen limitation and nitrogen excess.

- Complementation studies expressing *M. mazei glnK_1* in an *E. coli glnK*-mutant strain demonstrated that the archaeal GlnK protein is able to functionally substitute for the bacterial GlnK protein for growth on arginine as sole nitrogen source. Thus, the archaeal $GlnK_1$ protein appears to be involved in nitrogen regulation in *M. mazei*.

- *M. mazei* glutamine synthetase ($GlnA_1$) was identified as the first target protein of the archaeal PII-like protein by pull-down experiments using His_6-tagged $GlnK_1$ protein immobilized to a Ni-NTA matrix and *M. mazei* cell extracts. Reverse pull-down experiments confirmed the interaction between $GlnA_1$ and $GlnK_1$, which also represents the first description of a glutamine synthetase being directly regulated by a PII-like protein.

- Direct interaction between $GlnK_1$ and $GlnA_1$ was confirmed by gel filtration analysis and subsequent Western blotting of the peak fractions corresponding to $GlnA_1$ and $GlnA_1$/$GlnK_1$-complexes, respectively. Studying glutamine synthetase (GS) activity showed that $GlnA_1$/$GlnK_1$ complex formation results in an inhibition of $GlnA_1$ activity down to 70 %, indicating that upon a change in nitrogen availability $GlnK_1$ may regulate $GlnA_1$ activity by direct protein-protein-interaction.

- Gel filtration analysis indicated that purified $GlnA_1$ shows a dodecameric structure under native conditions, which has been postulated due to high homologies of *M. mazei* $GlnA_1$ to glutamine synthetases of the GSI-α subgroup. This higher oligomeric structure of $GlnA_1$ is apparently dependent on 2-oxoglutarate as demonstrated by gel filtration analysis of $GlnA_1$ in the presence or absence of the effector molecule. 2-oxoglutarate further effected $GlnA_1$ activity: The low basal GS activity (0.4 - 0.6 U/mg) was significantly increased (up to 16-fold) after preincubation with 2-oxoglutarate. Together with the effect of 2-oxoglutarate on integrity of the higher oligomeric structure of $GlnA_1$ this finding indicates a relation between the dodecameric structure and enzyme activity.

- A second effect of 2-oxoglutarate was obtained by analyzing complex formation between $GlnA_1$ and $GlnK_1$ in the presence of 2-oxoglutarate. Preincubation of $GlnA_1$ and $GlnK_1$ with 2-oxoglutarate resulted in a reduced yield of complexes as the amount of free

trimeric $GlnK_1$ increased. Furthermore the inhibitory effect of $GlnK_1$ on $GlnA_1$ activity was abolished by the addition of 2-oxoglutarate, corresponding to the 2-oxoglutarate effect on complex formation. Both the stimulating effect of 2-oxoglutarate and the inhibitory effect of purified $GlnK_1$ on $GlnA_1$ activity were confirmed in *M. mazei* cell extracts. On the basis of these findings we hypothezise that the GS enzyme activity of $GlnA_1$ is regulated by 2-oxoglutarate and $GlnK_1$, whereas 2-oxoglutarate has a dominant effect over $GlnK_1$.

- A genetic system for *M. mazei* was established by (i) optimizing the plating efficiency on solid medium and (ii) developing a reliable transformation method. By several plating and cultivation steps a potential cell wall mutant, *M. mazei**, was selected, which showed a higher resistance to mechanical stress and allowed plating efficiencies of 10 %. By modifying and optimizing the liposome-mediated transformation protocol developed for *Methanosarcina* strains and using the *M. mazei** strain we succeeded in genetically manipulating *M. mazei* strain Gö1. For the first time both stable transformation of the *E. coli-Methanosarcina* shuttle vector pWM321 and construction of chromosomal deletion mutants were successfully performed in *M. mazei*. The first *M. mazei* chromosomal mutant was generated by disrupting the *glnK₁*-gene by insertion of the puromycin-resistance cassette via double homologous recombination.

- Characterization of the *M. mazei glnK₁*-mutant strain concerning growth under nitrogen fixing conditions showed a partial reduced growth rate and a reduced cell yield indicating that the $GlnK_1$ protein is apparently not essential for nitrogen fixation but appears to be required for maximal growth under this condition. Transcriptional analysis by RT-PCR of selected genes known to be regulated by nitrogen did not show any differences between RNA derived from the wild type and the mutant strain both grown under nitrogen limitation. Therefore a function of $GlnK_1$ as potential transcription regulator was excluded.

INTRODUCTION

Besides carbon nitrogen is one of the most important elements required for life as it is crucial for biosynthesis e.g. of nucleotides, amino acids, amino sugars (lipopolysaccharids, peptidoglucan) and nicotinamideadeninnucleotide. Therefore biologically available nitrogen, also called fixed nitrogen, is essential for life. To provide cells with this nutrient many strategies for utilization of different nitrogen sources like ammonium, inorganic nitrogen compounds and molecular nitrogen have been developed. Ammonium is the preferred source of nitrogen as it can directly be converted into glutamine and glutamate by the enzymes glutamate dehydrogenase and glutamine synthetase/glutamate synthase at only low energetically costs. In contrast, the assimilation of inorganic oxidized nitrogen compounds requires reduction and amino acids as nitrogen source first have to be degraded by transferring the amino group prior assimilation (Reitzer 1996). An alternative way to provide cells with nitrogen is the process of nitrogen fixation, the enzymatic reduction of molecular nitrogen derived from the atmosphere into ammonium (Winogradsky 1893), which is limited to prokaryotes (Hill et al. 1981; Kennedy and Toukdarian 1987; Masepohl et al. 1988; Fischer 1994). Because of high energy demands (Burgess and Lowe 1996) and the fact that the corresponding key-enzyme, the nitrogenase, is highly sensitive towards oxygen, nitrogen fixation is a very energy consuming reaction for organisms. This demonstrates that organisms require strong regulatory mechanisms to modulate their way of nitrogen assimilation and to adapt to changing environmental nitrogen availabilities in order to achieve highest and economical growth rates at their present growth conditions. As ammonium can be directly converted into the amino acids glutamine and glutamate the key donors for aminogroups in biosynthetic reactions, it supports the cells with the highest growth rate. Growth with all other nitrogen sources results in reduced growth due to precedent degradation steps and higher energy demands and is therefore defined as nitrogen limited.

In order to achieve maximal growth rates under any nutritional conditions many different adaption strategies have been developed. Only few absolutely conserved mechanisms exist among the bacteria and archaea, but one of the most conserved proteins is found in the regulatory network of nitrogen metabolism. This family of small sensor proteins, commonly known as PII-like proteins, coordinates the transduction of the cellular nitrogen status and thereby modulates the regulation of the nitrogen metabolism. PII-like proteins are the gene products of *glnB*- and *glnK*-genes, respectively. Members of these proteins

have been identified not only in bacteria and archaea but were also recently found in plants representing the ubiquitous distribution of this protein family throughout the domains of life. The first protein identified of this family is the *Escherichia coli* GlnB, originally designated as PII-protein (Prival et al. 1973). In most organisms paralogous genes encoding for additional PII-like proteins have been identified. These genes were designated *glnK,* which are generally located next to a gene encoding for an ammonium transporter *amtB,* the two forming one operon. In *E. coli* the GlnB and GlnK proteins show high similarities (67 % identity on the amino acid sequence level) and both proteins are able to functionally substitute for each other in some respects (Arcondeguy et al. 2000). A characteristic ability of PII-like proteins is the switch between two different forms by covalent modification at a conserved residue in a certain protein region, the so called T-loop. The state of modification is dependent on the cellular nitrogen status and involves post-translational modification under nitrogen-limited conditions and demodification under nitrogen sufficiency. By changing its modification state the nitrogen status is signalled from the PII-protein to enzymes involved in nitrogen metabolism, mostly by direct protein-protein-interaction, thereby regulating their activities. PII modifications identified so far include uridylylation at the conserved tyrosin residue (Tyr51) located in the T-loop, which has been observed in enteric bacteria like *E. coli* and *Klebsiella pneumoniae* (Jiang et al. 1998a; Atkinson and Ninfa 1999) and adenylylation also at Tyr51 for the GlnK proteins of *Corynebacterium glutamicum* and *Streptomyces coelicolor* (Hesketh et al. 2002; Strösser et al. 2004). A different modification was identified in cyanobacteria e.g. *Synechococcus elongatus.* Here the PII proteins are modified by phosphorylation at a conserved serin residue (Ser49), also located in the T-loop (Forchhammer and Tandeau de Marsac 1995). One exception in the modification pattern known to date is the GlnY protein of *Azoarcus* sp. BH72 that exists exclusively in its modified (uridylylated) form independent of the actual nitrogen supply (Martin et al. 2000). Although in general, bacterial PII-like proteins are modified in response to nitrogen availability it also seems that in various systems PII-like proteins are not subjected to covalent modifications, such as in Prochlorophytes (Palinska et al. 2002), *Bacillus subtilis* (Detsch and Stulke 2003) and plants (Smith et al. 2004). Functions assigned for the *E. coli* GlnB protein are the regulation of the

GS $\longrightarrow$ GS(AMP)$_{12}$　　　NtrC-P $\longrightarrow$ NtrC

ATase　　　　　　　NtrB　　　　　High N

PII

3 UMP

UR/UTase　　　　　　3 UTP

3 PPi

PII(UMP)$_3$

ATase

GS $\longleftarrow$ GS(AMP)$_{12}$　　NtrC-P $\longleftarrow$ NtrC　　Low N
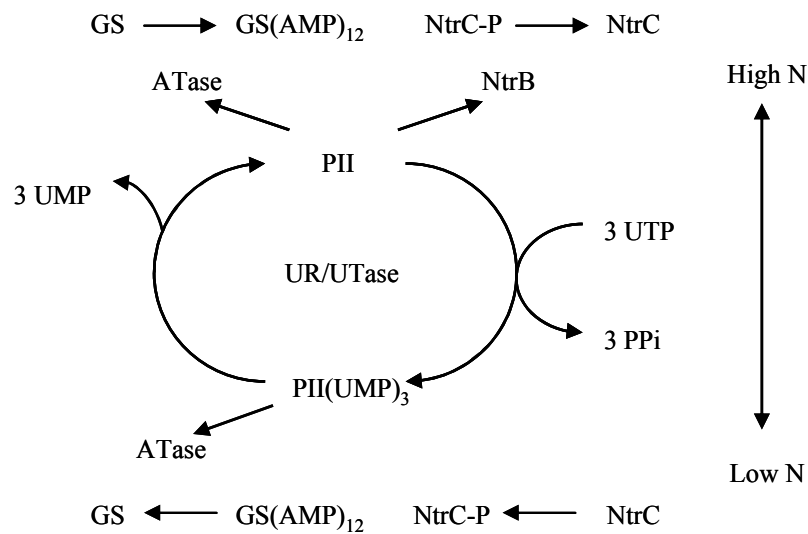
Fig. 1. Nitrogen regulatory system in enteric bacteria. The activities of both GS and NtrC are regulated in response to the intracellular nitrogen status. The uridylyltransferase/-removing enzyme UTase (*glnD* product) catalyzes the uridylylation and deuridylylation of PII (*glnB* product). The adenylyltransferase/-removing enzyme ATase (*glnE* product) catalyzes the adenylylation and deadenylylation of GS. NtrB catalyzes the phosphorylation and dephosphorylation of NtrC. (Arcondeguy et al. 2001) modified.

transcription activator NtrC by interacting with the corresponding histidine kinase NtrB (Jiang et al. 1998b): Under nitrogen sufficiency, the unmodified GlnB interacts with NtrB and thereby stimulates the phosphatase activity of NtrB resulting in a dephosphorylation of NtrC. Under nitrogen limitation, however, the uridylylated GlnB does not interact with NtrB, which thus acts as a kinase and NtrC is phosphorylated to its active form. Furthermore GlnB regulates, dependent on its modification state, the intracellular switch of the adenylyltransferase activity from adenylylation under nitrogen excess to deadenylylation under nitrogen limitation thereby controlling the glutamine synthetase activity (Jiang et al. 1998c; Reitzer 2003).

It has been shown that the *E. coli* GlnK protein acts as backup system and fine control for the PII regulatory cascade (Atkinson et al. 2002), however, previously it has been postulated that GlnK appears to have a distinct function as negative regulator for the AmtB ammonium transporter by being sequestered to the membrane in an AmtB-dependent manner upon an ammonium-upshift (Coutts et al. 2002; Javelle et al. 2004). Recently, it has also been demonstrated for *C. glutamicum* that after a shift to nitrogen sufficiency the

GlnK protein is sequestered to the membrane by interacting with the ammonium transporter AmtB (Strösser et al. 2004). In diazotrophic bacteria, GlnK is involved in the nitrogen dependent regulation of nitrogen fixation by transducing the signal of the internal nitrogen status to the inhibitor NifL and thereby regulating NifL-mediated inhibition of the *nif*-genes specific transcriptional activator NifA (Liang et al. 1992; Arsene et al. 1996; He et al. 1998; Arsene et al. 1999; Jack et al. 1999; Little et al. 2000; Rudnick et al. 2002; Drepper et al. 2003; Stips et al. 2004). Besides these interaction partners of GlnB and GlnK proteins in enteric bacteria, recently, a new receptor protein was identified for the PII protein of the cyanobacterium *S. elongatus*. Here, the activity of the N-acetylglutamate (NAG) kinase is enhanced by complex formation with PII (Heinrich et al. 2004). A similar mechanism was observed for the cyanobacterium *Synechococcus* sp. strain PCC7942, which led to the speculation that in general the regulatory PII/NAGK interaction is conserved in cyanobacteria (Burillo et al. 2004).

Genes encoding for PII-like proteins have also been identified in archaea but to date only few biochemical data are available for the respective proteins. Beside the "classical" PII-like proteins, GlnB and GlnK, a new group of PII-like signal molecules have been described in archaea; two *glnB*-like genes are located within the *nif*-gene cluster encoding for the nitrogenase enzyme complex (Kessler and Leigh 1999; Kessler et al. 2001). Due to their location between *nifH* and *nifD* these genes have been designated as *nifI$_1$* and *nifI$_2$* (Arcondeguy et al. 2001). Although they have been initially identified in genomes of methanoarchaea ongoing genome sequence projects revealed that *nifI$_1$/I$_2$* are also present in some bacterial species, e.g. *Clostridium acetobutylicum* and *Desulfovibrio gigas,* possibly acquired by horizontal gene transfer, for review see (Arcondeguy et al. 2001). Recently, it has been shown for *Methanococcus maripaludis* that after an ammonium-upshift both NifI-proteins form heteromeric complexes, which bind to the nitrogenase thereby inhibiting nitrogenase activity.

The main goal of this thesis was to elucidate the regulatory mechanisms of nitrogen assimilation by focussing on the function of PII-like proteins in methanogenic archaea, as only few was known about the regulation of nitrogen fixation and nitrogen assimilation in archaea in general and particularly in methanogens. As model organism the methylotrophic methanogenic *Methanosarcina mazei* strain Gö1 was chosen, which belongs to the order of Methanosarcinales and is able to use a wide substrate spectrum including H$_2$ plus CO$_2$, methanol, acetate and methylamines (Hippe et al. 1979; Deppenmeier et al. 1990). Reasons to choose this organism as model organism have been (i) that *M. mazei* has been

biochemically well studied in regard to methanogenesis and (ii) that the genome sequence was available allowing genome-wide functional analysis (Deppenmeier et al. 2002). The only disadvantage working with archaea has been the lack of efficient genetic methods, due to the special living conditions and unique features of these organisms. As methanogens are strictly anaerobic, plating under anaerobic conditions is problematic as agar plates are exposed to a low humidity as well as to adversarial pH and redox conditions that might inhibit growth of cells. Further problems to solve have been (i) the absence or inefficiency of antibiotics or other selection markers and (ii) the non existence of usable vectors or transposons for members of this domain. Besides, none of the methods for transformation, such as conjugation and transformation of purified DNA was functional in methanoarchaea. However, in recent years significant success has been achieved in developing new techniques and adapting already existing methods allowing genetic analysis in archaea (Tumbula et al. 1994; Argyle et al. 1996). The discovery that antibiotics such as puromycin, pseudomonic acid and neomycin are effective against archaea as well as the development of functional plasmid-based shuttle vectors helped in developing methods for genetic manipulation in archaea (Kiener et al. 1986; Possot et al. 1988; Argyle et al. 1996; Metcalf et al. 1997; Boccazzi et al. 2000). Finally, some years ago an efficient genetic approach especially for the genus *Methanosarcina* has been developed by Metcalf and co-workers (Metcalf et al. 1997). They established a liposome-mediated transformation protocol for transducing foreign DNA into *Methanosarcina* cells, by taking advantage of liposomes as DNA delivery vehicle: In a first step a liposome reagent forms complexes with the DNA to transform, which are then able to fuse with the membrane of *Methanosarcina* protoplasts. This method, which is not limited to autonomously replicating plasmids, but can also be used for linearized DNA and transposons, worked for most of the tested *Methanosarcina* strains but failed for *M. mazei* strain Gö1 (Metcalf et al. 1997). Though, at that time no specific attempt has been made to optimize the method for *M. mazei*.

The intention of this thesis was to get an insight into the regulation of nitrogen metabolism of *M. mazei* strain Gö1 by focussing on the contribution of the PII-like protein $GlnK_1$ the gene of which was identified in the genome sequence. One major goal was the characterization of the $GlnK_1$ protein concerning transcriptional regulation, synthesis and modification. The second goal was to identify potential interaction partners of $GlnK_1$ and to determine its potential regulatory function. For the latter genetic methods, effective

plating and a reliable transformation protocol had to be established for the organism in order to study $GlnK_1$ function by genetic approaches.

Chapter 1:

Functional organization of a single *nif* cluster in the mesophilic archaeon *Methanosarcina mazei* strain Gö1

SUMMARY

The mesophilic methanogenic archaeon *Methanosarcina mazei* strain Gö1 is able to utilize molecular nitrogen (N_2) as its sole nitrogen source. We have identified and characterized a single *nitrogen fixation* (*nif*) gene cluster in *M. mazei* Gö1 with an approximate length of 9 kbp. Sequence analysis revealed seven genes with sequence similarities to *nifH, nifI_1, nifI_2, nifD, nifK, nifE*, and *nifN* similar to other diazotrophic methanogens, and certain bacteria such as *Clostridium acetobutylicum* with the two *glnB*-like genes (*nifI_1, nifI_2*) located between *nifH* and *nifD*. Phylogenetic analysis of deduced amino acid sequences for the nitrogenase structural genes of *M. mazei* Gö1 showed that they are most closely related to *Methanosarcina barkeri nif2* genes, and also closely resemble those for the corresponding *nif* products of the Gram positive bacterium *C. acetobutylicum*. Northern blot analysis and reverse PCR analysis demonstrated that the *M. mazei nif* genes constitute an operon transcribed only under nitrogen starvation as a single 8 kb transcript. Sequence analysis revealed a palindromic sequence at the transcriptional start site in front of the *M. mazei nifH* gene, which may have a function in transcriptional regulatory processes of the *nif*-operon.

INTRODUCTION

Biological nitrogen fixation, the enzymatic reduction of atmospheric nitrogen (N_2) to ammonia, is not limited to the bacterial domain but is also observed in several methanogenic members of the archaeal domain. Nitrogenase, the enzyme complex of dinitrogenase and dinitrogenase reductase, is responsible for the reduction of molecular nitrogen; it is highly conserved in structure, function, and amino acid sequence across both domains (Lobo and Zinder 1992; Young 1992; Fischer 1994; Rees and Howard 1999). The dinitrogenase, which is an $\alpha_2\beta_2$ heterotetramer containing the P-cluster and the FeMo-cofactor, is encoded by *nifD* and *nifK*; the nitrogenase reductase is a homodimer with a

single [4Fe-4S]-cluster linking the subunits, and is encoded by *nifH* (Georgiadis et al. 1992; Rees and Howard 1999) and therein cited papers). In Bacteria*,* the genes *nifH, nifD* and *nifK,* which encode for the molybdenum-containing nitrogenase are typically found together in a single operon and are physically adjacent to other *nif* genes as part of a larger *nif* regulon. Downstream of *nifK,* the genes *nifE* and *nifN,* which are essential for FeMo-cofactor assembly (Dean et al. 1993), are found in a separate operon. In Archaea, genes homologous to the bacterial *nif* genes have been identified, and nitrogen fixation has been observed in several methanogenic species (Lobo and Zinder 1992; Young 1992; Bult et al. 1996; Chien and Zinder 1996; Haselkorn and Buikema 1996; Kessler et al. 1997) (Smith et al. 1997). The discovery of genes homologous to *nifH, nifD,* and *nifK* suggests that the basic mechanism of nitrogen fixation is similar in Bacteria and Archaea and predicts that most methanogenic nitrogenases contain a molybdenum-cofactor (Chien and Zinder 1996; Kessler et al. 1997). It was very recently shown, that unique among the archaea, *Methanosarcina acetivorans*, appears to contain all three types of nitrogenases, the molybdenum nitrogenase and two alternative nitrogenases (Galagan et al. 2002). In methanogenic archaea the nitrogen fixation genes *nifH, nifD, nifK, nifE* and *nifN* are present in the same order as in bacteria (Dean and Jacobson 1992). However, in contrast, (i) methanogenic *nif* gene promoters are typical archaeal promoters, and the apparatus of transcription is similar to that of *Eucarya* (Langer and Zillig 1993; Marsh et al. 1994; Langer et al. 1995; Qureshi et al. 1995; Hausner et al. 1996; Thomm 2000; Bell and Jackson 2001), (ii) the archaeal *nif* genes are present in a single operon, and (iii) all diazotrophic methanogens contain two open reading frames inserted between *nifH* and *nifD* that show a strong similarity to *glnB* (Sibold et al. 1991; Merrick and Edwards 1995; Arcondeguy et al. 2001; Kessler et al. 2001). Recently, this *nif* gene organization with the two *glnB*-like genes, which have been renamed into *nifI₁* and *nifI₂* (Arcondeguy et al. 2001), has also been found in *Clostridium acetobutylicum* (Nolling et al. 2001) and *C. beijerinckii* (Chen et al. 2001). The presence of the *nifI* ORFs within the bacterial *nif* operon may be either the result of a horizontal interdomain gene transfer or this respective arrangement is ancestral and other bacteria have lost it.

As with bacterial nitrogen fixation*, nif* gene transcription in the methanogenic archaea is regulated by the availability of nitrogen (Souillard and Sibold 1989; Chien and Zinder 1994; Kessler et al. 1998). However, because of the major differences between archaeal and bacterial transcription, it is likely that the mechanisms of methanogenic *nif* gene regulation differ significantly from bacterial *nif* regulation. Transcriptional regulation of *nif*

genes in response to the nitrogen status of the cells has been studied in detail for *Methanococcus maripaludis* and *M. barkeri* 227*;* experimental evidence was obtained by Leigh and his coworkers that nitrogen metabolism genes in *Methanococcus* and *Methanobacterium* species are coordinately regulated at the transcriptional level by a common repressor binding site (Cohen-Kupiec et al. 1997; Kessler and Leigh 1999). In *M. barkeri* 227, *nif* regulation is also mediated by a negative mechanism; however, in contrast to *Methanococcus,* this mechanism does not appear to be based on an operator site (Chien et al. 1998).

Our goal is to analyze the regulatory network of nitrogen metabolism and nitrogen fixation in *Methanosarcina mazei* strain Gö1. This mesophilic archaeon belongs to the methylotrophic methanogens of the order *Methanosarcinales* and is able to grow on H_2 plus CO_2, methanol, methylamines and acetate. The pathways of methanogenesis from these substrates have been analyzed in detail in recent years (Deppenmeier et al. 1990; Thauer 1998; Ferry 1999) and the genome of *M. mazei* strain Gö1 has been sequenced by the Göttingen Genomics Laboratory (Deppenmeier et al. 2002). In the following, we report on the arrangement and expression of *nif* genes in *M. mazei*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Methanosarcina mazei* strain Gö1 (DSM3647) was obtained from the DSM. pTZ19R sequencing vector (Pharmacia) and pSK^+ Bluescript (Stratagene, La Jolla, US) was used for subcloning and DNA sequencing.

Growth. *M. mazei* Gö1 was grown without shaking at 37 °C, in 5 ml or 25 ml closed growth tubes on 150 mM methanol in a minimal medium described previously generally under a nitrogen gas atmosphere containing 20% CO_2 (Deppenmeier et al. 1990). For nitrogen-limiting growth conditions, ammonium was omitted from the medium. Control growth experiments for nitrogen fixation were performed using either an argon atmosphere or a hydrogen atmosphere containing 20% CO_2. In general, growth was monitored by determining the optical density of the cultures at 600 nm (O.D.$_{600}$).

Cloning and nucleotide sequencing. The complete genomic sequence from *M. mazei* strain Gö1 was determined by a whole-genome-shotgun approach (Deppenmeier et al. 2002). The generated sequence readings were assembled into *contigs* with P. Greens PHRAP assembling tools and have been edited with GAP which is part of the STADEN

package software (Staden et al. 2000). Sequence analysis was performed with the Genetics Computer Group (GCG) program package (Devereux et al. 1984). The nucleotide sequences for *nifH, nifI₁, nifI₂, nifD, nifK, nifE*, and *nifN* have been submitted to GenBank (accession number AY029234).

RNA isolation and Northern blot analyses. *M. mazei* Gö1 cells from exponentially growing cultures (5 ml or 50 ml, O.D. $_{600}$ = 0.3 - 0.4) grown with N_2 or 10 mM NH_4^+ as nitrogen source, were anaerobically centrifuged in the growth tubes at 3000 x g for 10 min, and resuspended in 30 mM sodium acetate, pH = 5.2. After incubation with 1.5% sodium dodecyl sulfate, RNA was extracted using the RNeasy Kit (Qiagen, Santa Clarita, CA) according to the protocol of the manufacturer and treated with DNAse1. Following ethanol precipitation in the presence of 4 M LiCl the RNA pellet was resuspended in 30 or 60 µl RNAse-free water and stored at -70° C. RNA (9 - 12 µg) was separated by electrophoresis using a 1% denaturing agarose gel containing 6% formaldehyde, and then transferred to nylon membranes (Hybond-N, Amersham/Pharmacia, Piscataway NJ) by vacuum blotting according to the manufacturer's direction. After 3 min UV cross-linking, Northern hybridization was performed to locate the mRNA of interest (Sambrook 1989). Filters were hybridized overnight at 55 °C with [α–^{32}P] ATP-labelled *nifH, nifK* and *nifN* probes in the presence of 5 x SSC, 0.02% SDS and 0.1% laurylsarcosine (1 x SSC is 0.15 M NaCl plus 0.15 M sodium citrate). After hybridization, the membranes were washed twice with 2 x SSC / 0.1% SDS solution at room temperature for 5 min, twice with 2 x SSC / 0.1% SDS at 55 °C for 30 min, and twice with 0.1 x SSC / 0.1% SDS at room temperature for 30 min. The hybridized mRNA was detected and analyzed by using a PhosphorImager and the ImageQuant 1.2 software (Molecular Dynamics). The RNA marker standard used was obtained from New Englands Biolabs (UK).

Generating DNA probes. DNA probes for *nifH, nifK, nifN* and for 16S rRNA were amplified by PCR using genomic DNA from *M. mazei* Gö1 as template. The oligonucleotides used were: *nifH* sense primer (5'GCAATCTACGGAAAGGGCGG) and antiprimer (5'CATCAGTT CCCCACTGGCAAC), *nifK* sense primer (5'CCCAT-GTGAAGAGATAACCAGAG) and antiprimer (5'CGTCTTCAATGATAACTCCGA-CG), *nifN* sense primer (5'GAAGTGCCCTTGCCTTTAAAGG) and antiprimer (5'CCGGACTTTCAGGTTTTTCCG), *glnK₁* sense primer (5'TAGGATAGAGAATTCCTACTGGTGGTC) and antiprimer (5'CCATACAGTGT

AAGCTTCGTTTATAGCC) and 16S rDNA sense primer (5'GCAGCAGGCGCGAAAAC) and antiprimer (5'CGTTTACGGCTGGGACTA). Reactions were carried out in 100 μl volumes using Taq polymerase (New England Biolabs, UK) and primers at a concentration of 0.3 μM. The annealing temperature was 58 ° (49 °C for 16S rDNA) and synthesis was carried out for 30 s for 25 cycles. The PCR products, 450 bp (*nifH*), 417 bp (*nifK*), 438 bp (*nifN*), 415 bp (*glnK₁*) and 420 bp (16S rDNA), respectively, were purified by gel electrophoresis and extraction using the QIAQuick extraction kit (Qiagen, Santa Clarita CA). 1 - 1.5 μg purified PCR products were labeled with α-P^{32}ATP using the random labeling system from Gibco BRL (Random Primers Labeling System) according the protocol of the manufacturer.

Reverse transcription PCR. Transcriptional analysis by reverse transcription PCR (RT-PCR) was carried out on DNA-free RNA extracted from cells grown with N_2 or 10 mM NH_4^+ as nitrogen source by phenol/chloroform extraction and DNAseI treatment (Sambrook 1989). Control PCR reactions using RNA in the absence of reverse transcriptase showed that the isolated RNA preparations were free of genomic DNA. The RT-PCR reactions were carried out as recommended by the supplier (Qiagen, Santa Clarita CA) using 0.1 μg RNA, 0.6 μM sense primer and antiprimer for *nifH, nifK, nifN, glnK₁*, and 16S rDNA (see above) and the OneStep RT-PCR Kit (Qiagen, Santa Clarita CA). The control RT-PCR of 16S rDNA was carried out from 10 ng RNA with the respective primers. The annealing temperatures used were 58 °C (*nifH, nifK, nifN, glnK₁*) and 49 °C (16S rDNA). Products for each primer pair and growth condition were separated on 1.5% agarose gels and quantified using the GelDoc2000 Quantity One software (Bio-Rad Laboratories, Hercules CA).

Determination of the transcriptional start site. The *nif*-transcriptional start site was determined by using the 5'-RACE system, as recommended by the supplier (Gibco BRL) using 1 μg of total RNA (DNA-free) from cells grown under nitrogen starvation and specific primers, *nifH*-GSP1 (5`TGCCCCTGCCTGCACATCC) and *nifH*-GSP2 (5`CTCGGGCCCTCCTGATTCC), which hybridize to bases +297 to +278 and to bases +269 to +250 of *nifH*, respectively. The obtained PCR-product (310 bp) was cloned into pSK$^+$ Bluescript (Stratagene, La Jolla, US) and sequenced in both directions using an ABI PRISM 377 DNA sequencer.

RESULTS AND DISCUSSION

Our goal in this work has been to study nitrogen regulation in the methanogenic archaeon *Methanosarcina mazei* strain Gö1. By analyzing the sequences obtained from the genome sequencing project of *M. mazei* Gö1, we have identified a single nitrogen fixation (*nif*) gene cluster. We have characterized growth of *M. mazei* under nitrogen starvation and analyzed the structure and transcriptional regulation of this *nif* cluster.

Diazotrophic growth of *M. mazei* Gö1.

In order to determine if *M. mazei* is able to fix molecular nitrogen and to use it as the sole nitrogen source, we analyzed growth under nitrogen-limiting conditions. Growth experiments were performed under anaerobic conditions in defined nitrogen-depleted mineral medium under a N_2/CO_2 (80% / 20%) gas atmosphere, with methanol as carbon source. For nitrogen-sufficient growth, the medium was supplemented with 10 mM ammonium or 4 mM glutamine as nitrogen source (see Materials and Methods). When incubated under nitrogen-limiting conditions, with molecular nitrogen in the gas phase as the sole nitrogen source, *M. mazei* showed significant growth (Figure 1A). No growth was observed in controls with nitrogen-depleted medium using an argon/CO_2 (80% / 20%) or a hydrogen/CO_2 (80% / 20%) gas atmosphere (Figures 1B and 1C), indicating that growth of *M. mazei* observed with molecular nitrogen in the gas atmosphere results from nitrogen fixation. The doubling time under these nitrogen-limiting conditions was determined to be 13 h, versus 10 h, when grown under nitrogen-sufficient conditions (Figure 1A). In addition to this decrease in growth rate, nitrogen-limited cultures showed a prolonged lag-phase and the cell yield was approximately two-fold lower than under nitrogen-sufficient growth conditions (maximal optical density of $O.D._{600} = 0.35$ to 0.4 for nitrogen-limited conditions, vs. $O.D._{600} = 0.75$ for nitrogen-sufficient growth, Figure 1A). This reduced yield is likely due to the ATP expenditure of N_2 fixation and to the limited solubility of N_2 in the medium; as the cultures were incubated without shaking, diffusion of N_2 into the medium appeared to be insufficient to support high density cultures at higher optical densities.
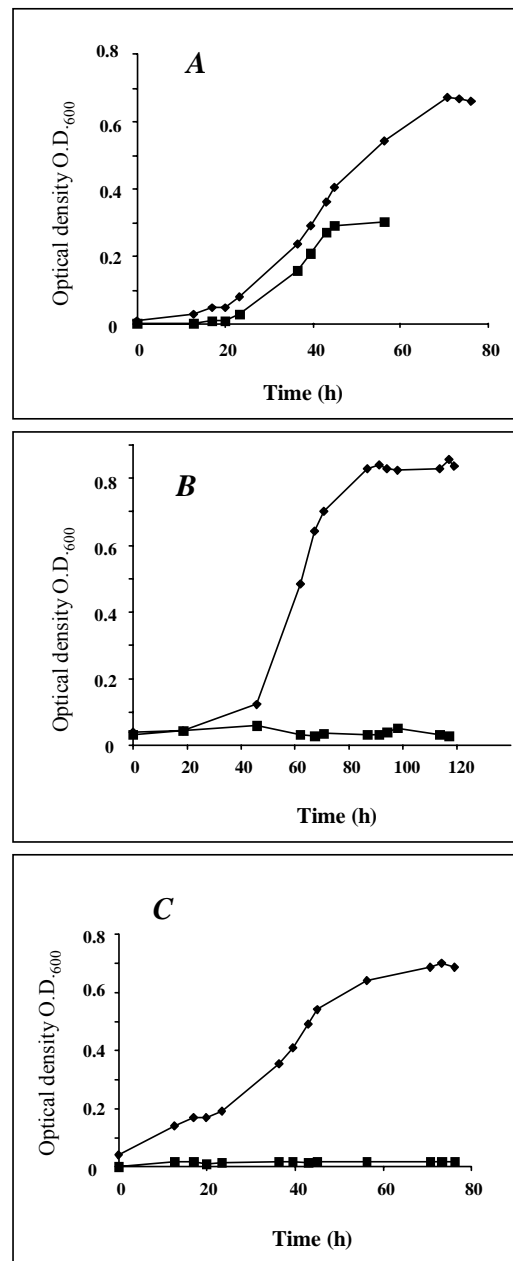
Fig. 1. Growth of *M. mazei* under different nitrogen availabilities. Cells were grown on 150 mM methanol in 5 ml minimal medium under a nitrogen gas atmosphere containing 20% CO_2 (**A**), a hydrogen gas atmosphere containing 20% CO_2 (**B**), or an argon gas atmosphere containing 20% CO_2 (**C**). The medium was supplemented with 10 mM ammonium (diamonds) or contained no additional sufficient nitrogen source (squares).

Sequencing and nucleotide analysis of the *nif* gene cluster from *M. mazei* strain Gö1.
The entire genome of *M. mazei* strain Gö1 has been sequenced by the Göttingen Genomics Laboratory (Deppenmeier et al. 2002). The assembled sequence data were searched for the presence of regions coding for methanogenic or bacterial subunits of nitrogenase. A single *nif* gene cluster, with an approximate length of 9 kbp, was detected. The fragment comprises seven open reading frames, each preceded by a putative ribosome-binding site and starting with the initiation codon ATG (or, in one case GTG), and terminated by stop codons TAA or TGA. Sequence analysis of this *nif* gene cluster was performed with the Genetics Computer Group (GCG) program package (Devereux et al. 1984), and revealed five genes with sequence similarities to nitrogen fixation genes *nifH, nifD, nifK, nifE* and *nifN*. In addition, two genes located between *nifH* and *nifD* showed sequence similarities to bacterial *glnB* genes (Sibold et al. 1991; Merrick and Edwards 1995; Arcondeguy et al. 2001) and were therefore designated *nifI$_1$* and *nifI$_2$*, respectively. Additional open reading frames, in the adjacent regions on either side of the cluster have been identified as tentative flavine containing oxidoreductase (upstream of *nifH*) and a potential periplasmic molybdate binding protein plus a molybdenum transporter (downstream of *nifN*). The gene order of the *M. mazei* nitrogen fixation genes is the same as in other diazotrophic methanogenic archaea, and differs from most bacterial *nif* gene clusters in that the two *nifI*-genes are located between *nifH* and *nifD* (Sibold et al. 1991; Chien and Zinder 1994; Chien and Zinder 1996; Kessler et al. 1998; Arcondeguy et al. 2001). Phylogenetic analysis of the deduced amino acid sequence of the *M. mazei* Gö1 *nif* genes showed that they are most closely related to *Methanosarcina barkeri nif2* genes suggesting that *M. mazei* Gö1 contains a molybdenum-containing nitrogenase (Table 1).

Table 1. Comparison of the deduced amino acid sequence of *M. mazei nif*-genes with those of other methanogenic and bacterial nitrogen fixing microorganisms. Percentage of identity was calculated using the GCG program (Devereux et al. 1984).

	M. mazei Gö1	*M. barkeri* (nif2)	*M. acetivorans*	*Mc. maripaludis*	*Clostridium acetobutylicum*	*C. pasteurianum*	*Klebsiella pneumoniae*
nifH	100 %	91 %	90 %	60 %	69 %	68 %	62 %
glnB'	100 %	88 %	90 %	56 %	49 %	-	-
glnB''	100 %	48 %	76 %	43 %	48 %	-	-
nifD	100 %	87 %	88 %	39 %	52 %	52 %	40 %
nifK	100 %	91 %	91 %	45 %	51 %	49 %	38 %
nifE	100 %	83 %	87 %	45 %	49 %	53 %	34 %
nifN	100 %	75 %	85 %	40 %	41 %	-[a]	26 %

[a] sequence not available in public databases

Beside the high similarity to the *nif2* gene cluster of *M. barkeri* and the *nif* cluster of *M. acetivorans* the gene products of the *M. mazei* nitrogenase structural genes further showed a significantly higher similarity to the corresponding *nif* gene products of the Gram-positive bacterium *Clostridium acetobutylicum* than to their archaeal counterparts in *Methanococccus maripaludis* and *Methanothermobacter thermoautotrophicus*. In addition, it was recently found that the *nif* cluster of *C. acetobutylicum* shows the same gene organization with the two *nifI*-genes located between *nifH* and *nifD* (Nolling et al. 2001). These findings suggest that the nitrogenase subunits of the *Methanosarcina* species are more closely related to the clostridial nitrogenases, than to the archaeal nitrogenases of *Methanococcus* and *Methanothermobacter*.

The deduced amino acid sequences of the two *glnB*-like *nifI*-genes showed considerable similarity to the corresponding gene products from the *nif* regions of other diazotrophic methanogens and *C. acetobutylicum* (Figure 2). The strongest similarities were observed in the B-loop region and the C-terminal regions just after the B-loop. In the T-loop regions, only the N-terminal regions showed a strong similarity (amino acid 33 to 42). In general, the GlnB-like proteins encoded by homologues of the *glnB* family located within *nif* clusters do not contain the conserved uridylylation site (tyrosine 51) of bacterial GlnB-proteins in the T-loop, which was originally shown by Sibold et al. (Sibold et al. 1991), and differ significantly from bacterial GlnB-proteins in the region N-terminal of amino acid residue 51 (amino acid 43 to 50). These findings suggest that NifI-proteins encoded in *nif* clusters are not likely to be covalently modified by uridylylation. However, the conserved tyrosine at position 49 in *M. mazei* $NifI_1$ and its homologues might be target for modification, if the NifI- proteins are modified at all. In addition to amino acid 49, amino acids in position 3, 16, 17 and position 51 and 54 in the T-loop are conserved within the $NifI_1$ proteins and within the $NifI_2$ proteins, respectively (Figure 2). The alignment further indicates, that the $NifI_2$ protein of *M. mazei* and its homologues contain additional amino acids in their C-terminal loop region, whereas only the B-loop of *C. acetobutylicum* $NifI_1$ contains three additional amino acids. Taking together, these *nifI* genes appear to represent a separate *glnB*-like family in addition to the GlnB and GlnK homologues. Experimental data indicate that the *nifI*-genes are not involved in the regulation of *nif* gene expression at the transcriptional level in *Methanococcus maripaludis* (Kessler et al. 1998). However, Leigh and coworkers have recently presented evidence that both *nifI*-genes are required for posttranslational inhibition by ammonium of the *Methanococcus maripaludis* nitrogenase ("ammonium switch-off") (Kessler and Leigh 1999; Kessler et al. 2001).

T -Loop

B-Loop

M. mazei glnB'	M Q M I R A I I R P G M E P K V V E S L E K E G C I S L T K
M. barkeri orf105	M Q M I R A I I R P G M E T K V I E C L E K E G C I S L T K
Mc. maripaludis glnBi	M K M I R A V V R P S K A E E V V D A L A E S G C L A L T K
C. acetobutylicum glnB'	M L M I K A I L R P Q K V T N V L S E L S D A G F P A V T K
M. mazei glnB"	V K E I T A I I R M N R V Q K T K D V L L E C G F P S F T V
M. barkeri orf125	M K E I T A I I R M N K A Q R T K D V L L E C G F P S F T I
Mc. maripaludis glnBii	M K E I I A I I R P S K M A Q T K T V L E G L G F P A M T A
C. acetobutylicum glnB"	M K E I M A I I R M N M V E K T K E A L L K G G N P A I T C
K. pneumoniae glnB	M K K I D A I I K P F K L D D V R E A L A E V G I T G M T V

M. mazei nifI₁	M E V F G R G K Q K G I H I A D V H Y D E - - - - - - - - -
M. barkeri orf105	M E V F G R G K Q K G I H I A D I S Y D E - - - - - - - - -
Mc. maripaludis glnBi	M D V I G R G K Q K G I K I D Q I Y Y D E - - - - - - - - -
C. acetobutylicum glnB'	F S V V G R G K Q R G V K V G D I Y Y D E - - - - - - - - -
M. mazei nifI₂	R R V M G R G K Q K G L C F E F N P P L P E P E K E A E T C
M. barkeri orf125	R R V M G R G K Q R G L C H E F N P P L P D P E K E A E T C
Mc. maripaludis glnBii	N R V L G R G K Q K A I V G E L G F E V D N K E L L - - - -
C. acetobutylicum glnB"	L K V L G R G R Q K V D F S M I E D Y I P N L M D Q K M A E
K. pneumoniae glnB	T E V K G F G R Q K G H T E L Y R G A E Y M V D - - - - - -
	51

M. mazei nifI₁	- - - - - - - - - L Q K T M L M M V V E D E H R D R V V Q T I
M. barkeri orf105	- - - - - - - - - L Q K T M L L M V V E D E H K D R A I K T I
Mc. maripaludis glnBi	- - - - - - - - - L P K T M L M L V V E D D T A E N V I E L I
C. acetobutylicum glnB'	- - - - - - - - I P K E M L L I V V N D E D K D D V V N I I
M. mazei nifI₂	- - - - - I - H F I P K R M F T I V V D D E N V N E V V R K I
M. barkeri orf125	- - - - - I - R F I P K R M F T I V V D D E N V S E V V Q K I
Mc. maripaludis glnBii	N Q P G D M R Y I P K T M L T L I V P D E D A S L V V E A I
C. acetobutylicum glnB"	E L S E I H R L I S K R L I I I L A K D E D V K E I V D E I
K. pneumoniae glnB	- - - - - - - - F L P K V K I E I V V T D D I V D T C V D T I

M. mazei nifI₁	M E A R T G - - - K Y G D G R I F V N P V E E A Y T I - -
M. barkeri orf105	M E S A R T G - - - K Y G D G R I F V T P V E E A Y T I - -
Mc. maripaludis glnBi	T K T A Y T G - - - S F G Y G K I F V S P V D E A Y T V - -
C. acetobutylicum glnB'	A K N A K T G E K G A F G D G K I F I V P V E Q A Y T I - -
M. mazei nifI₂	I E V N Q T G - - - H P G D G K I F V S S I S E A Y R I R T
M. barkeri orf125	I E V N Q T G - - - N A G D G K I F V S D V T E A I R I R T
Mc. maripaludis glnBii	M K V N K S G - - - Q Y G D G K I F V C P I E D I T V R T
C. acetobutylicum glnB"	I E V N R T G - - - N P G D G K I F V I N I A D A M R I R T
K. pneumoniae glnB	I R T A Q T G - - - K I G D G K I F V F D V A R V I R I R T

M. mazei nifI₁	- R T G K P G L
M. barkeri orf105	- R T G K P G L
Mc. maripaludis glnBi	- R T R S C G L
C. acetobutylicum glnB'	- S S G K A G L
M. mazei nifI₂	G E S G E A T V N K E M V
M. barkeri orf125	G E S G E A T V N K S W Y N G S R N R F K N
Mc. maripaludis glnBii	S E R G E A I
C. acetobutylicum glnB"	E E T G D M A I
K. pneumoniae glnB	G E E D D A A I

Fig. 2. Alignment of the two *glnB*-like genes of *M. mazei* located in the *nif* gene cluster in comparison with the corresponding *glnB*-like genes in *Mc. maripaludis*, *M. barkeri*, and *C. acetobutylicum,* and *glnB* from *Klebsiella pneumoniae*. The predicted secondary structural elements, the T-loop and the B-loop, are indicated above the sequence. The boxed areas show the tyrosine residue (Tyr51) which is the conserved site of uridylylation in bacteria and the corresponding amino acid residues of the *glnB*-like genes at position 51.

Interestingly, *nifI₂* homologues of *M. mazei* and *M. barkeri* differ from all known *nifI₂* homologues in that both deduced proteins contain additional amino acids in the C-terminal loop region, which or are not found in PII-like proteins (Figure 2).

**Transcriptional organization and regulation of the *nif* gene cluster in *M. mazei*.**

In order to analyze the promoter region we determined the *nif*-transcriptional start site by using the 5'-RACE method and RNA extracted from cells grown under nitrogen starvation with molecular nitrogen as the sole nitrogen source as described in Methods. The transcriptional start site was localized 42 bp upstream of the putative translational start site of *nifH* (Figure 3). A potential archaeal consensus promoter sequence is centered 38 to 25 bp upstream from the 5' end of the transcriptional start site, and 81 to 68 bp from the translational start codon ATG of *nifH*. It contains a typical archaeal factor-B-recognition element (BRE) [GAAA] and a potential TATA-box [TTTAAATA] (Figure 3). No similar promoter sequences were obtained at appropriate locations 5' of the other genes. Downstream of *nifN*, a potential transcriptional termination site was identified, containing
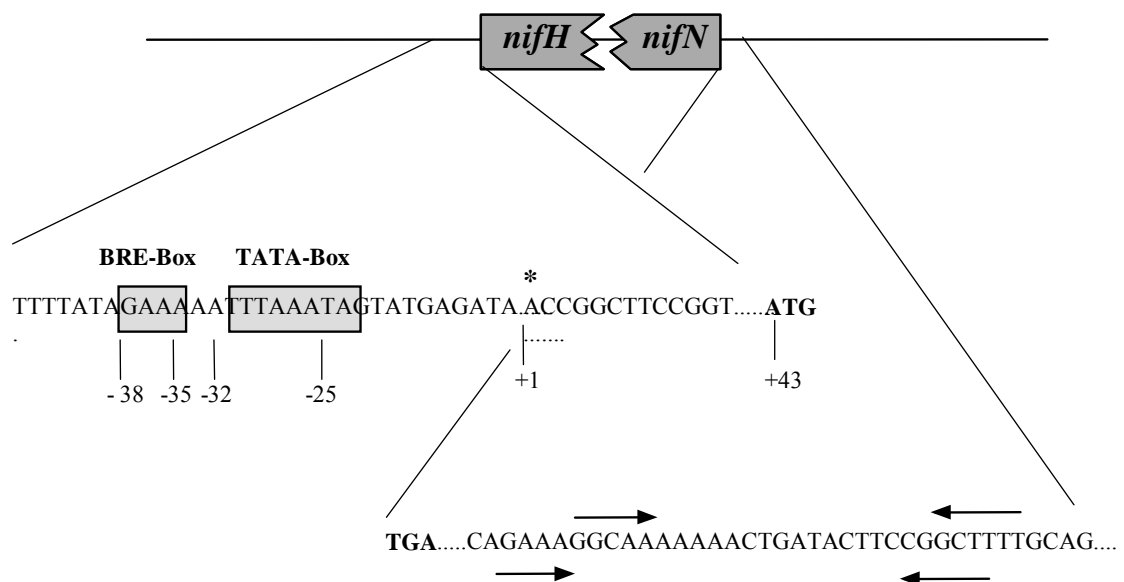
Fig. 3. Diagram of the putative promoter region upstream of *nifH* and putative terminator region downstream of *nifN*, within the *M. mazei nif* gene cluster. Key: *, transcriptional start site; TATA-box, archaeal TATA-box promoter element; BRE, factor B recognition element; stem loop structures are marked by arrows indicating the length and the orientation of the stems. Sequences are numbered relative to the mRNA initiation site determined by the 5' RACE method.

two potential stem loops followed by a T-rich region (Figure 3). These findings indicate that the *nif* genes in *M. mazei* are organized in one operon, with the two *nifI* genes included.

To examine for further evidence for an operon organization of the *nif* gene cluster, and to study the potential transcriptional regulation by combined nitrogen, we performed Northern blot analyses. RNA was extracted from cells grown under nitrogen starvation, with molecular nitrogen as the sole nitrogen source, and under nitrogen sufficiency in the presence of 10 mM ammonium. A single transcript was detected in mRNA isolated from cells grown under nitrogen starvation when an internal fragment derived from *nifH* was
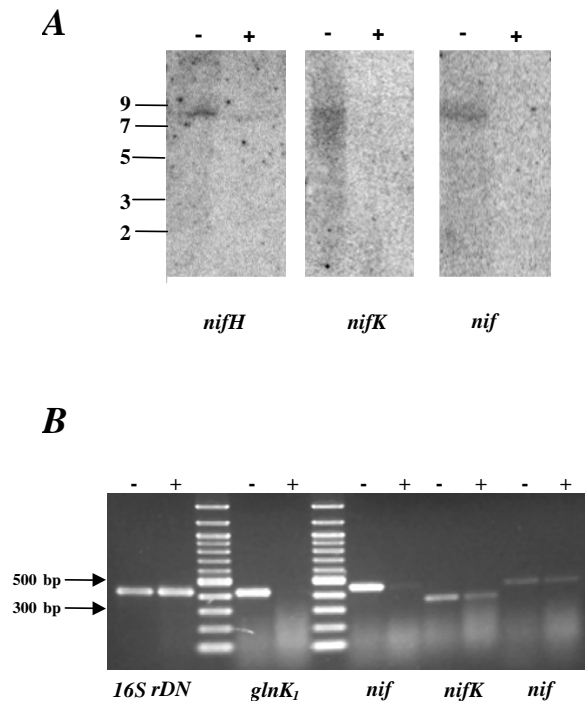
Fig. 4. Transcriptional analysis of the *M. mazei nif* gene cluster. (**A**) Northern blot analysis of total RNA isolated from *M. mazei* cells grown under nitrogen limitation (N$_2$) and nitrogen sufficiency (NH$_4^+$) by using probes for *nifH*, *nifK*, and *nifN*. Each lane was loaded with 0.25 µg total RNA from cells grown under nitrogen limitation (-) or nitrogen sufficiency (+); numbers of the left are molecular sizes in kilobases. (**B**) RT-PCR analysis. Reverse transcription was carried out on 0.1 µg RNA isolated from cells grown under nitrogen limitation (-) or nitrogen sufficiency (+) using the OneStep RT-PCR Kit from Qiagen and the respective primers as described in Materials and Methods. Control PCR reactions using RNA in the absence of reverse transcriptase showed that the isolated RNA preparations were free of genomic DNA. As a control a 16S rDNA-specific RT-PCR was carried out on 10 ng RNA for each growth condition. Products of the expected size (450 bp (*nifH*), 417 bp (*nifK*), 438 bp (*nifN*), 415 bp (*glnK$_1$*) and 420 bp (16S rDNA), respectively) were separated 1.5% agarose gels and visualized by ethidium bromide staining.

used as the hybridization probe (see Materials and Methods). As shown in Figure 4A, we observed the presence of the same single transcript in RNA isolated from cells grown under nitrogen-starvation conditions, using internal probes derived from *nifK* or *nifN*. The length of this single transcript was calculated to be approximately 8 kb, which is in accordance with the expected length of a *nifHI₁I₂DKEN* transcript. This indicates that the seven genes are co-transcribed from a single promoter (Figure 5). When the cells were grown under nitrogen sufficient conditions, however, no transcript was detected in the total RNA suggesting that transcription is regulated by ammonium and only occurs under nitrogen starvation (Figure 4A). The transcriptional regulation by nitrogen availability was further confirmed by reverse transcriptase PCR (Figure 4B). Only when RNA was extracted from cells grown under nitrogen limitation, the products of the expected sizes of the control gene *glnK₁*, (415 bp), which is known to be regulated by ammonium (Ehlers et al. 2002a), and *nifH* (450 bp) were amplified using the OneStep RT-PCR Kit (Qiagen) and the respective *glnK₁* and *nifH* primers. Using the same amounts of RNA extracted from nitrogen-limited cells the *nifK* product (417 bp) and the *nifN* product (438 bp) were significantly less amplified as the *nifH* product. Using RNA extracted from cells grown under nitrogen sufficiency, small amounts of the *nifH, nifK* and *nifN* products were obtained - equivalent to 1%, 18%, and 50% of the amount obtained from RNA extracted from nitrogen-limited cells, respectively (Figure 4B). The control RT-PCR using 16S rDNA-specific primers confirmed that equal amounts of RNA were used for nitrogen limitation and nitrogen sufficiency. These findings indicate that transcription of the *nif*-operon is not completely repressed under nitrogen sufficiency and the transcript is rapidly degraded beginning at the 3' end. The apparent degradation of the transcript also explains the difficulties in detecting the 8 kb transcript in the Northern blot analyses (Figure 4A).

The organization of the seven genes of the *nif* gene cluster in one operon in *M. mazei* is similar to the *nif* gene organization in *Mc. maripaludis*, but differs from the corresponding *nif2* gene cluster in *M. barkeri,* which appears to be organized in two transcriptional units (Chien and Zinder 1996; Kessler et al. 1998). The sequence at the transcriptional start site in front of the *nifH*-gene in *M. mazei,* shows a nearly palindromic sequence <u>ACCGG</u>CTT<u>CCGGT</u> (see Figure 3).
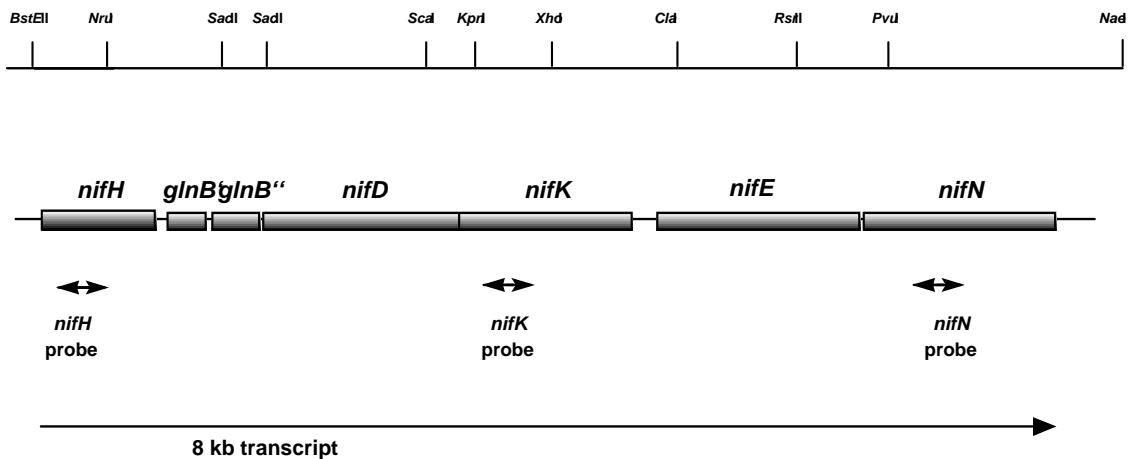
Fig. 5. Organization of the *nif*-gene cluster from *M. mazei* Gö1. The sizes of the boxes are proportional to the lengths of the genes. Probes used in Northern blot analysis, the single transcript observed, and the restriction sites are indicated.

For *Mc. maripaludis* Leigh and his coworkers identified a palindromic operator sequence located immediately 3' to the transcriptional start site for *nifH* (<u>CGGAA</u>AGAAGC<u>TTCCG</u>) (Cohen-Kupiec et al. 1997). Thus, the palindromic sequence in front of the *M. mazei nifH* gene may have a function in regulatory processes of *nif*-gene transcription. If this is the case, the mechanism of transcriptional regulation of the *nif* cluster in *M. mazei* may be more similar to the regulation in *Mc. maripaludis* and may differ from that in *M. barkeri*, which misses the operator sequence (Chien and Zinder 1996).

Conclusion:

We have identified a single ammonium-regulated *nif* gene cluster in the mesophilic methanogenic archaeon *M. mazei* strain Gö1 encoding for a molybdenum-containing nitrogenase most closely related to the molybenum nitrogenases of *M. acetivorans* and of *M. barkeri* 227. However, no additional *nif*-gene cluster, encoding a potential alternative nitrogenase, was observed in the complete *M. mazei* genome sequence. This is in contrast to *M. acetivorans* and *M. barkeri,* which contain three and at least two sets of *nif* genes, respectively (Chien et al. 2000; Galagan et al. 2002); ERGO database (Integrated Genomics, Inc.) (http://www.integratedgenomics.com) and ORNL database

(http://genome.ornl.gov/microbial/mbar). We cannot rule out that *M. mazei* has lost the genes encoding for the alternative nitrogenases. However, as the genome size of *M. mazei* is smaller compared to the two other organisms, the number of nitrogenases found in the organisms might correlate with the genome size of the respective organism.

Chapter 2:

Characterization of GlnK$_1$ from *Methanosarcina mazei* strain Gö1: Complementation of an *Escherichia coli glnK* mutant strain by *M. mazei* GlnK$_1$

ABSTRACT

Trimeric PII-like signal proteins are known to be involved in bacterial regulation of ammonium assimilation and nitrogen fixation. We report here the first biochemical characterization of an archaeal GlnK protein from the diazotrophic methanogenic Archaeon *Methanosarcina mazei* strain Gö1; and show that *M. mazei* GlnK$_1$ is able to functionally complement an *Escherichia coli glnK* mutant for growth on arginine. This indicates that the archaeal GlnK protein substitutes for the regulatory function of *E. coli* GlnK. *M. mazei* GlnK$_1$ is encoded in the *glnK$_1$amtB$_1$* operon, which is transcriptionally regulated by the availability of combined nitrogen and is only transcribed in the absence of ammonium. The deduced amino acid sequence of the archaeal *glnK$_1$* shows 44 % identity to the *E. coli* GlnK and contains the conserved tyrosine residue (Tyr 51) in the T-loop structure. *M. mazei glnK$_1$* was cloned and overexpressed in *E. coli,* and GlnK$_1$ was purified to apparent homogeneity. A molecular mass of 42 kDa was observed under native conditions indicating that its native form is a trimer. GlnK$_1$-specific antibodies were raised and used to confirm the *in vivo* trimeric form by Western analysis. *In vivo* ammonium up-shift experiments and analysis of purified GlnK$_1$ indicated significant differences to the *E. coli* GlnK. First, GlnK$_1$ from *M. mazei* is not covalently modified by uridylylation under nitrogen-limitation. Second, heterotrimers between *M. mazei* GlnK$_1$ and *K. pneumoniae* GlnK are not formed. Because *M. mazei* GlnK$_1$ was able to complement growth of an *E. coli glnK* mutant with arginine as the sole nitrogen source, it is likely that uridylylation is not required for its regulatory function.

INTRODUCTION

PII-like proteins, which are highly conserved nitrogen signaling molecules, are found in all three domains of life (Merrick and Edwards 1995; Hsieh et al. 1998; Ninfa and Atkinson

2000). The mechanism of signal transduction is best understood in enteric bacteria. It involves covalent modification of the PII protein, encoded by *glnB,* by the uridylyltransferase/uridylyl-removing enzyme, the *glnD* gene product (Merrick and Edwards 1995). The internal glutamine pool, the primary signal for the nitrogen status of the cells, modulates the activity of the GlnD protein (Ikeda et al. 1996; Jiang et al. 1998a; Ninfa and Atkinson 2000; Schmitz 2000), which subsequently transduces the signal through uridylylation or deuridylylation to the *glnB* gene product (GlnB). The unmodified form of GlnB acts as a signal for nitrogen excess, whereas the uridylylated GlnB is a signal for nitrogen starvation (for review see Merrick and Edwards 1995; Ninfa et al. 1995; Porter et al. 1995; Jiang et al. 1998a; Jiang et al. 1998b; Jiang et al. 1998c). Depending on its uridylylation status, GlnB regulates phosphorylation of the nitrogen regulatory protein NtrC by the histidine kinase NtrB and adenylylation of glutamine synthetase by the adenylyltransferase (ATase) (Jaggi et al. 1996; Jiang et al. 1997; Wyman et al. 1997; Jiang et al. 1998a; Jiang et al. 1998b; Jiang et al. 1998c).

It was recently discovered that *Escherichia coli* and many other members of the Proteobacteria encode two PII-like signal transduction proteins, GlnB and its paralogue GlnK (de Zamaroczy et al. 1996; van Heeswijk et al. 1996; de Zamaroczy 1998; Qian and Tabita 1998; Jack et al. 1999; Michel-Reydellet and Kaminski 1999; Martin et al. 2000; Ninfa and Atkinson 2000; Arcondeguy et al. 2001). The *glnK*-genes in enteric bacteria are organized in conjunction with a gene encoding an ammonium transporter (*amtB*). In contrast to the *glnB*-gene, enteric *glnK* genes are under the control of the general nitrogen regulatory system, and are therefore only expressed under nitrogen starvation (van Heeswijk et al. 1996; Jack et al. 1999; Ninfa and Atkinson 2000; Arcondeguy et al. 2001). However, GlnK is covalently modified by uridylylation, as is GlnB, in response to the internal nitrogen status; this occurs at the conserved tyrosine residue located in the T-loop (Y51) by the uridylyltransferase/uridylyl-removing enzyme GlnD (van Heeswijk et al. 1996; Jiang et al. 1998a; Atkinson and Ninfa 1999). Crystallographic analysis of the two PII-like trimeric proteins revealed that while GlnK and GlnB are structurally very similar, the conformation of the three loops (T-, C- and B-loop) can differ significantly (Jaggi et al. 1996; Xu et al. 1998). The T-loop is thought to be the site of interaction with other proteins involved in signal transduction; the conformation of the T-loop can thus determine the specific interactions of the PII-like proteins (Jiang et al. 1997; Arcondeguy et al. 2000). Recently, it has been discovered that in several diazotrophic bacteria the GlnK proteins are involved in regulation of nitrogen fixation (He et al. 1998; Arcondeguy et al. 1999; Jack et

al. 1999; Souza et al. 1999; Arcondeguy et al. 2000; Halbleib and Ludden 2000; Steenhoudt and Vanderleyden 2000). For the diazotroph *Klebsiella pneumoniae,* genetic experiments indicate that the uridylylation status of GlnK is not essential for its nitrogen signaling function in nitrogen fixation (He et al. 1998; Arcondeguy et al. 1999), whereas this may be of importance in other diazotrophic bacteria (Little et al. 2000).

Interestingly, GlnB homologues have also been identified in *Archaea*, suggesting that GlnB-like proteins are likely to play a key role in nitrogen sensing and regulation in *Archaea* as well. The first GlnB homologues found in diazotrophic methanogenic archaea are encoded within the *nif*-cluster between the structural genes for nitrogenase *nifH* and *nifD* (Chien and Zinder 1994; Chien and Zinder 1996; Kessler et al. 1998). These regulatory genes, formerly named *glnB*$_i$ and *glnB*$_{ii}$, are novel homologues of the *glnB* family and have been recently renamed into *nifI*$_1$ and *nifI*$_2$ (Arcondeguy et al. 2001); their regulatory function is presumed to be the switch-off of nitrogen fixation in the presence of ammonium, as shown for *Methanococcus maripaludis* (Kessler and Leigh 1999; Kessler et al. 2001). In addition, genomic sequencing has revealed additional *glnB*-like genes in methanogenic archaea not associated with *nif* genes (Bult et al. 1996; Klenk et al. 1997; Smith et al. 1997; Kessler and Leigh 1999). These *glnB*-like genes are linked to genes that encode for a putative ammonium transporter. In this respect they most resemble the bacterial *glnK* genes and may consequently have a regulatory function in nitrogen regulation, as has been shown for the bacterial GlnK proteins (reviewed in (Merrick and Edwards 1995; He et al. 1998; Jack et al. 1999; Little et al. 2000; Arcondeguy et al. 2001).

The mesophilic methanogenic Archaeon *Methanosarcina mazei* strain Gö1, which belongs to the methylotrophic methanogens of the order *Methanosarcinales*, is able to grow on H$_2$ plus CO$_2$, methanol, methylamines or acetate as sole carbon and energy source (Deppenmeier et al. 1996; Thauer 1998; Ferry 1999). We recently showed that *M. mazei* is able to use molecular nitrogen as sole nitrogen source and characterized a single nitrogen fixation (*nif*) gene cluster in *M. mazei* Gö1 (Ehlers et al. 2002b). In addition to the two *glnB*-like genes located between *nifH* and *nifD,* we have now identified two *glnK*-like genes in *M. mazei*. In this report we have characterized one of these methanogenic GlnK-like proteins, GlnK$_1$, and have investigated its potential role in nitrogen regulation. This is to our knowledge the first biochemical characterization of an archaeal GlnK-like protein.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The bacterial strains and plasmids used in this work are listed in Table 1. Plasmid DNA was transformed into *E. coli* cells according to the method of Inoue *et al*. (Inoue et al. 1990) and into *Klebsiella pneumoniae* cells by electroporation. The *glnK*::KIXX allele was transferred from *K. pneumoniae* UNF3433 (Jack et al. 1999) into *K. pneumoniae* wild-type by P1-mediated transduction with selection for kanamycin resistance, resulting in RAS31.

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

Strain or plasmid	Relevant genotype	Source or description
Strains		
Methanosarcina mazei strain Gö1	Wild-type	DSM (DSM364)
Klebsiella pneumoniae strains		
Klebsiella pneumoniae M5al	Wild-type	
RAS43	*Klebsiella pneumoniae* M5al / pRS149	This study
UNF3433	Δ(*his-nifH*)*2639 nifH*::MudAp*lac* Δ*lac-2002 sbl-300*::Tn*10 glnk*::KIXX	(Jack et al. 1999)
RAS31	*Klebsiella pneumoniae* M5al, but *glnK*::KIXX	This study
RAS32	RAS31 / pRS149	This study
RAS33	RAS31 / pRS161	This study
RAS44	RAS31 / pRS154	This study
RAS45	RAS31 / pRS155	This study
Escherichia coli strains		
NCM1529	*araD139*Δ(*argF-lacU*)*169 fth D5301 gyrA219 non-9 rpsL150 ptsF25 relA1 deoC1 trpDC700putPA1303*::[Kan[r]-(*nifH'-'lacZ*)] (wild type)	(He et al. 1998)
NCM1971	NCM1529 but *glnK*::Spc[r]	(He et al. 1998)
NCM1686	NCM1529 but *glnD99*::Tn*10*	(He et al. 1998)
HS9060	Δ*glnK* / Δ*glnB*	V. Weiss, unpublished
RAS40	HS9060 (Δ*glnB* Δ*glnK*) / pRS149	This study

RAS41	HS9060 ($\Delta gln\ B\Delta glnK$) /pRS149 plus pRIL	This study
RAS42	HS9060 ($\Delta glnB\ \Delta glnK$) / pRS155	This study
RAS37	NCM1971 / pRS149	This study
RAS38	NCM1971 / pRS154	This study
RAS39	NCM1971 / pRS161	This study

Plasmids

pRS63	pKK223-3 plus *lacI^Q* inserted in the *Sph*I and *Xma*III site	This study
pRS149	*Ms. mazei glnK$_1$* controlled by the *tac* promoter (pRS63 derivative)	This study
pRS161	*Ms. mazei glnK$_1$* controlled by the *tet* promoter (pACYC184 derivative)	This study
pRS154	*K. pneumoniae glnK* controlled by the *tet* promoter (pACYC184 derivative)	This study
pRS155	*K. pneumoniae glnK* controlled by the *tac* promoter (pRS63 derivative)	This study
pMmdb57	pTZ19R containing *Ms. mazei glnK$_1$amtB$_1$* operon on a 2.3 kbp fragment.	This study
pRIL	pACYC-based plasmid containing extra copies of the *argU, IleY* and *leuW* tRNA	Stratagene
pJES1208	*glnD*' under heat induceable *lac* promoter	S. Kustu, unpublished
pTZ19R	Sequencing vector	Pharmacia/ Amersham

Construction of plasmids. Plasmid pRS149, which contains *M. mazei glnK$_1$* under the control of the *tac* promoter, was constructed as follows. A 0.4 kbp PCR fragment carrying *glnK$_1$* was generated using chromosomal *Methanosarcina mazei* DNA as template and a set of primers, which were homologous to the *glnK$_1$* flanking 5´- and 3´-regions with additional *Eco*RI and *Hind*III synthetic restriction recognition sites (underlined) (5'TAGGATAGA<u>GAATTC</u>CTACTGGTGGTC3', sense primer (MmGlnK); 5' CCATACAGTGT<u>AAGCTT</u>CGTTTATAGCC3', antisense primer (MmGlnK2)). The 0.4 kbp PCR product was cloned into the *Eco*RI and *Hind*III sites of pRS63 and the correct insertion was analyzed by sequencing. pRS155 encoding *K. pneumoniae glnK* under the control of the *tac* promoter was constructed by PCR-cloning. A PCR-generated *Eco*RI restriction site upstream of the start codon was used to clone the gene into the expression vector pRS63 restricted with *Eco*RI and *Sma*I. pRS161 and pRS154 contain the *M. mazei glnK$_1$* and *K. pneumoniae glnK* genes, respectively, inserted into the *Eco*RV and *Sal*I sites of pACYC184 and thereby expressed from the *tet* promoter.

Cloning and nucleotide sequencing. The complete genomic sequence from *M. mazei* strain Gö1 was determined by a whole-genome shotgun approach. Chromosomal DNA was isolated according to the method described by Ausubel et al. (Ausubel et al. 1987)**,** physically sheared by a HPLC-pump and fractionated by gel electrophoreses. 2 - 5 kbp fragments were ligated into pTZ19R sequencing vector and the resulting recombinant plasmids transformed into *E. coli* DH5α. More than 20,000 clones from small insert libraries (inserts of approximately 2.5 kbp) representative of the whole genome were purified using a QIAGEN Biorobot 9600 and sequenced in both directions using LICOR IL 4200 and ABI PRISM 377 DNA sequencers. The generated sequences were assembled into *contigs* with P. Greens phrap assembling tools and have been edited with GAP which is part of the STADEN package software (53). Sequence analysis was performed with the Genetics Computer Group (GCG) program package (Devereux et al. 1984). Plasmid Mmdb57 contains the *glnK$_1$amtB$_1$* operon on a 2.2 kbp fragment.

Protein purification. For GlnK purifications, plasmids pRS149 and pRS155 were transformed into *E. coli* HS9060 (V. Weiss, unpublished) with chromosomal deletions in *glnB* and *glnK*, to avoid contamination by *E. coli* PII-like proteins. For expression, 1 l cultures were grown aerobically in LB medium at 37 °C. Expression of proteins was induced with 150 µM isopropyl-ß-D-thiogalactopyranoside (IPTG) when cultures reached a turbidity at 600 nm of 0.6. Cell extracts were prepared by disruption of cells in 50 mM Tris/HCl pH 7.6 using a French pressure cell followed by centrifugation at 20,000 x g. *M. mazei* GlnK$_1$ and *K. pneumoniae* GlnK were purified from the supernatant by mercapthoethanol precipitation (22 % (vol/vol)) followed by intensive dialysis of the supernatant with 50 mM Tris/HCl pH 7.6 containing 0.1 mM EDTA and subsequent purification by anion exchange chromatography. The supernatant was applied to a Q-Sepharose FF (XK26, Pharmacia/Biotech) and chromatographed with a linear gradient from 0 to 0.5 M NaCl (total volume 300 ml). GlnK fractions eluted at 180 - 255 mM NaCl in a total volume of 40 ml and were reapplied to Q-Sepharose FF (XK16 Pharmacia/Biotech) after 1:4 dilution with 50 mM Tris/HCl pH 7.6. The *K. pneumoniae* GlnK fractions, which eluted at 290 mM NaCl, showed apparent homogeneity and were stored at - 70 °C in 50 mM Tris/HCl, pH 7.6 containing 50 % glycerol. *M. mazei* GlnK$_1$ fractions from the second Q-Sepharose FF were further purified by gel filtration on Sephacryl S200 (100 cm x 12 mm; Pharmacia/Biotech) to achieve homogeneity. A polyclonal rabbit antiserum was raised to the *M. mazei* GlnK$_1$ protein by Goetek

(Göttingen, Germany), which was specific for GlnK$_1$ from *M. mazei* and did not cross react with either *K. pneumoniae* or *E. coli* GlnK.

<u>GlnD' Purification</u>: pJES1208 containing *glnD'* (1-1077bp) was transformed into the *glnD* mutant *E. coli* strain NCM1686 (Hsieh et al. 1998). The resulting strain was grown in 1 l LB medium at 30 °C. When the cells reached a turbidity of 0.6 at 600 nm, GlnD' expression was achieved by heat induction at 43 °C for 30 min followed by an incubation at 37 °C for 2 h. Cell extract was prepared by disruption of the cells in 50 mM Tris/HCl pH 7.5 using a French pressure cell followed by centrifugation at 20,000 x g. Purification of GlnD' to 95 % apparent homogeneity was achieved by anion exchange chromatography on Q-Sepharose FF (XK26, Pharmacia/Biotech, 60 ml) using a linear gradient from 0 to 1 M KCl (total volume 500 ml). The GlnD' protein eluted at 230 mM KCl and was further purified by gel filtration on Sephacryl S200 (Pharmacia/Biotech) using 50 mM Tris/HCl pH 7.5 containing 200 mM KCl. The purified fractions were concentrated and stored at - 70°C.

***In vitro* uridylylation.** Purified GlnK$_1$ protein fractions synthesized under nitrogen sufficiency appeared to be in their unmodified trimeric form as determined by native gelelectrophoresis. *In vitro* uridylylation was performed at 30 °C using purified *E. coli* GlnD' containing only the uridylylase transferase activity. The 200 µl test assay contained 50 mM Tris/HCl, pH 7.5, 100 mM KCl, 10 mM MgCl$_2$, 1 mM dithiothreitol, 0.285 µM GlnD', 1 mM UTP, 0.1 mM ATP, and either 0.67 µM *K. pneumoniae* GlnK or 0.67 µM *M. mazei* GlnK$_1$. Uridylylation was initiated by addition of 50 µM 2-ketoglutarate to the reaction. Concentrations of GlnD' are stated in terms of a monomer; the concentration of the GlnK fractions are stated in terms of the trimer. Samples were removed from the assay at various times, treated with 25 mM EDTA to stop the uridylylation reaction, and analyzed by native gel electrophoresis.

Alternatively radioactive labeled UTP was used. The standard uridylylation reactions were performed as above but contained 0.4 mM UTP and 0.08 MBq [α–^{32}P] UTP with a specific activity of 110 TBq/mmol and were started with 5 mM 2-ketoglutarate. After 20 sec, 10 min and 60 min samples were removed from the assay treated with 25 mM EDTA, separated on a denaturing 12.5 % polyacrylamide gel and analyzed using a PhosphoImager and the ImageQuant 1.2 software (Molecular Dynamics). The gel was subsequently stained for protein with Coomassie Brilliant Blue.

Analysis of *M. mazei* GlnK$_1$ uridylylation by native gel electrophoresis. For the analysis of GlnK modifications, the different mobilities of the unmodified and uridylylated protein in non-denaturing polyacrylamide gels were investigated (Forchhammer and Hedler 1997). Native gel electrophoresis was performed by using 12.5% polyacrylamide gels (29:1, acrylamide:bisacrylamide) with 5% stacking gels. The buffer for the running gels was 187.5 mM Tris/HCl, pH 8.9, the buffer for the stacking gels was 62.5 mM Tris/HCl, pH 7.5; and the running buffer was 82.6 mM Tris/HCl, pH 9.4, containing 33 mM glycine. Gels were run using a BioRad Miniprotein I electrophoresis apparatus and were either stained with Coomassie Brilliant Blue or were subsequently transferred on nitrocellulose membranes for western blot analysis. In general, uridylylated forms of PII-like proteins show higher mobilities in non-denaturing polyacrylamide gels resulting in a protein band with an apparent lower molecular mass than the respective non-modified protein.

Heterotrimerization of *M. mazei* GlnK$_1$ with GlnK from *K. pneumoniae*. Potential heterotrimer formation was analyzed *in vitro* by denaturation of 0.25 μM *M. mazei* GlnK$_1$ in the presence of 0.25 μM *K. pneumoniae* GlnK with 6 M urea for 20 min on ice. *K. pneumoniae* GlnK was either unmodified or completely uridylylated (see *in vitro* uridylylation). After subsequent renaturation by dialysis for 16 h into 50 mM Tris/HCl pH 7.6 containing 0.1 mM EDTA trimers were analyzed by native gel electrophoresis and western blot analysis.

Growth. *E. coli glnK* mutant strains carrying plasmids encoding *M. mazei glnK$_1$* and *K. pneumoniae glnK* were grown under aerobic conditions at 37° C in minimal medium supplemented with 0.1 mM tryptophan and 0.5 % glucose as the sole energy and carbon source (He et al. 1997). Precultures for growth experiments on the limiting nitrogen source arginine were grown in medium supplemented with 4 mM glutamine, which was completely utilized during growth of the precultures. The main cultures (30 ml) supplemented with 10 mM arginine as the sole nitrogen source were grown in 100 ml flasks at 37° C under vigorous shaking. Expression of the *M. mazei glnK$_1$* gene was not induced with IPTG (isopropyl-ß-D-thiogalactopyranoside). During growth, samples were taken for monitoring growth at 600 nm and for protein expression. Protein expression was monitored by western blot analyses using polyclonal rabbit antiserum against *M. mazei* GlnK$_1$ and *K. pneumoniae* GlnK. Main cultures of *E. coli* HS9060 (ΔglnB / ΔglnK)

carrying *M. mazei glnK*$_1$ or *K. pneumoniae glnK* were grown under the same conditions but with additional 0.02 % glycine in the minimal medium.

K. pneumoniae wild-type strains were grown under anaerobic conditions with N$_2$ as gas phase at 30° C in 40 ml minimal medium supplemented with 0.4 % sucrose as sole carbon source. In strains carrying *M. mazei glnK*$_1$, GlnK$_1$ expression was induced with 10 or 50 µM IPTG. Growth was monitored by determination of the turbidity of the culture at 600 nm and cell extracts were prepared for western blot analysis.

M. mazei strain Gö1 was grown without shaking at 37 °C in 5 ml closed growth tubes on 150 mM methanol in a minimal medium described previously under a nitrogen gas atmosphere containing 20 % CO$_2$ (Deppenmeier et al. 1990). For nitrogen-limiting growth conditions, ammonium was omitted from the medium. For the ammonium upshift experiments, 50 ml cultures were grown under nitrogen-limiting conditions until the cells reached a turbidity of 0.4 at 600 nm. The cells were then harvested and resuspended under anaerobic conditions in minimal medium containing 100 µM or 15 mM ammonium without a carbon source, and incubated for additional 1 or 3 h at 37 °C. Cell extracts were prepared for western blot analyses by disruption in 50 mM Tris/HCl pH 7.6 by sonification.

Western blot analyses. Cells were grown under the different growth conditions described. When exponentially growing cultures reached a turbidity of 0.4 to 0.7 at 600 nm, 1 ml samples were harvested and concentrated 20-fold into sodium dodecyl sulfate (SDS) gel-loading buffer (Laemmli 1970). Samples were separated by SDS/polyacrylamide (12.5 %) gel electrophoresis. For analysis by native gel electrophoresis followed by western blotting, cell extracts were prepared from 50 ml cultures by disruption in 50 mM Tris/HCl pH 7.6 by sonification. Proteins were transferred to nitrocellulose membranes as described previously, and the membranes were exposed to polyclonal rabbit antisera directed against the GlnK proteins of *K. pneumoniae* and *M. mazei*. Protein bands were detected with secondary antibodies directed against rabbit immunoglobulin G and coupled to horseradish peroxidase (BioRad Laboratories) using either the Colour Development Reagent system (BioRad Laboratories) or the ECL-Plus system (Amersham Pharmacia Biotech) for detection. Purified GlnK from *K. pneumoniae,* purified GlnK$_1$ from *M. mazei,* and prestained protein markers (New England Biolabs, UK) were used as standards.

RNA isolation. *M. mazei* Gö1 cells (5 ml) from exponentially growing cultures (turbidity at 600 nm = 0.3 - 0.4), grown with N$_2$ or 10 mM NH$_4^+$ as nitrogen source, were

anaerobically centrifuged in the growth tubes at 1000 x g for 10 min and resuspended in 30 mM sodium acetate, pH 5.2. After incubation with 1.5 % sodium dodecyl sulfate, RNA was extracted using the RNeasy Kit (Qiagen) according to the protocol of the manufacturer and treated with DNAseI. Following ethanol precipitation in the presence of 4 M LiCl, the RNA pellet was resuspended in 30 μl RNAse-free water and stored at -70° C.

Northern blot analyses. RNA (9 - 12 μg) was separated by electrophoresis using a 1% denaturing agarose gel containing 6 % formaldehyde, and then transferred to nylon membranes (Hybond-N, Amersham/Pharmacia) by vacuum blotting according to the manufacturer's direction. After 3 min UV cross linking, Northern hybridization was performed to locate the mRNA of interest (Sambrook 1989). Filters were hybridized overnight at 55 °C with $[\alpha\text{--}^{32}P]$ ATP-labeled $glnK_1$ probe in the presence of 5 x SSC, 0.02 % SDS and 0.1% laurylsarcosine (1 x SSC is 0.15 M NaCl plus 0.15 M sodium citrate). After hybridization, the membranes were washed twice with 2 x SSC / 0.1 % SDS solution at room temperature for 5 min, twice with 2 x SSC / 0.1 % SDS at 55 °C for 30 min and twice with 0.1 x SSC / 0.1 % SDS at room temperature for 30 min. The hybridized mRNA was detected and analyzed by using a PhosphorImager and the ImageQuant 1.2 software (Molecular Dynamics). The standard RNA marker used was obtained from New Englands Biolabs (UK).
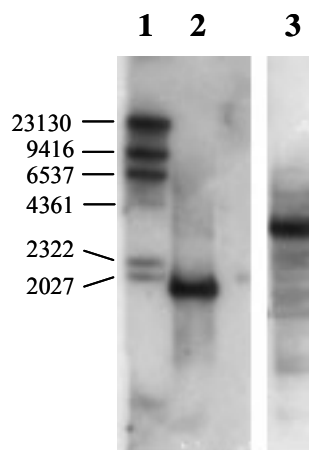
Fig. 1. Specificity of the *M. mazei glnK* probes. Genomic *M. mazei* DNA was completely digested by *Pst*I. Southern hybridization was performed as described by Sambrock et al. (Sambrook et al. 1989) using $glnK_1$-DNA probe (lane 2) and $glnK_2$-DNA probe (lane 3) (see Materials and Methods); lane 1, labeled DNA marker.

Generating the $glnK_1$-DNA probe: $glnK_1$ was amplified by PCR using genomic DNA from *M. mazei* Gö1 as template and the oligonucleotides MmGlnK and MmGlnK2 (see above). Reactions were carried out in 100 µl volumes using Vent polymerase (New England Biolabs, UK) and primers at a concentration of 0.12 µM. The annealing temperature was 59 °C and synthesis was carried out for 30 s, for 30 cycles. The 415 bp PCR product was purified by gel electrophoresis and extraction using the QIAQuick extraction kit (Qiagen). 1 - 1.5 µg purified PCR products were labeled with [α-P^{32}] ATP using the random labeling system from Gibco (Random Primers Labeling System) according the protocol of the manufacturer. The specificity of the $glnK_1$-DNA probe was tested by Southern hybridization (Sambrook 1989) with *M. mazei* DNA completely digested by *Pst*I. Under the conditions used for Northern blot analysis, the hybridization with the labeled $glnK_1$ probe resulted in only one hybridization signal (1.8 kbp) and did not cross react with the fragment hybridizing with the labeled $glnK_2$ probe (3.1 kbp) (see Fig. 1). $glnK_2$ probe (394 bp) was generated and labeled as described for the $glnK_1$ probe using a set of $glnK_2$ specific primers.

Determination of the transcriptional start site. The $glnK_1$-transcriptional start site was determined by using the 5'-RACE system, as recommended by the supplier (Gibco BRL) using 1 µg of total RNA (DNA-free) from cells grown under nitrogen starvation and specific primers, $glnK_1$-GSP1 (5`CCAACGTAACCGTCACTGCC3`) and $glnK_1$-GSP2 (5`GCCTCAATTGTTGGCTCAAG3`), which hybridize to bases +265 to +245 and to bases +226 to +205 of $glnK_1$, respectively. The obtained PCR-product (243 bp) was cloned into pSK$^+$ Bluescript (Stratagene, La Jolla, US) and sequenced in both directions using an ABI PRISM 377 DNA sequencer.

Data deposition. The nucleotide sequence for *M. mazei* $glnK_1amtB_1$ has been submitted to Genbank under accession number AF36724.
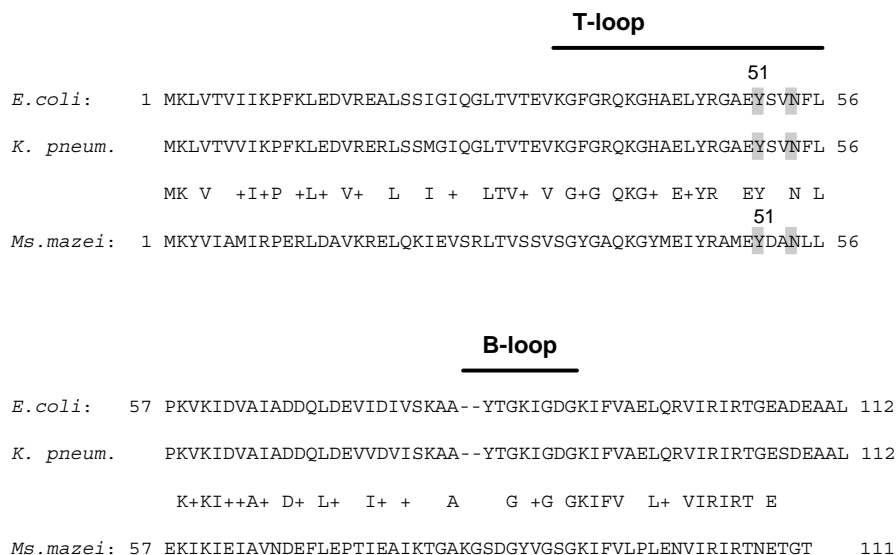
RESULTS

In the genome sequence of the diazotrophic methanogenic archeaon *Methanosarcina mazei* strain Gö1, we identified two *glnB*-like genes, which are not located within the *nif*-cluster and most closely resemble the bacterial *glnK*-gene. Bacterial GlnB-like proteins are known

to be involved in general nitrogen regulation and some of these have been shown to have a specific role in nitrogen fixation (Merrick and Edwards 1995; He et al. 1998; Jack et al. 1999; Arcondeguy et al. 2001). Based on this knowledge the present work was designed to characterize the identified *M. mazei* GlnK$_1$ protein and examine the potential regulatory function of the archaeal GlnK protein.

Cloning, sequencing, and transcriptional analysis of the *glnK$_1$amtB$_1$* operon in *M. mazei* strain Gö1.

The entire genome of *M. mazei* strain Gö1 has been sequenced by the Genomics Laboratory Göttingen. In the course of sequencing, the preliminary data were checked for the presence of *glnB*-like genes. In addition to *nifI$_1$* and *nifI$_2$*, which are localized between *nifH* and *nifD* (Ehlers et al. 2002b), we identified two *glnB*-like open reading frames, the gene products of which showed high similarity to bacterial GlnB proteins. In contrast to *nifI$_1$* and *nifI$_2$*, both of these additional *glnB*-like genes were organized in an operon in conjunction with a gene, the product of which showed a high similarity to bacterial ammonium transporters. This structural organization prompted us to designate the operons *glnK$_1$amtB$_1$* and *amtB$_2$glnK$_2$*. We concentrated on characterizing *glnK$_1$amtB$_1$*, which is located 8 kbp downstream of the *nif* gene cluster.

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                                    T-loop
                                    ────────────────
                                                  51
E.coli:     1 MKLVTVIIKPFKLEDVREALSSIGIQGLTVTEVKGFGRQKGHAELYRGAEYSVNFL 56

K. pneum.     MKLVTVVIKPFKLEDVRERLSSMGIQGLTVTEVKGFGRQKGHAELYRGAEYSVNFL 56

            MK V +I+P +L+ V+  L  I +  LTV+ V G+G QKG+ E+YR  EY  N L

                                                  51
Ms.mazei:   1 MKYVIAMIRPERLDAVKRELQKIEVSRLTVSSVSGYGAQKGYMEIYRAMEYDANLL 56



                            B-loop
                            ──────────
E.coli:    57 PKVKIDVAIADDQLDEVIDIVSKAA--YTGKIGDGKIFVAELQRVIRIRTGEADEAAL 112

K. pneum.     PKVKIDVAIADDQLDEVVDVISKAA--YTGKIGDGKIFVAELQRVIRIRTGESDEAAL 112

            K+KI++A+ D+ L+  I+ +   A    G +G GKIFV  L+ VIRIRT E

Ms.mazei:  57 EKIKIEIAVNDEFLEPTIEAIKTGAKGSDGYVGSGKIFVLPLENVIRIRTNETGT    111
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Fig. 2. Sequence comparison. Alignment of the amino acid sequences of *M. mazei* GlnK$_1$, *E. coli* GlnK, and *K. pneumoniae* GlnK. Arginine at position 54 and the functional tyrosin at position 51, which is known to be uridylylated in GlnK of *K. pneumoniae* and *E. coli*, are indicated with gray boxes. The loop regions are indicated above the corresponding amino acids. The two additional amino acids of *M. mazei* GlnK$_1$, which are not found in *K.pneumoniae* and *E. coli* GlnK, are indicated with a gray box.

Sequence analysis revealed that the $glnK_1$ gene (342 bp) codes for a polypeptide of 114 amino acids with a predicted molecular mass of 12.744 kDa, which showed 44 % and 43 % identity, respectively, to the bacterial GlnK proteins of *Escherichia coli* and *Klebsiella pneumoniae*. Interestingly, the conserved tyrosine residue of the T-loop (Y51), the site of modification by uridylylation in the bacterial PII proteins, is also present in the archaeal $GlnK_1$ protein of *M. mazei* (Fig. 2). In addition, the amino acid residue in position 54 of *M. mazei* $GlnK_1$ is asparagine (N54), which has recently been shown to be a critical amino acid in the T-loop of *K. pneumoniae* GlnK in distinguishing GlnB and GlnK in respect to the ability of GlnK to regulate nitrogen fixation and is possibly directly involved in the interaction with NifL/NifA (Arcondeguy et al. 2000). $AmtB_1$ encodes for a polypeptide of a predicted molecular mass of 41.556 kDa (396 amino acids), the amino acid sequence of which showed 38 % identity to the bacterial AmtB-protein of *Escherichia coli*.

Transcriptional analysis of the $glnK_1amtB_1$ operon in Northern blot analysis using a homologous $glnK_1$ probe, revealed the presence of one main transcript of approximately 2.3 kb length, but only for cultures grown under nitrogen-limiting conditions (Fig. 3). This indicates that transcription of $glnK_1amtB_1$ plus the open reading frame downstream from $amtB_1$ (*orfX*) (Fig. 4), coding for a hypothetical protein, is initiated at a single
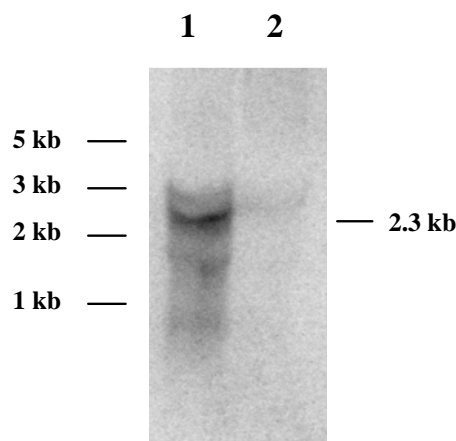
Fig. 3. Transcriptional analysis of $glnK_1amtB_1$. Northern blot hybridizations of total RNA isolated from *M. mazei* Göl grown on N_2 as the sole nitrogen source (lane 1) or ammonium-supplemented medium (lane 2) by using radioactively labeled $glnK_1$ probe. Numbers at left are molecular sizes in kilobases, the estimated size of the hybridizing mRNA species is indicated on the right.

transcriptional start site and is repressed by ammonium, as is the case in bacteria. The two additional smaller transcripts, which appear in very low amounts under nitrogen limitation, may result from termination after *amtB₁* (in case of the approx. 1.5 kb transcript) and / or from specific degradation of the 2.3 kb transcript. In order to confirm the presence of a single transcriptional start site and to analyze the promoter region we determined the *glnK₁*-transcriptional start site by using the 5'-RACE method and RNA extracted from cells grown under nitrogen starvation as described in Methods. The transcriptional start site was localized 17 bp upstream of the putative translational start site of *glnK₁*. 65 to 31 bp upstream from the 5' end of the transcriptional start site of the *glnK₁amtB₁*-operon a potential archaeal consensus promoter sequence was identified. It contains a typical archaeal factor-B-recognition element (BRE) [CGAAA] and a potential TATA-box [TTTAGATA] (see Fig. 4). No similar promoter sequences were obtained at an appropriate location 5' of *amtB₁*.
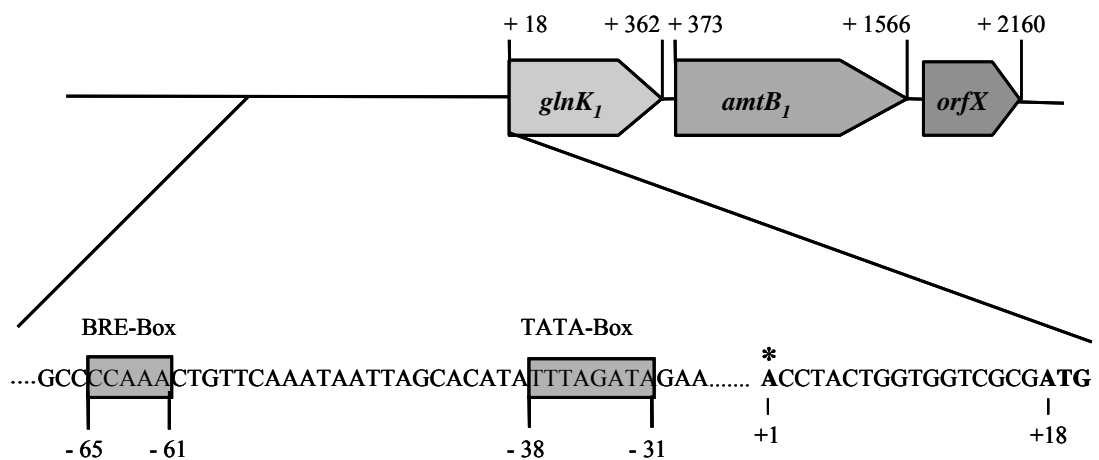
Fig. 4. Diagram of the putative promoter region upstream of *glnK₁amtB₁*. Key: *, transcriptional start site; TATA-box, archaeal TATA-box promoter element; BRE, factor B recognition element. Sequences are numbered relative to the mRNA initiation site determined by the 5' RACE method (see Materials and Methods).

Purification and characterization of heterologously expressed *M. mazei* GlnK₁.

We cloned *glnK₁* from *M. mazei*, placed it under the control of the *tac* promoter and overproduced the corresponding protein in an *E. coli* background with chromosomal deletions in *glnK* and *glnB* to avoid contamination of bacterial PII-proteins (RAS40, see Materials and Methods). Overproduction of the archaeal protein was evaluated by gel electrophoretic analysis of the induced cell extracts. In general, expression resulted in an overproduction of *M. mazei* GlnK₁ protein up to approximately 4 % of the cell extract protein. GlnK₁ expression in RAS41 in the presence of an additional pACYC-based plasmid containing extra copies of seldom tRNA genes, did not significantly increase expression levels of GlnK₁. Thus, the archaeal protein was overexpressed in RAS40 and purified from cell-free extracts by mercapthoethanol precipitation, anion exchange chromatography and gel filtration to apparent homogeneity as described in Materials and Methods. Subsequent characterization of purified *M. mazei* GlnK₁ by gel electrophoresis under denaturating and native conditions revealed that GlnK₁ has a molecular mass of approximately 14 kDa, which is constistent with the predicted molecular mass based on the DNA sequence, and is a trimer in its native state.

Analysis of GlnK₁ under different nitrogen availabilities.

In the nitrogen regulatory system, bacterial PII proteins are covalently modified by a uridylyltransferase in response to nitrogen limitation at a conserved tyrosine residue (Y51) located in the T-loop. Within the *M. mazei* genome no open reading frame was identified, the deduced amino acid sequence of which showed significant similarities to the enteric uridylyltransferase (GlnD). However, *E. coli* GlnD has been shown to heterologously uridylylate a variety of PII-like proteins at the conserved tyrosine residue (Y51); even tyrosine residue (Y51) of the PII-like protein from S*ynechococcus* PCC 7942 is uridylylated by *E. coli* GlnD, although *in vivo* the PII protein is modified by phosphorylation at a serine residue (S49) in response to nitrogen limitation (Forchhammer and Tandeau de Marsac 1995; Forchhammer and Hedler 1997). In order to examine if tyrosine 51 in *M. mazei* GlnK₁ is modified by *E. coli* GlnD, we performed *in vitro* uridylylation assays with purified GlnK₁ protein from *M. mazei*. Additionally we analyzed *M. mazei* GlnK₁ in *in vivo* ammonium up-shift experiments of nitrogen-limited *M. mazei* cultures to study whether GlnK₁ is modified in response to the internal nitrogen status of the cells.
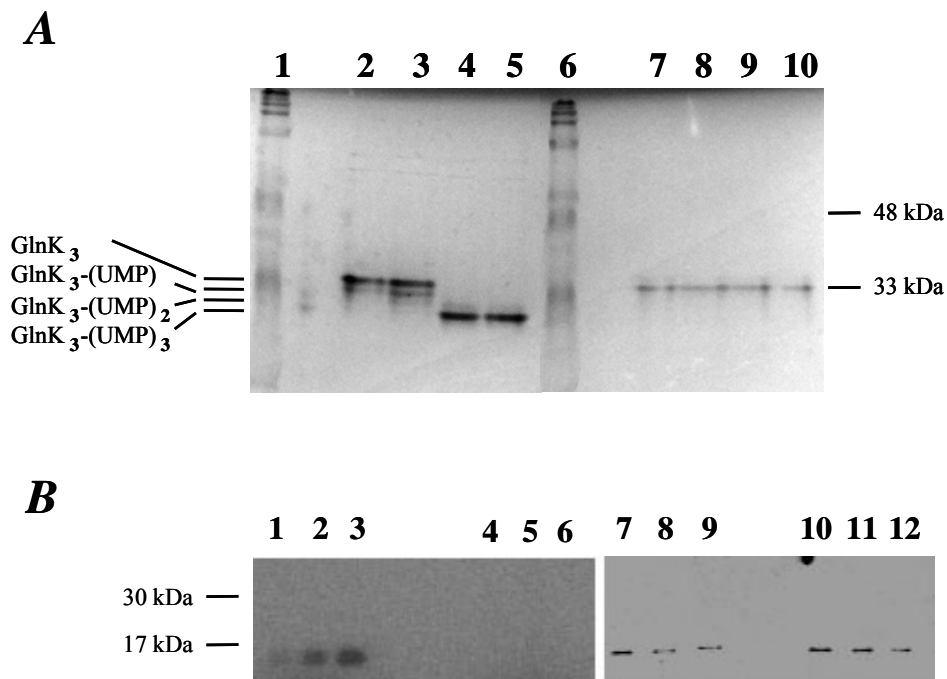
Fig. 5. GlnD-dependent modification of GlnK proteins. *In vitro* uridylylation of 0.67 μM purified *K. pneumoniae* GlnK and *M. mazei* $GlnK_1$ using *E. coli* GlnD' (0.285 μM). (A) The standard uridylylation reactions were performed at 30° C and started with the addition of 50 μM 2-ketoglutarate as described in methods. Samples were removed from the assay after 5 s, 20 s, 10 min and 60 min, treated with 25 mM EDTA to stop the reaction, and the reaction products were separated and analysed by non-denaturating 12.5 % PAGE Gels were stained for protein with Coomassie Brilliant Blue. Lanes 1 and 6, prestained high molecular mass standards (NewEngland, Biolabs); lanes 2 to 5, *K. pneumoniae* GlnK; lanes 7 to 10, *M. mazei* $GlnK_1$. The electrophoretic mobility of *K. pneumoniae* GlnK trimers uridylylated to different amounts are indicated. (B) The standard uridylylation reactions were performed as described above but contained of 0.4 mM UTP and 0.08 MBq [α–^{32}P] UTP with a specific activity of 110 TBq/mmol and were started with 5 mM 2-ketoglutarate. After 20 sec, 10 min and 60 min samples were removed and treated with 25 mM EDTA, separated on a denaturing 12.5 % polyacrylamide gel and analyzed using a PhosphoImager and the ImageQuant 1.2 software (Molecular Dynamics) (lanes 1 to 6). The gel was subsequently stained for protein with Coomassie Brilliant Blue (lanes 7 to 12). Lanes 1 to 3 and lanes 7 to 9, the respective samples of *K. pneumoniae* GlnK; lanes 4 to 6 and lanes 10 to 12, the respective samples of *M. mazei* $GlnK_1$.

As PII-like proteins are trimers in their native state, they can appear in the cell in four trimer conformations uridylylated to different amounts: unmodified trimers (PII_3), trimers with one (PII_3-(UMP)) or two monomers uridylylated (PII_3-(UMP)$_2$), or completely uridylylated trimers (PII_3-(UMP)$_3$). The uridylylation status of PII-like proteins can be determined by native gel electrophoresis. In general, uridylylated forms of the PII-like

proteins show higher mobilities in non-denaturing polyacrylamide gels, resulting in a protein band with an apparent lower molecular mass than the respective non-modified protein (see Fig. 5A lanes 2 to 5, which show the *K. pneumoniae* GlnK trimers uridylylated to different amounts). In order to determine potential modification by uridylylation, purified *M. mazei* GlnK$_1$, which was heterologously synthesized in the *E. coli* Δ*glnB*/Δ*glnK* background under nitrogen sufficiency, was analyzed by native gel electrophoresis. In comparison with the unmodified and the modified trimeric forms of *K. pneumoniae* GlnK, it appeared to be in its unmodified trimeric form (Fig. 5A compare line 7 with lanes 2 and 5). Using purified *E. coli* GlnD' enzyme, which contains only the uridylylase transferase activity, we analyzed whether *M. mazei* GlnK$_1$ synthesized under nitrogen sufficiency can be heterologously modified by uridylylation *in vitro*. The uridylylation was performed at 30 °C in the presence of 2-ketoglutarate, UTP and ATP, and the grade of uridylylation was analyzed after various incubation times by native gel electrophoresis (see Materials and Methods). As a control, unmodified *K. pneumoniae* GlnK protein was modified in a separate uridylylation assay under the same conditions. The time dependence of the respective uridylylation assays is depicted in Figure 5A. Trimeric unmodified *K. pneumoniae* GlnK was completely uridylylated within 10 min (Fig. 5A, lane 4), whereas *M. mazei* GlnK$_1$ appeared to remain unmodified by characterization of its mobility on native gels even after 60 min incubation in the uridylylation assay (Fig. 5A, lane 10). Also subsequent silver staining the gel or western analysis did not indicate the presence of an additional *M. mazei* GlnK$_1$ trimer conformation (data not shown). To rule out that uridylylation of *M. mazei* GlnK$_1$ does not alter the mobility of the trimeric protein in native gels, we used radioactive labeled UTP in the *in vitro* uridylylation assays (see Materials and Methods). Analysis of those uridylylation assays of *K. pneumoniae* GlnK and *M. mazei* GlnK$_1$, shown in Fig. 5B, confirmed that *M. mazei* GlnK$_1$ is not modified by *E. coli* GlnD'. This failure to heterologously uridylylate the *M. mazei* GlnK$_1$ *in vitro* may result from the specificity of the *E. coli* GlnD' protein, or from the absence of a metabolic signal specific to *Methanosarcina*.

As the *in vitro* assay might miss a *Methanosarcina*-specific metabolic signal, we analyzed potential *in vivo* modifications of chromosomally-expressed GlnK$_1$ by ammonium up-shift experiments with exponentially-growing *M. mazei* cells. In order to monitor chromosomally-expressed GlnK$_1$ under different nitrogen availabilities by immunological means, we raised a specific polyclonal rabbit antiserum against *M. mazei* GlnK$_1$ (see Materials and Methods). *M. mazei* cells were grown in minimal medium with either
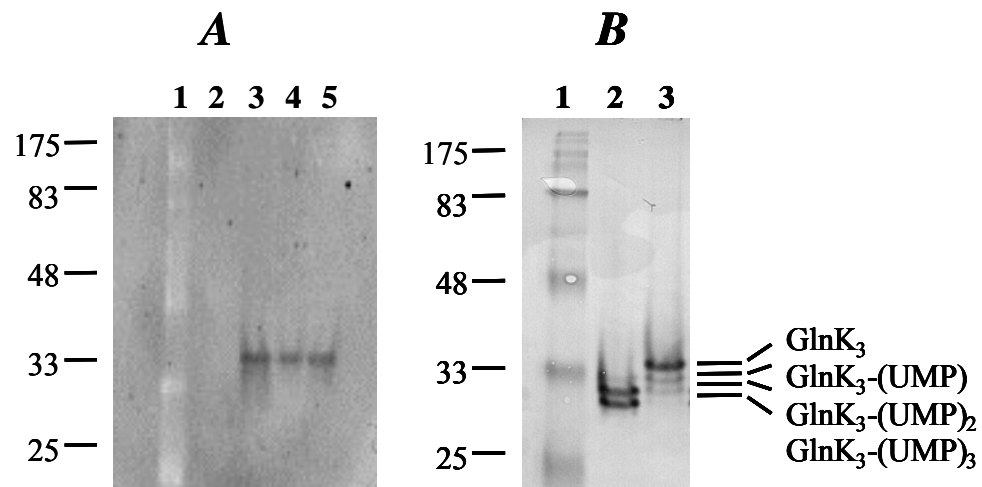
Fig. 6. Western blot analysis of chromosomally expressed GlnK₁ of *M. mazei*. The expression and modification of the GlnK₁ protein in *M. mazei,* as dependent on the nitrogen availability, was studied. Cell extracts were separated by non-denaturing PAGE, and the mobility of GlnK₁ was analyzed by immunoblot analysis. (**A**) *M. mazei* cells were grown in minimal medium supplemented with 15 mM ammonium (lane 2) or with molecular nitrogen as the sole nitrogen source (lane 3 and 4). Lane 5, *M. mazei* cells grown under nitrogen limitation but shifted to 15 mM ammonium for 3 h (ammonium up-shift); the ECL-Plus system (Amersham Pharmacia Biotech) was used for immunological detection. (**B**) As a control, modification of *K. pneumoniae* GlnK, expressed under nitrogen-limiting conditions, then exposed to ammonium, was analysed. Lane 2, GlnK modification by uridylylation, under nitrogen-limiting conditions before the ammonium up-shift; lane 2, GlnK modification, 3 h after the ammonium up-shift; the Colour Development Reagent system (BioRad Laboratories) was used for immunological detection. Lane 1 in A and B, prestained high molecular mass standards (NewEngland, Biolabs).

molecular nitrogen as the sole nitrogen source (nitrogen-limiting) or with 15 mM ammonium. For the ammonium up-shift experiments, exponentially-growing nitrogen-limited *M. mazei* cells were harvested under anaerobic conditions, resuspended in anaerobic minimal medium, containing no carbon source but supplemented with 15 mM ammonium and incubated further at 37°C for 1 or 3 h. Analyses of chromosomally-synthesized GlnK₁ protein fractions of the different cell extracts by native gel electrophoresis and subsequent Western blot analysis are shown in Fig. 6. Expression of GlnK₁ in *M. mazei* is detectable only under nitrogen limitation with molecular nitrogen as the sole nitrogen source (Fig. 6A, lanes 3 and 4). Upon the ammonium upshift, no modification of chromosomally-expressed GlnK₁ was detected by a change of its migration behavior, as is seen for *K. pneumoniae* GlnK. *M. mazei* GlnK₁ under nitrogen-limiting

conditions showed the same migration behavior as the unmodified trimeric form of *K. pneumoniae* GlnK and was not affected by the ammonium up-shift (Fig, 5A, lanes 4 and 5 compared to Fig. 5B, lanes 2 and 3). Also shifts to much lower ammonium concentrations (100 μM) and further incubation for 1 or 3 h did not result in any changes of the GlnK$_1$ migration behavior (data not shown). This finding that *M. mazei* GlnK$_1$ is not uridylylated upon an ammonium up-shift indicates that the archaeal GlnK$_1$ protein - if modified at all - may be modified in a different manner in response to the presence of ammonium.

Analysis of heterotrimer formation of *M. mazei* GlnK$_1$ with *K. pneumoniae* GlnK

Formation of heterotrimers between different PII-like proteins in one species and also between PII-like proteins from different species have been recently demonstrated (Forchhammer et al. 1999; van Heeswijk et al. 2000). Furthermore, in *E. coli* and *K. pneumoniae* it has been proposed that the formation of functionally inactive heterotrimers between GlnK and PII monomers have a significant function in nitrogen regulation upon an ammonium up-shift (Arcondeguy et al. 1999; Forchhammer et al. 1999; Arcondeguy et al. 2000). In order to analyze whether *M. mazei* GlnK$_1$ is able to form heterotrimers with *K. pneumoniae* GlnK, we studied heterologous expression of *M. mazei* GlnK$_1$ in *K. pneumoniae* and heterotrimer formation *in vitro*.

M. mazei GlnK$_1$ was heterologously expressed in a *K. pneumoniae* wild-type strain growing with molecular nitrogen as the sole nitrogen source. It has been shown that GlnK is required for nitrogen fixation in *K. pneumoniae* (He et al. 1998; Jack et al. 1999), thus growth under those conditions requires functional GlnK trimers. The heterologous expression of *M. mazei* GlnK$_1$ in *K. pneumoniae* (RAS43) did not affect growth or nitrogen fixation of *K. pneumoniae* (data not shown). This indicates that heterologous expression either resulted in *M. mazei* GlnK$_1$ homotrimers, which do not affect the native *K. pneumoniae* GlnK trimers, or resulted in the formation of functional heterotrimers between *K. pneumoniae* GlnK and *M. mazei* GlnK$_1$. Immunological analyses of the trimeric GlnK-proteins in cell extracts using specific polyclonal antibodies directed against *M. mazei* GlnK$_1$ or *K. pneumoniae* GlnK$_1$, showed that no heterotrimers were formed (Fig. 7A). The cell extract of RAS43, grown with molecular nitrogen as the sole nitrogen source, contained completely uridylylated homotrimers of *K. pneumoniae* GlnK, which did not react with the antibodies directed against *M. mazei* GlnK$_1$, and unmodified homotrimers of

A

GlnK$_3$
GlnK$_3$-(UMP)$_3$

B

GlnK$_3$
GlnK$_3$-(UMP)$_3$
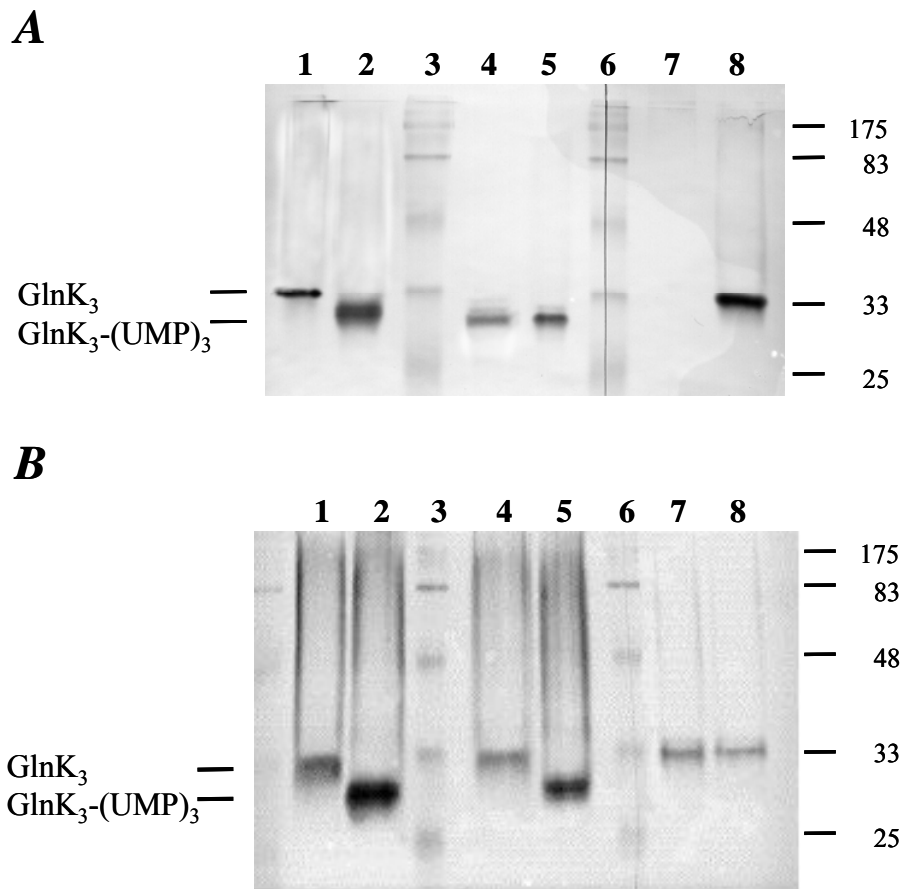
Fig. 7. *In vivo* **and** *in vitro* **analysis of trimer formation between** *K. pneumoniae* **GlnK and** *M. mazei* **GlnK$_1$.** (**A**) *In vivo* analysis: *K. pneumoniae* cells were grown at 30 °C in minimal medium with molecular nitrogen as the sole nitrogen source. In RAS43, the synthesis of *M. mazei* *glnK$_1$* was induced with 10 μM IPTG. Cell extracts of exponentially growing cells were separated by non-denaturating PAGE. Potential formation of heterotrimers between *K. pneumoniae* GlnK and *M. mazei* GlnK$_1$ was analyzed by immunoblot analysis using specific polyclonal antibodies directed against *K. pneumoniae* GlnK (lane 1 to 5) and *M. mazei* GlnK$_1$ (lane 7 and 8). lanes 4 and 7, *K. pneumoniae* wild-type cell extract (approx. 15 μg); lanes 5 and 8, *K. pneumoniae* cell extract with additional expressed *M. mazei* GlnK$_1$, RAS43 (approx. 17 μg). (**B**) *In vitro* analysis: 0.25 μM *M. mazei* GlnK$_1$ was denatured with 6 M urea in the presence of 0.25 μM *K. pneumoniae* GlnK$_1$, which was either unmodified or completely uridylylated as described in Methods. After renaturation by dialysis for 16 h, trimers formed were analyzed by native gel electrophoresis and western blot analysis using specific polyclonal antibodies directed against *K. pneumoniae* GlnK (lane 1 to 5) and *M. mazei* GlnK$_1$ (lane 7 and 8). lanes 4 and 7, denaturation and renaturation of *M. mazei* GlnK$_1$ in the presence of unmodified *K. pneumoniae* GlnK; lanes 5 and 8, denaturation and renaturation of *M. mazei* GlnK$_1$ in the presence of completely uridylylated *K. pneumoniae* GlnK. As controls, lanes 1 and 2 contained, respectively, 0.2 μg completely deuridylylated and uridylylated *K. pneumoniae* GlnK trimers. Lanes 3 and 6, prestained high molecular mass standards (NewEngland, Biolabs).

M. mazei GlnK$_1$, which did not react with the antibodies directed against *K. pneumoniae* GlnK (Fig. 7A, lanes 5 and 8, respectively). No heterotrimers were detectable, which one would expect to react with both specific polyclonal antibodies, and to migrate between the two homotrimeric forms, GlnK$_3$-(UMP)$_3$ from *K. pneumoniae,* and (GlnK$_1$)$_3$ from *M. mazei*.

In addition to these *in vivo* studies, we analyzed heterotrimer formation *in vitro* by denaturation by 6 M urea and subsequent renaturation of GlnK trimers by dialysis (see Materials and Methods). 0.25 µM *M. mazei* GlnK$_1$ was denatured and renatured in the presence of equal amounts of *K. pneumoniae* GlnK. In addition to unmodified *K. pneumoniae* GlnK (GlnK$_3$) we used completely uridylylated *K. pneumoniae* GlnK (GlnK$_3$-(UMP)$_3$) to distinguish between homotrimers and heterotrimers based on their different migration behavior in native gel electrophoresis. Heterotrimers should be detectable with both specific polyclonal antibodies and should migrate between the two homotrimeric forms, GlnK$_3$-(UMP)$_3$ in the case of *K. pneumoniae* and (GlnK$_1$)$_3$ in the case of *M. mazei*. The immunological analysis of the resulting trimers confirmed our *in vivo* data: No heterotrimers between *M. mazei* GlnK$_1$ and uridylylated *K. pneumoniae* GlnK were detectable (Fig. 7B, lane 5 and 8). These data strongly indicate that *M. mazei* GlnK$_1$ is not able to oligomerize with enteric PII-like proteins.

Functional analysis of *M. mazei* GlnK$_1$ in an *E. coli glnK* mutant strain.
Archaeal PII proteins are likely to play a crucial role in nitrogen sensing and regulation comparable to that of the bacterial PII proteins. Because of the similarities in structural organization and transcriptional regulation, we studied the possibility that *M. mazei* GlnK$_1$ can functionally substitute for bacterial *E. coli* GlnK. As the *E. coli glnK* mutant strain NCM1971 (Ikeda et al. 1996) showed a small but significant growth phenotype on NB solid medium, we studied aerobic growth of this strain in minimal medium in the presence of the limiting nitrogen source arginine. Under these growth conditions, the *glnK* mutant strain showed significantly lower growth rates and lower cell densities than the respective parental strain, NCM1529 (Fig. 8A). As some components of the arginine catabolism in *E. coli* are expressed in a NtrC-dependent manner (Zimmer et al. 2000) and GlnK is required for fine control of NtrC phosphorylation (Atkinson and Ninfa 1998), the observed growth defect on arginine apparently results from decreased arginine degradation in the absence of GlnK. Unexpectedly, expression of *M. mazei* GlnK$_1$, either from the *tet* promoter on a low
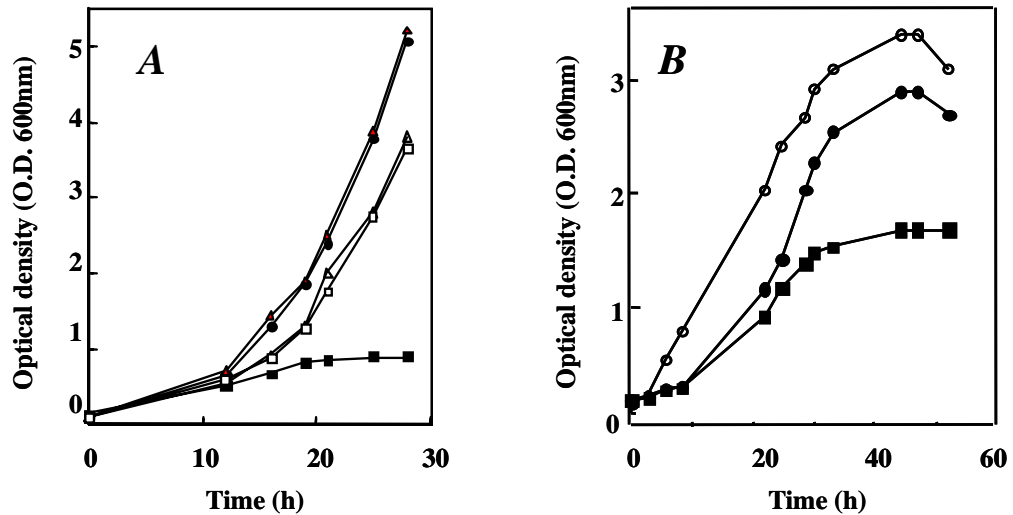
Fig. 8. Growth of *E. coli* mutant strains on the limiting nitrogen source arginine. A, the *E. coli glnK* mutant NCM1971 and derivatives were grown at 37 °C under aerobic conditions in minimal medium supplemented with 0.5 % glucose as the C-source and 10 mM arginine as the sole nitrogen source (see Materials and Methods). (closed circles), NCM1529 (parental strain); (closed squares), NCM1971 (*glnK* mutant); (closed triangles), RAS38 (*K. pneumoniae glnK* controlled by the *tet* promoter); (open triangles), RAS39 (*M. mazei glnK*$_1$ controlled by the *tet* promoter); (open squares), RAS37 (*M. mazei glnK*$_1$ controlled by the *tac* promoter). **B**, the *E. coli glnB/glnK* double mutant HS9060 and derivatives were grown under the same conditions as under A, but the medium was additionally supplemented with 0.02 % glycine. (closed squares), HS9060; (closed circles), RAS40 (*M. mazei glnK*$_1$ controlled by the *tac* promoter); and (open circles), RAS42 (*K. pneumoniae glnK* controlled by the *tac* promoter). The medium was not supplemented with IPTG to ensure very low induction level of *K. pneumoniae glnK* and *M. mazei glnK*$_1$.

copy plasmid (RAS39), or from the *tac* promoter (RAS37), was able to restore the wild-type growth phenotype of the *E. coli glnK* mutant strain on arginine as the sole nitrogen source. Restored growth was comparable to that seen with the addition of *K. pneumoniae glnK* on the plasmid (RAS38) and showed the some growth rate as determined for the wild type (Fig. 8A). To demonstrate that the observed complementation is based on expression of *M. mazei glnK*$_1$ in *E. coli glnK* mutant strains, we monitored *E. coli* GlnK and *M. mazei* GlnK$_1$ in the respective strains by immunological means. Using specific polyclonal antibodies, *M. mazei* GlnK$_1$ was detected in RAS39 and RAS37, whereas *E. coli* GlnK was not detectable in any of the complemented *glnK* mutant strains (data not shown). These results strongly suggest that the archaeal *M. mazei* GlnK$_1$ is able to functionally

complement *E. coli* GlnK for growth on arginine as the sole nitrogen source, and most likely acts in *E. coli* in the same way as the bacterial nitrogen-regulatory protein GlnK. The fact that *M. mazei* GlnK$_1$ is apparently not modified by uridylylation in response to nitrogen availability further suggests that the GlnK nitrogen signaling function in *E. coli* is independent of the uridylylation state of the trimeric protein, as is apparently the case for *K. pneumoniae* GlnK regarding its control in regulating NifLA activity (He et al. 1998; Arcondeguy et al. 1999).

To rule out that the restoration of growth on arginine by *M. mazei* GlnK$_1$ is not based on *M. mazei* GlnK$_1$ affecting GlnB functions, we studied growth on arginine of an *E. coli* Δ*glnB*/Δ*glnK* double mutant strain (HS9060) and derivatives containing *M. mazei glnK$_1$* (RAS40) or *K. pneumoniae glnK* (RAS42) under the control of the *tac* promoter. As the *E. coli* Δ*glnB/*Δ*glnK* double mutant strain was not able to grow in the minimal medium used for the *glnK* mutant strain, the respective medium was additionally supplemented with 0.02 % glycine. No IPTG was added to ensure very low induction levels of *K. pneumoniae glnK* and *M. mazei glnK$_1$*. Expression of *M. mazei* GlnK$_1$ was able to restore the wild-type growth rate of the Δ*glnK*/Δ*glnB* double mutant strain on arginine, as was the expression of *K. pneumoniae* GlnK. Cultures containing *M. mazei* GlnK$_1$ (RAS40) did not reach the same maximum turbidity obtained for the double mutant strain expressing *K. pneumoniae* GlnK (RAS42) (Fig. 8B).

Effects of *M. mazei* GlnK$_1$ on nitrogen fixation in *K. pneumoniae*.

M. mazei glnK$_1$ is able to complement an *E. coli glnK* mutant strain for growth on arginine. The amino acid sequence of the *M. mazei* GlnK$_1$ T-loop shows 52 % similarity to that of *K. pneumoniae* (Fig. 2). However, it contains the conserved amino acid residue asparagine in position 54 (N54), which has been recently shown to be a critical residue in distinguishing GlnB and GlnK with respect to the ability of GlnK to regulate nitrogen fixation (Arcondeguy et al. 2000). We therefore studied the ability of *M. mazei glnK$_1$* to complement a *K. pneumoniae glnK* mutant strain for growth on molecular nitrogen as the sole nitrogen source.

In order to monitor the effect of GlnK$_1$ on nitrogen fixation by directly analyzing growth on molecular nitrogen as the sole nitrogen source, we constructed a chromosomal *glnK* deletion in the wild-type strain *K. pneumoniae* M5a1 by transducing the *glnK* null allele of the *glnK* mutant strain UNF3433 (Jiang et al.1998a) as described in Methods. The resulting strain was designated RAS31. As expected from previous studies of *nif* inductions
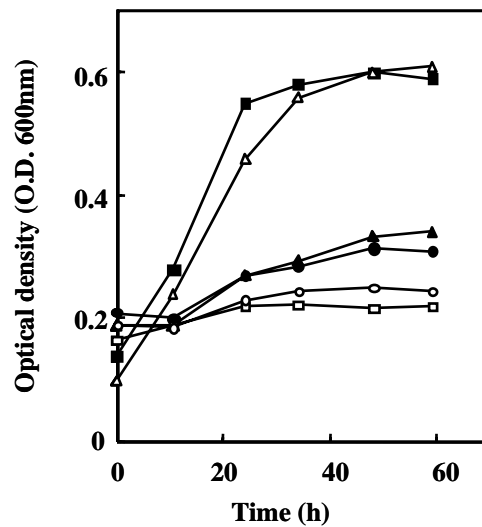
Fig. 9. Growth of *K. pneumoniae glnK* mutant strains on molecular nitrogen as the sole nitrogen source. *K. pneumoniae* wild-type, *K. pneumoniae glnK* mutant strain RAS31 and derivatives were grown at 30 °C under anaerobic conditions in minimal medium supplemented with 0.4 % sucrose as the C-source under a nitrogen atmosphere (see Materials and Methods). In RAS32, expression of *M. mazei* $GlnK_1$ was induced with 10 or 50 μM IPTG. (closed squares), *K. pneumoniae* M5a1 (parental strain); (open squares), *glnK* mutant strain of *K. pneumoniae* M5a1 (RAS31); (open triangles), RAS44 (*K. pneumoniae glnK* controlled by the *tet* promoter); (open circles), RAS33 (*M. mazei glnK*$_1$ controlled by the *tet* promoter); (closed circles), RAS32 (*M. mazei glnK*$_1$ controlled by the *tac* promoter) in the presence of 10 μM IPTG; (closed triangles), RAS32 in the presence of 50 μM IPTG.

determined with *lacZ*-reporter fusions (He et al. 1998; Jack et al. 1999), anaerobic growth on minimal medium with molecular nitrogen as the sole nitrogen source was completely abolished in the *glnK* mutant strain RAS31 (Fig. 9). Additional expression of *M. mazei* $GlnK_1$ from a low copy plasmid (RAS33) did not affect the mutant growth phenotype of RAS31 on molecular nitrogen. Unphysiological high induction of *M. mazei glnK*$_1$ in the presence of up to 50 μM IPTG (RAS32) did result in slow but significant growth (Fig. 8). The doubling time of RAS32 on molecular nitrogen in the presence of 50 μM IPTG was calculated to be 32 h, compared to 9 h for the parental strain. Thus, *M. mazei* $GlnK_1$ appeared to weakly complement a *K. pneumoniae glnK* mutant for diazotrophic growth. However, as the expression level of $GlnK_1$ was unphysiological high these findings indicate that *M. mazei* $GlnK_1$ is not able to complement for functions of *K. pneumoniae* GlnK with respect to its regulatory function in nitrogen fixation.

DISCUSSION

We recently showed that the mesophilic methanogenic archaeon *M. mazei* is able to use molecular nitrogen as the sole nitrogen source (Ehlers et al. 2002b). In addition to the two *glnB*-like genes within the *nif* cluster, we have now identified two additional *glnB*-like genes in *M. mazei* at two different locations on the chromosome by analyzing the genome sequence. Based on their structural organization these genes have been designated $glnK_1$ and $glnK K_2$. In order to determine whether *M. mazei* GlnK proteins may have a function in nitrogen regulation, we have studied the $glnK_1amtB_1$ operon, which is located 8 kbp upstream of the *nif* operon, and characterized native and purified heterologously synthesized $GlnK_1$.

Analyses of *M. mazei* $GlnK_1$ in comparison to bacterial PII-like proteins.

Transcriptional analysis by Northern blotting showed that the $glnK_1amtB_1$ operon from *M. mazei* is transcriptionally regulated through ammonium availability and only transcribed in the absence of a combined nitrogen source (Fig. 3), as is known for enteric $glnK_1amtB_1$ operons. This regulation of $GlnK_1$ expression was confirmed by analysing protein synthesis of $GlnK_1$ in *M. mazei* under different nitrogen availabilities (Fig. 6). The mechanisms in *M. mazei* resulting in transcriptional regulation of the $glnK_1amtB_1$ operon in response to nitrogen availability, however, have yet to be elucidated. Concerning potential mechanisms of transcriptional regulation in response to ammonium in methanogenic archaea, a common repressor binding-site sequence (GGAA(N6)TTCC) has been identified in *Methanococcus maripaludis* in the promoter region of the *nif* and *glnA* operon indicating a coordinated transcriptional regulation in *M. maripaludis* via an operator site (Kessler and Leigh 1999). This repressor binding-site sequence was further found in the genome of *Methanobacterium* and *Methanococcus* species, located in front of several genes involved in nitrogen metabolism including an *amtBglnB* operon (Bult et al. 1996; Cohen-Kupiec et al. 1997; Smith et al. 1997). However, in *M. mazei,* neither the *nif* operon nor the $glnK_1amtB_1$ operon showed this repressor binding-site sequence ((Ehlers et al. 2002b); and this study). In *Methanosarcina barkeri* 227, the *nif2* operon is also missing the operator sequence, but is transcriptionally regulated through ammonium availability (Chien and Zinder 1996; Chien et al. 1998). Thus, these findings indicate the mechanism of transcriptional regulation through ammonium availability in *M. mazei* and *M. barkeri* may differ from the mechanisms in *Methanococcus* and *Methanobacterium*.

Amino acid sequence analysis showed that *M. mazei* GlnK$_1$ contained the conserved tyrosine (Y51) located in the T-loop structure of the protein, which - in enteric GlnB and GlnK proteins - is modified by uridylylation in response to the nitrogen status of the cell (Merrick and Edwards 1995; Atkinson and Ninfa 1999; Arcondeguy et al. 2001). Two lines of evidence strongly indicate that the archaeal GlnK protein, unlike the enteric GlnK proteins, is not modified by uridylylation in response to the nitrogen status of the cells. First, *in vitro* uridylylation assays using GlnD' from *E. coli* showed that a covalent modification by uridylylation was not detectable for the purified, heterologously expressed *M. mazei* GlnK$_1$ (Fig. 5). This failure to uridylylate the *M. mazei* GlnK$_1$ protein *in vitro* may be due to the specificity of the *E. coli* GlnD protein. If this is the case it is of special importance as *E. coli* GlnD has been shown to heterologously uridylylate a variety of bacterial PII-like proteins. In this respect, it is of interest that even the PII-like protein from *Synechoccoccus* PCC 7942, which has been shown to be modified by phosphorylation at a serine residue (Ser49), can be heterologously uridylylated by the *E. coli* GlnD protein at the conserved tyrosine residue (Tyr51) (Forchhammer and Tandeau de Marsac 1995; Forchhammer and Hedler 1997). This indicates that the archaeal GlnK protein differs from bacterial PII-like proteins in a manner that does not allow recognition by the *E. coli* uridylyltransferase GlnD, even though the conserved tyrosine residue is present. Alternatively, a *Methanosarcina* specific metabolic signal may be required for covalent modification of the archaeal GlnK protein in response to nitrogen availability. Consequently, we secondly looked for covalent modifications in response to nitrogen availability *in vivo,* with ammonium up-shift experiments of *M. mazei* cultures in which GlnK$_1$ was chromosomally expressed. These *in vivo* ammonium up-shift experiments clearly confirmed the *in vitro* results by heterologous uridylylation (Fig. 6). This is consistent with our finding that no open reading frame was identified in the *M. mazei* genome with a deduced amino acid sequence similar to the enteric uridylyltransferase (GlnD). Furthermore, we can also exclude that the *M. mazei* GlnK$_1$ is *in vivo* modified by phosphorylation in response to nitrogen availability - as occurs with the *Synechococcus* PII protein - as an increase in the electrophoretic mobility of the phosphorylated form of the protein would also result (Forchhammer and Tandeau de Marsac 1995; Forchhammer and Hedler 1997). These findings strongly indicate that if there is a covalent modification in response to nitrogen availability in *M. mazei* GlnK$_1$, the methanogenic protein is modified in a manner different from the bacterial GlnB-like proteins.

In addition to the absence of modification in response to the internal nitrogen status, we obtained *in vivo* and *in vitro* evidence that *M. mazei* GlnK$_1$ does not form heterotrimers with GlnK from *K. pneumoniae* (Fig. 7) or *E. coli* (data not shown). In this respect the archaeal GlnK protein again differs from most bacterial PII-like proteins, for which heterotrimerization is observed even among species which are phylogenetically distant (Forchhammer and Hedler 1997). The two additional amino acids in the *M. mazei* GlnK$_1$ B-loop region, which or are not found in PII-like proteins in enterics (Fig. 2) and which are either part of the second α-helix or part of the B-loop, might be of importance for differences in the overall structure and thus for the failure to form heterotrimers. These findings indicate that the structure of the bacterial and archaeal GlnK core proteins, which are presumed to be responsible for interaction between GlnK monomers, differs significantly.

Regulatory function of *M. mazei* GlnK$_1$ in nitrogen regulation.

To date, no genetic system has been established in *M. mazei* for introducing chromosomal deletions in order to perform functional analysis of *M. mazei* proteins. Thus, in order to study the potential regulatory function of GlnK$_1$ in nitrogen regulation, we studied the ability of *M. mazei* GlnK$_1$ to restore function to an *E. coli glnK* mutant. In a result both interesting and unexpected, expression of *M. mazei* GlnK$_1$ restored growth of an *E. coli glnK* mutant on the limiting nitrogen source arginine (Fig. 7A). This is, to our knowledge, the first report of a functional complementation of a bacterial *glnK* mutant by an archaeal GlnK protein. Taking into account, that *M. mazei* GlnK$_1$ differs from bacterial PII-like proteins in its modification in response to nitrogen availability and in its ability to form heterotrimers, the ability to restore growth of an *E. coli glnK* mutant on arginine is of significant interest. The functional complementation of the *E. coli glnK* mutant strongly indicates that *M. mazei* GlnK$_1$ is involved in nitrogen regulation. It further suggests that the uridylylation status of the protein is not of importance for the regulatory function of GlnK in *E. coli,* as *M. mazei* GlnK$_1$ is not uridylylated in response to nitrogen availability. This is consistent with the results obtained for the function of GlnK uridylylation on *K. pneumoniae* NifLA regulation: uridylylation is apparently not required for relief of NifL inhibition (He et al. 1998; Arcondeguy et al. 1999). However, uridylylation is important for GlnB function, thus *M. mazei* GlnK$_1$ cannot substitute for GlnB functions in an *E. coli*

$\Delta glnB/\Delta glnK$ double mutant to the same amount as does *K. pneumoniae* GlnK, which is uridylylated under nitrogen-limiting conditions (Fig. 7B).

M. mazei $GlnK_1$ contains the conserved asparagine 54 in the T-loop, which has been shown to be a crucial amino acid which discriminates GlnK from GlnB in *K. pneumoniae* in respect to the GlnK ability to regulate nitrogen fixation (Arcondeguy et al. 2000). However, *M. mazei* $GlnK_1$ is not able to substitute for *K. pneumoniae* GlnK with respect to its signaling function in the nitrogen fixation regulatory system (Fig. 8). This indicates, that *M. mazei* $GlnK_1$ is missing the specific GlnK function necessary for transducing the nitrogen signal to the bacterial *nif* system. The finding that *M. mazei* $GlnK_1$ is not uridylylated cannot account for the failure, as the uridylylation state of *K. pneumoniae* GlnK is not essential for the signal transduction (He et al. 1998; Arcondeguy et al. 1999; Jack et al. 1999). It is more likely that the failure is based on the specificity of the regulatory system of nitrogen fixation in *K. pneumoniae* itself, in which GlnK transduces the nitrogen signal to the *nif* system by interacting with the transcriptional activator NifA or with its antagonist NifL (He et al. 1998; Jack et al. 1999; Arcondeguy et al. 2000; Schmitz et al. 2002). The transcriptional regulation of methanogenic *nif* genes, however, appears to be based in general on a negative mechanism (Blank et al. 1995; Cohen-Kupiec et al. 1997; Chien et al. 1998; Kessler et al. 1998; Kessler and Leigh 1999). Thus, if $GlnK_1$ functions in the transcriptional regulation of nitrogen fixation in *M. mazei* at all, specific interacting partners and metabolic signals may be required for $GlnK_1$. Additionally, we cannot exclude that the second operon, $amtB_2glnK_2$ in *M. mazei* (data not shown) may be of importance for *nif* transcriptional regulation.

In summary our findings strongly indicate that $GlnK_1$ is involved in nitrogen regulation in *M. mazei*. However, the archaeal GlnK protein differs from most bacterial PII-like proteins in a manner that does not allow (i) recognition by the *E. coli* uridylyltransferase GlnD even though the conserved tyrosine 51 is present and (ii) heterotrimerization with bacterial GlnK proteins, suggesting that the structure of the bacterial and archaeal GlnK core proteins differ significantly. Hence, it can be speculated that archaeal GlnK proteins which are presumed to be involved in nitrogen regulation, evolved differently from bacterial PII-like proteins concerning their structural features depending on the archaeal-specific interacting partners and requirements.

Chapter 3:

Development of genetic methods and construction of a chromosomal *glnK₁* mutant in *Methanosarcina mazei* strain Gö1

ABSTRACT

Until now the methanogenic archaeon *Methanosarcina mazei* strain Gö1 was genetically not accessible due to inefficient plating on solid medium and lack of an effective transformation method. Here, we report on the first significant improvement of the plating efficiency up to 10 %, achieved by selecting for a spontaneous mutant of *M. mazei*, which showed higher resistance to mechanical stress, and plating the cells in 0.5 % top agar using trimethylamine as carbon and energy source under a H_2S containing atmosphere (0.01%). Using this mutant we succeeded in establishing a liposome-mediated transformation protocol, which allowed genetic manipulation of *M. mazei* strain Gö1 for the first time. We further report on the construction of the first chromosomal deletion mutant of *M. mazei* by means of homologous recombination. Characterizing this mutant strain showed that *M. mazei* lacking a functional *glnK₁*-gene exhibited a partial growth defect under nitrogen-limitation with molecular nitrogen as sole nitrogen source. Quantitative reverse transcriptional (RT)-PCR analysis however, showed that genes involved in nitrogen assimilation or nitrogen fixation are not differentially transcribed in the *glnK₁* mutant compared to the wild type. Thus, we propose that the archaeal $GlnK_1$-protein is not directly involved in the transcriptional regulation of genes involved in nitrogen metabolism, but affecting components of the nitrogen metabolism on the post-translational level.

INTRODUCTION

Methanosarcina mazei strain Gö1, which belongs to the methylotrophic methanogenic *Archaea,* is able to grow on H_2 plus CO_2, methanol, acetate and methylamines as sole carbon and energy source (Hippe et al. 1979; Deppenmeier et al. 1990). We have recently

shown that *M. mazei* is also able to fix molecular nitrogen (N$_2$) when growing under nitrogen limitation and characterized a single nitrogen fixation (*nif*) gene cluster in *M. mazei* (Ehlers et al. 2002a; Ehlers et al. 2002b). This potential to use a wide spectrum of carbon and nitrogen sources reflects the organism's high ability to adapt to changes of environmental conditions. This is also indicated by the whole genome information, the analysis of which showed that *M. mazei* is a metabolically versatile methanogen and might have acquired new pathways for energy generation from acetate and methylamines along with additional genes for environmental adaptation by lateral gene transfer (Deppenmeier et al. 2002). However, the further understanding of genes' functions and their importance for the organism's adaptation and survival in its environment is not easy to gain, as the genetic work with this methanogenic archaeon has been highly limited.

In recent years significant progress has been made in developing new techniques and adapting already existing ones that allow genetic analysis in *Archaea* (Tumbula 1994; Argyle et al. 1996). The main problems were (i) the absence or inefficiency of antibiotics or other selection markers and (ii) the absence of suitable vectors or transposons for members of this domain. Furthermore, none of the existing methods for bacterial DNA transformation, such as conjugation, chemical transformation or electroporation of purified DNA was successful for methanoarchaea. The discovery that antibiotics such as puromycin, pseudomonic acid and neomycin can be used as selection marker in *Archaea* as well as the construction of functional *Escherichia coli* shuttle vectors significantly helped in developing methods for genetic manipulation in *Archaea* (Kiener et al. 1986; Possot et al. 1988; Argyle et al. 1996; Metcalf et al. 1997; Boccazzi et al. 2000; Pritchett et al. 2004). Finally, in the recent years an efficient transformation method has been specifically established for the genus *Methanosarcina* (Metcalf et al. 1997). By taking advantage of liposomes as DNA delivery vehicle it is now possible to transfer DNA into *Methanosarcina* cells. In a first step, complexes are formed between a liposome reagent and the DNA to transform, which are then able to fuse with the cytoplasmic membrane of *Methanosarcina* protoplasts. This newly established liposome-mediated transformation method is not limited to autonomously replicating plasmids, but can also be used for transforming linear DNA for homologous recombination and for transposon mutagenesis approaches (Metcalf et al. 1997; Zhang et al. 2000; Zhang et al. 2002). The transformation protocol was successful for most of *Methanosarcina* strains tested, but failed for *M. mazei* Gö1, though no specific attempts have been made to optimize the method for this particular strain (Metcalf et al. 1997; W. Metcalf, unpublished). A further basic problem concerning

the genetic accessibility of *M. mazei* Gö1 has been its inefficient plating capacity. Thus, our main focus of this work was to significantly improve the plating efficiency of *M. mazei* and optimize the liposome-mediated transformation protocol for this particular *Methanosarcina* strain. During those studies we succeeded in constructing a *glnK₁*-mutant strain of *M. mazei*, which is to our knowledge the first definite *M. mazei* mutant strain constructed.

MATERIALS and METHODS

Bacterial Strains and Plasmids. Strains and plasmids used in this study are listed in Table 1. Plasmid DNA was transformed into *E. coli* according to the method of Inoue et al. (Inoue et al. 1990) and into *M. mazei* as described below.

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Genotype or description	Source or reference
Strains		
Methanosarcina mazei strain Gö1	Wild type	DSM No. 3647
*M. mazei**	potential cell wall mutant	This work
M. mazei ΔglnK₁⁻*	*M. mazei*,* but *glnK₁::pac*	This work
Methanosarcina acetivorans C2A	Wild type	DSM No. 2834
Escherichia coli DH5α	general cloning strain	(Miller and Mekalanos 1988)
E. coli DH5α/λpir	general cloning strain	(Miller and Mekalanos 1988)
WM629	DH5α/λpir/pWM321	(Metcalf et al. 1997)
Plasmids		
pBluescript SK⁺	general cloning vector	Stratagene, La Jolla, US
pWM321	*E. coli-Methanosarcina* shuttle vector, carrying *pac*-cassette	(Metcalf et al. 1997)
pRS204	*pac*-cassette cloned into pSL1180	This work
pRS208	pBluescript SK⁺, but *Eco*RI site closed	This work
pRS217	*glnK₁* fragment in pRS208	This work
pRS220	pRS217, but *glnK₁::pac*	This work

Growth. *M. mazei* wild type and mutant strains were grown in minimal medium under a nitrogen gas atmosphere in 5- or 50-ml closed growth tubes, which were incubated at 37 °C without shaking, as described previously (Deppenmeier et al. 1990; Ehlers et al. 2002b). The medium was complemented with 150 mM methanol and 40 mM acetate or 25 mM trimethylamine (TMA) as sole carbon sources and reduced with 2 mM cystein and 1 mM sodium sulfite. *M. acetivorans* was grown in high salt (HS) medium on 40 mM acetate and 150 mM methanol as described by Metcalf et al. (Metcalf et al. 1997). For nitrogen limited growth, the ammonium was omitted from the media and molecular nitrogen in the gas phase served as sole nitrogen source (Ehlers et al. 2002b). In general the *Methanosarcina* cultures were supplemented with 100 µg / ml ampicillin to prevent bacterial contamination. For mutant selection 2.5 µg / ml puromycin was added to the medium. Growth was monitored by determining the optical density of the cultures at 600 nm (O.D.$_{600}$).

Construction of plasmids. The fragment containing the *M. mazei glnK$_1$* gene with additional 1000 bp up- and downstream regions was amplified using chromosomal *M. mazei* DNA as template and the following primers with additional synthetic *Sac*I restriction recognition sites (underlined): glnK$_1$ forward 5'CTGCTTCCTCAAA<u>GAGCTC</u>TTTCC 3' and glnK$_1$ reverse 5' CTCCGT<u>GAGCTC</u>CAAATGCATC 3'. The obtained 2317 bp PCR product was cloned into a pBluescript SK$^+$ derivative with the *Eco*RI site within the multiple cloning site closed yielding plasmid pRS217. The *Eco*RI site located 118 bp upstream of the *glnK$_1$*-gene on pRS217 was mutated using the Chameleon$^®$ double stranded, site-directed mutagenesis kit (Stratagene), the mutation primer (5'ATTCGTTATTGCATTCGATCAACCCG 3') and the selection primer (5' GGGGCCCGGTAACCAGCTTTTGTTCC 3') resulting in an unique *Eco*RI site on pRS217 within the *glnK$_1$*-gene. The plasmid obtained was designated as pRS219. Finally the 1.8 kbp *Eco*RI fragment from pRS204 containing the *pac*-cassette under the control of the constitutive promoter (p*mcr)* and terminator (t*mcr)* from the *mcr*-gene of *Methanococcus voltae* was cloned into the unique *Eco*RI site of pRS219 within the *glnK$_1$* gene producing plasmid pRS220.

Liposome-mediated transformation. All steps were performed inside an anaerobic cabinet (Coy Laboratory Products Inc., US) containing an atmosphere devoid of O$_2$ consisting of approximately 80 % N$_2$ (5.0) and 20 % CO$_2$ (5.0). Residual O$_2$ introduced into the cabinet was consumed by additional H$_2$ using palladium catalyst STAK-PAK (Coy

Laboratory Products Inc., US) and the actual portion of H_2 amounted to approximately 1 to 2 % in the gas phase. The mutant strain *M. mazei** was isolated by repeated plating *M. mazei* in top agar and transferring the obtained single colonies into liquid medium. After modifying and optimizing the transformation protocol for *Methanosarcina* strains described by Metcalf et al. (Metcalf et al. 1997), *M. mazei** was generally transformed as follows. Exponentially growing *M. mazei** cells were harvested by centrifugation for 20 min at 5,000 rpm. The cells were resuspended in 0.85 M carbonate-buffered sucrose solution (pH = 7.3) to a final density of 1 x 10^{10} cells / ml to obtain protoplasts. 2 µg of the plasmid DNA to be transformed was diluted in 50 µl 0.85 M sucrose solution and mixed with 30 µl liposome DOTAP reagent (Boehringer, Germany) and 70 µl 0.85 M sucrose solution followed by an incubation at 37 °C for at least 30-40 min to allow the formation of DNA-liposome complexes. 990 µl of the *M. mazei* protoplasts were added to this DNA-liposome mixture and incubated at 37 °C. After 4 - 6 h, 500 µl of the transformation mixture were transferred into 5 ml minimal medium supplemented with 25 mM TMA and 0.5 M sucrose. After further incubation for 12-16 h, 0.2 ml of the transformation culture were transferred into 5 ml minimal medium containing 2.5 µg puromycin to select for puromycin resistant clones. *M. acetivorans* was transformed as described by Metcalf et al. (Metcalf et al. 1997) with the modifications described by Bocazzi et al. (Boccazzi et al. 2000).

Southern blot analysis. The DNA probe for *glnK₁* was generated as follows: *glnK₁* was amplified from genomic *M. mazei* DNA using homologous primers as recently described (Ehlers et al. 2002a). The PCR reaction was carried out in a 25 µl standard reaction using the PCR DIG Labeling Mix (Boehringer, Germany) that provides incorporation of Dig-11-dUTP into the PCR amplificon. The obtained PCR product was purified by gel extraction. The probe for the *pac*-cassette was obtained by labelling the 1.8 kb *Eco*RI fragment from pRS204 containing the *pac*-cassette using the random primed DIG DNA Labeling Kit (Boehringer, Germany). Southern hybridization was performed as described (Sambrook 1989) and detection using CSPD was carried out according to protocol of the manufacturer (Boehringer, Germany).

Western blot analysis. Western blot analysis was carried out by separating *M. mazei* *ΔglnK₁* crude extracts by 12.5% SDS-PAGE followed by protein transfer on nitrocellulose membranes (BioTrace®NT, Pall Life Science). Membranes were exposed to polyclonal

rabbit antiserum directed against the GlnK$_1$ protein of *M. mazei* (Ehlers et al. 2002a); protein bands were detected by secondary antibodies directed against rabbit immunoglobulin G and coupled to horseradish peroxidase (Bio-Rad Laboratories) by using the ECL-Plus system (Amersham/Bioscience). Quantification of proteins was performed using the Storm PhosphoImager and the ImageQuant software (Molecular Dynamics).

Table 2. Primers used for quantitative RT-PCR analysis.

Description	Gene / Protein	Sequence 5' → 3'
Mm orf 272 for.rt	MM1621 putative COBW protein	TAGGAGGTTTTCTCGGAAGCG
Mm orf 272 rev.rt	MM1621 putative COBW protein	AAGCGTATCTCCATCAAGCCC
Mm orf 484 for.rt	MM2181 fructose-1,6-bisphosphatase	GCCTCCATGAGAAGAATGCTC
Mm orf 484 rev.rt	MM2181 fructose-1,6-bisphosphatase	CTTCAAGGTCTCCAACTCCTG
Mm orf 943 for.rt	MM1215 hexulose-6-phosphate synthase	TCAAGAGCGAGGGCATGAATG
Mm orf 943 rev.rt	MM1215 hexulose-6-phosphate synthase	GCACTACCGAGAACAATAGCC
Mm nifH for.rt	*nifH* / nitrogenase iron protein	CCACGCAGAATCTTACTGCAG
Mm nifH rev.rt	*nifH* / nitrogenase iron protein	AGCACGGTTTTCTGGTTCAGG
glnK1 for.rt	*glnK$_1$* / GlnK$_1$ protein	CGATGGAATATGATGCAAACCTGC
glnK1 GSP1A	*glnK$_1$* / GlnK$_1$ protein	CCAACGTAACCGTCACTGCC
glnK2 for.rt	*glnK$_2$* / GlnK$_2$ protein	CCCTGAAACTGCACCAGGTAA
glnK2 rev.rt	*glnK$_2$* / GlnK$_2$ protein	TTTCCTCTCCACTGCTGTACG
glnA1 for.rt	*glnA$_1$* / glutamine synthetase	GGGAGGATACTTCGATTTCGC
glnA1 GSP1A	*glnA$_1$* / glutamine synthetase	TGATGGGAGGCTTCTATCTGG

RNA preparation and quantitative reverse transcription(RT)-PCR. Total RNA was prepared from *M. mazei* wild type and *ΔglnK* mutant cells grown under nitrogen limitation by phenol extraction as described recently (Sambrook 1989). Contaminating genomic DNA was removed by DNaseI treatment. Quantitative RT-PCR assays were performed using the QuantiTect Probe RT-PCR Kit (Qiagen, Hilden) and the iCycler (BioRad) and at least three independent RNA preparations for each strain. The reactions were carried out with 400 ng of total RNA following the manufacturer's protocol. The primers used for RT-PCR reactions are listed in Table 2. To determine the fold change in gene expression the calculated threshold cycle (C_t) for each gene of interest was normalized to the corresponding C_t of three constitutively transcribed genes of *M. mazei* (MM1621, MM2181, MM1215) amplified from the same RNA. The x-fold change was calculated as described (Talaat et al. 2002) using the following formula: Fold change $= 2^{-\Delta\Delta Ct}$.

RESULTS and DISCUSSION

Our goal was to establish genetic methods for the strictly anaerobic methanogenic archaeon *M. mazei* strain Gö1 that will allow us to perform functional analysis of genes and regulatory elements using genetic approaches. By selecting for a spontaneous mutant strain with higher resistance to mechanical stress and optimizing the liposome-mediated transformation protocol developed recently (Metcalf et al. 1997), the organism is now genetically accessible and we succeeded in generating a *M. mazei glnK₁* null mutant.

Improvement of the plating efficiency for *M. mazei* strain Gö1 on solid medium. Plating experiments of the strict anaerobe were routinely performed with exponentially growing cultures inside an anaerobic cabinet under a N_2/CO_2-atmosphere as described in Materials and Methods. Direct plating of *M. mazei* cultures on solid minimal medium (MM) or high salt (HS) medium, both supplemented with 150 mM methanol and 40 mM acetate as the sole carbon sources, and incubating the plates at 37 °C under the N_2/CO_2 atmosphere present in the anaerobic cabinet, resulted only in a few single colonies on the plates. In order to improve the low plating efficiency of the organism, we optimized growth conditions by varying the medium composition, carbon source, agar concentration and the composition of the gas atmosphere, under which the plates were incubated for growth. Using 25 mM trimethylamine (TMA) as carbon and energy source in solid minimal
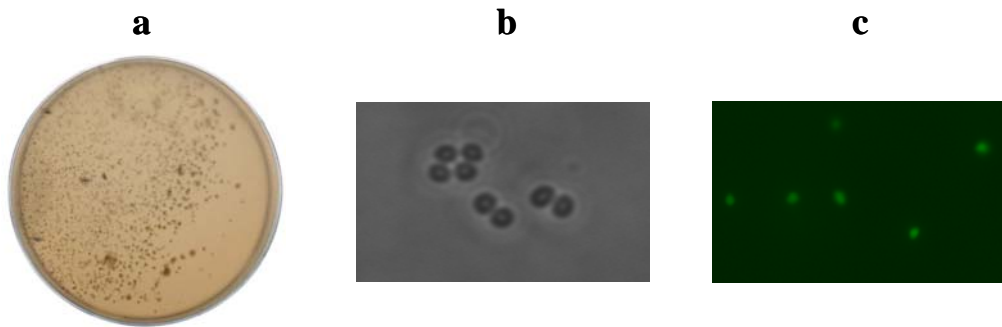
Fig. 1. *M. mazei* cells grown on solid medium. (a) *M. mazei** cells grown on minimal medium complemented with 25 mM TMA as carbon and energy source. Cells were plated in 0.5 % top agar and incubated under a $N_2/CO_2/H_2S$ (79.9%:20%:0.1%) gas atmosphere. **(b)** Resuspended *M. mazei** cells grown on solid medium analyzed by light microscopy. **(c)** Cells grown on solid medium and recultured in liquid medium analyzed by fluorescence microscopy.

medium showed a first significant improvement and succeeded in a plating efficiency of approximately 0.1 %. This may be based on an increased growth rate on TMA compared to growth on methanol, which might in addition evaporate over the incubation time. As an alternative to direct plating on solid medium, diluted cultures were poured in 0.5 % top agar on 1.5 % bottom agar, both consisting of minimal medium with 25 mM TMA, to reduce the mechanical stress during the plating procedure. After incubating those plates in an intra-chamber incubator under a gas atmosphere consisting of 79.9 % N_2, 20 % CO_2 and 0.1 % H_2S, which provides the cells with a lower reducing potential (Metcalf et al. 1998), plating efficiencies between 2 and 3 % were achieved. Routinely, single colonies were transferred into 0.5 ml liquid minimal medium containing 25 mM TMA. In general, approximately 80 % of those single colonies could be cultured in liquid medium and analysis by light and fluorescence microscopy confirmed that they consisted of *M. mazei* cells (Fig. 1).

In order to select for a spontaneous mutant with higher resistance to mechanical stress, *M. mazei* was repeatedly plated in top agar and the resultant single colonies were transferred into liquid medium followed by plating the respective cultures again in top agar. One of those strains obtained, apparently a spontaneous cell wall mutant, had acquired higher resistance to mechanical stress and showed significantly higher plating efficiencies (up to
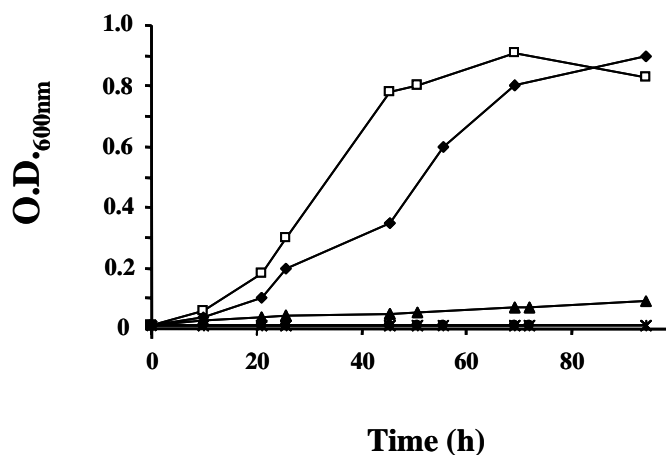
Fig. 2. Growth of *M. mazei* in the presence of puromycin. *M. mazei* was grown in liquid minimal medium (70 ml) with 150 mM methanol and 40 mM acetate as carbon sources under nitrogen sufficiency at 37 °C. The medium was supplemented with different concentrations of puromycin. No addition of puromycin (open squares), 1 µg / ml puromycin (closed squares), 2 µg / ml puromycin (closed triangles), 3 µg / ml puromycin (crosses), 4 µg / ml puromycin (stars) and 5 µg / ml puromycin (closed circles).

10 %). This strain was designated *M. mazei** and was used in all following experiments. It showed regularly formed, white to brown coloured colonies inside of the top agar that were visible after 10 days of incubation at 37 °C (Fig. 1A). The methane produced was recognizable by small gas bubbles inside of the top agar, which were already visible after seven days of incubation, indicating that *M. mazei* cells were growing. Although the maximum plating efficiency for *M. mazei** in top agar was significantly lower than that obtained for *M. acetivorans*, it was sufficient that colonies could be obtained, which is of importance for genetic approaches.

Puromycin as selection marker for *M. mazei*. The autonomous replicating *E. coli* shuttle vectors constructed for *Methanosarcina acetivorans,* which are based on the naturally occurring *M. acetivorans* plasmid pC2A (Sowers and Gunsalus 1988; Metcalf et al. 1997), contain the puromycin resistance marker developed by Gernhard et al. (Gernhardt et al. 1990). In order to test whether *M. mazei* strain Gö1 is sensitive towards puromycin, growth in the presence of different puromycin concentrations was analyzed in liquid minimal medium with methanol and acetate as carbon source. As evident from Fig. 2, the presence

of 1 μg puromycin / ml medium resulted in a significant decrease of the growth rate. Higher concentrations of the antibiotic led to complete growth inhibition indicating that *M. mazei* is sensitive towards puromycin. Thus, a concentration of 2.5 μg / ml puromycin was used for all following transformation experiments to select for puromycin resistant *M. mazei* clones.
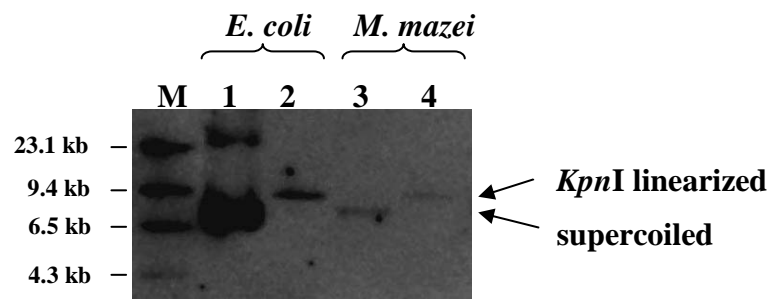
Optimizing the liposome-mediated transformation assay for *M. mazei*. The recently developed liposome-mediated transformation method for transferring DNA into species of the genus *Methanosarcina* initially failed for *M. mazei* strain Gö1 (Metcalf et al. 1997). In order to make *M. mazei* genetically accessible too, we used the mutant strain *M. mazei** instead of the wild type strain to increase the plating efficiency on solid medium after the transformation procedure, and optimized the original protocol by varying several parameters of the method.

*M. mazei** cells were generally grown in 25 and 50 ml minimal medium with 25 mM TMA as carbon and energy source to an optical density of approximately $OD_{600} = 0.6 - 0.7$. All the following transformation steps were performed in the absence of oxygen inside an anaerobic cabinet under a $N_2/CO_2/H_2$ atmosphere as described in Materials and Methods. Compared to the original method, the following modifications were tested: (i) Using at least 10-fold higher concentrations of protoplasts, (ii) increasing the incubation time of the liposome/DNA mixture up to 30 - 40 min and the incubation temperature to 37 °C, and (iii)

Table 3. Comparison of the modified method for liposome-mediated transformation for *M. mazei* strain Gö1 with the original protocol (Metcalf et al. 1997).

Parameters	Original method (Metcalf et al. 1997)	Method used for *M. mazei** in this study
Cell concentration of protoplasts	1×10^9 / ml	1×10^{10} / ml
Amount of plasmid DNA	2 μg	2 μg
Liposomes-DNA mixture		
Incubation time	15 min	30 – 40 min
Incubation temperature	25 °C	37 °C
Liposomes/DNA plus protoplasts		
Incubation time	4 h	6 h
Incubation temperature	25 °C	37 °C
Medium used after transformation	high salt medium + 125 mM MeOH + 40 mM acetate	minimal medium + 25 mM TMA + 0.5 M sucrose

increasing the incubation of protoplasts with the liposome-DNA complexes to at least 6 h. Finally, we changed the composition of the medium added to the cells after the 6 h transformation step, which proved to be one of the most important modifications for optimizing the transformation procedure. Instead of the high salt medium, which was less efficient for the recovery of *M. mazei* cells, minimal medium containing 25 mM TMA as the preferred carbon source was used, which was further supplemented with 0.5 M sucrose to keep the osmotic pressure approximately constant during the recovery step. After 16 h incubation, the recovered cells were transferred into liquid minimal medium containing 25 mM TMA and 2.5 µg / ml puromycin and were incubated further for 48 h to select for puromycin resistant transformants. Those transformed cultures were finally plated in

top agar containing 2.5 µg / ml puromycin as described above. Only the combination of all those variations (summarized in Tab. 3) succeeded in transforming *M. mazei** cells with an apparent transformation efficiency of 3.6×10^3 transformants per µg of DNA per 5.3×10^7 cells; no puromycin resistant transformants were obtained when only one of those parameters was altered.

Fig. 3. Southern Blot analysis of the purified shuttle vector pWM321 amplified in *M. mazei** **and *E. coli*.** *M. mazei** was transformed with the shuttle vector pWM321 using the liposome-mediated transformation protocol as described in Materials and Methods. Plasmid DNA was purified from *M. mazei**/pWM321 and *E. coli*/pWM321 using the QIAprep Spin Miniprep Kit (Qiagen). Purified plasmids (lanes 1 and 3) and *Kpn*I restricted plasmid DNA (lanes 2 and 4) were analyzed by Southern blot using a DIG-labelled *pac*-probe. Detection using CSPD as substrate was carried out according to the protocol of the manufacturer (Boehringer, Germany). Lanes 1 and 2, pMW321 isolated from *E. coli* as control; lanes 3 and 4, pMW321 DNA purified from *M. mazei**/pWM321.

The successful transformation with the *E. coli* / *M. acetivorans* shuttle vector pWM321 (Metcalf et al. 1997) was confirmed by Southern blot analysis. Plasmid-DNA isolated from puromycin resistant *M. mazei* clones showed the same size and restriction pattern as the control plasmid purified from the respective *E. coli* clone (Fig. 3). We further successfully retransformed the pWM321 shuttle vector purified from puromycin resistant *M. mazei* clones into both *E. coli* and *M. mazei*. These findings indicate that pWM321 is stably amplified and appears not to be significantly modified in *M. mazei*. However, after several frequent steps of subculturing *M. mazei*/pWM321 in liquid medium in the presence of puromycin, the shuttle vector showed the tendency to delete larger parts of the vector. This may indicate a potential incompatibility with some regions of the bacterial part of the shuttle vector.
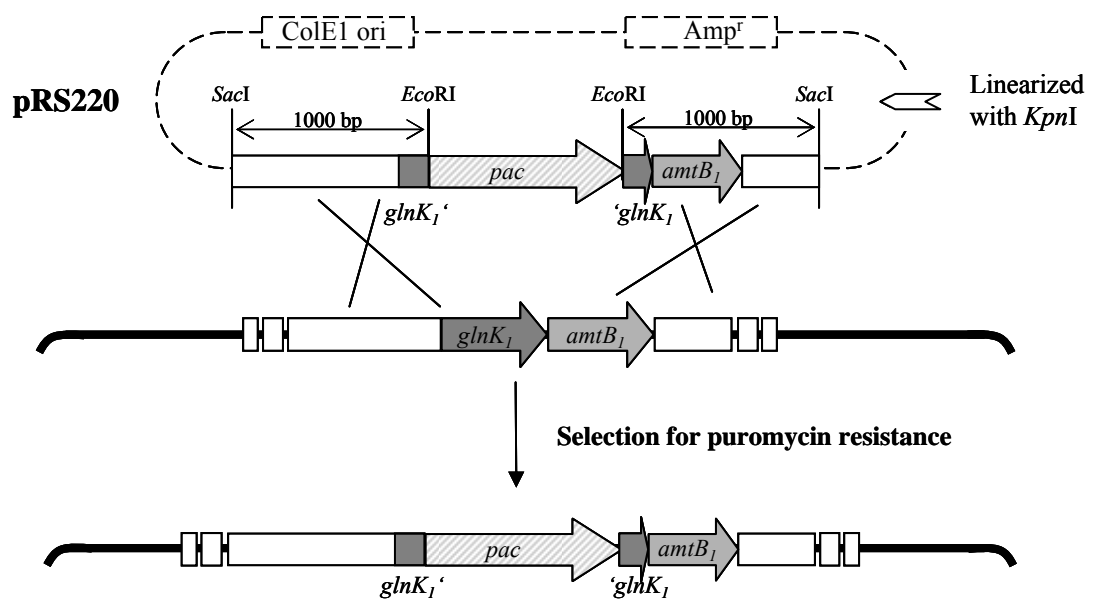
Fig. 4. Strategy for the *glnK₁*-gene disruption. Integration of the *pac*-cassette into the *Eco*RI site of the chromosomal *M. mazei glnK₁*-gene was accomplished through double homologous recombination using the non-replicable plasmid pRS220. *M. mazei** cells were transformed with *Kpn*I linearized plasmid pRS220 using the liposome-mediated protocol for *M. mazei* and selected for puromycin resistant transformants (see Materials and Methods).

Construction of a *glnK₁* null mutant of *M. mazei* by homologous recombination.

We previously characterized the GlnK₁ protein of *M. mazei* concerning its expression regulation, modification and ability to complement an *E. coli glnK* mutant strain. Although the archaeal protein differs from most bacterial PII-like proteins in several aspects, we obtained conclusive evidence that GlnK₁ is involved in the regulation of nitrogen metabolism in *M. mazei* (Ehlers et al. 2002a). In order to be able to study the regulatory function of GlnK₁ by a genetic approach, we constructed a *glnK₁* mutant strain of *M. mazei*. A *glnK₁* insertion mutant was constructed on a plasmid by inserting the *pac*-cassette encoding for puromycin resistance (Gernhardt et al. 1990) into the internal *Eco*RI site of the *M. mazei glnK₁*-gene as described in Materials and Methods. The obtained plasmid, designated pRS220, contained the interrupted *glnK₁*-gene flanked by additional 1000 bp of the down- and upstream region respectively, to allow introduction into the *M. mazei* chromosome by double homologous recombination (Fig. 4). The *Kpn*I-linearized plasmid pRS220, which is not replicable in *M. mazei,* was introduced into *M. mazei** and recombinant strains generated by means of a double cross over were identified by the ability to grow on solid medium in the presence of puromycin. Single colonies were transferred and cultivated in liquid minimal medium containing puromycin and the disruption of the *glnK₁*-gene from several independently obtained mutant strains was analyzed by Southern blot analysis using two hybridization probes, directed against the *glnK₁*-gene and against the *pac*-cassette, respectively. This analysis confirmed the
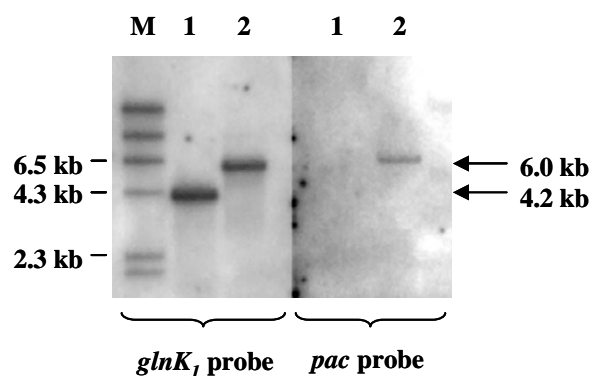

Fig. 5. Southern Blot analysis of the *M. mazei* *glnK₁*-mutant strain. Genomic DNA of the parental *M. mazei** and the *glnK₁*-mutant strain was digested to completeness with *Pvu*II. Southern Blot hybridization was performed using DIG-labelled probes directed against the *pac*-cassette and the *glnK₁*-gene. Lane 1, *M. mazei* wild type DNA; lane 2, DNA purified from the *M. mazei glnK₁*-mutant strain.

integration of the *pac*-cassette (approx. 1.8 kb) into the chromosomal *glnK₁*-gene of all the respective mutants obtained (Fig. 5). Western blot analysis further verified the absence of GlnK$_1$ in the mutant strain under nitrogen limitation, conditions under which GlnK$_1$ is highly expressed in *M. mazei*. The obtained *glnK₁* null mutant strain is the first definite chromosomal mutant constructed for *M. mazei* strain Gö1, illustrating that the optimized transformation method not only allows introduction of plasmid DNA but also generation of *M. mazei* mutant strains. With this tool we are now able to study the function of genes and regulatory elements by genetic approaches.

Characterization of the *glnK₁* mutant strain.

In order to address the question, how GlnK$_1$ is involved in nitrogen regulation, we studied the *glnK₁* mutant *M. mazei* strain. When cells were growing in minimal medium with ammonium as sufficient nitrogen source no phenotype of the mutant strain was detectable. The growth rate determined for the mutant strain in the presence of puromycin did not differ from that of the wild type strain (*M. mazei**) growing in the absence of puromycin (Fig. 6A). In contrast, a partial growth defect of the mutant strain was observed when cells were growing under nitrogen limitation with molecular nitrogen as sole nitrogen source (Fig. 6B). Under those nitrogen fixing conditions, the mutant showed a reduced growth rate (0.09 h^{-1}) compared to the parental strain and to *M. mazei** /pWM321 (0.14 h^{-1}), and in addition a reduced growth yield. This finding indicates that the GlnK$_1$-protein is required for maximal growth under nitrogen limitation, though it is not essential for nitrogen fixation, as the mutation did not abolish growth under nitrogen fixing conditions. At the current experimental status, we cannot rule out that the observed growth phenotype might be also based on the absence of the ammonium transporter AmtB$_1$, as the *pac*-cassette inserted into the *glnK₁*-gene has a polar effect on the *amtB₁*-gene. The absence of AmtB$_1$ may lead to a significantly decreased uptake of ammonium during the initial phase of ammonium-limited growth, resulting in a prolonged lag-phase, however, the absence of AmtB$_1$ should not directly account for the decrease in growth rate under nitrogen fixing conditions. In order to get a first impression, whether GlnK$_1$ effects transcription of genes involved in nitrogen metabolism, we quantified transcription of nitrogen-regulated genes in wild type and the *glnK₁* mutant grown under different nitrogen availabilities. Transcription of genes encoding for glutamine synthetase (*glnA₁*) and nitrogenase (*nifH*), which are known to be significantly upregulated under nitrogen limitation (Ehlers et al. 2002b; K. Veit and R.A. Schmitz, unpublished results), was studied by quantitative reverse
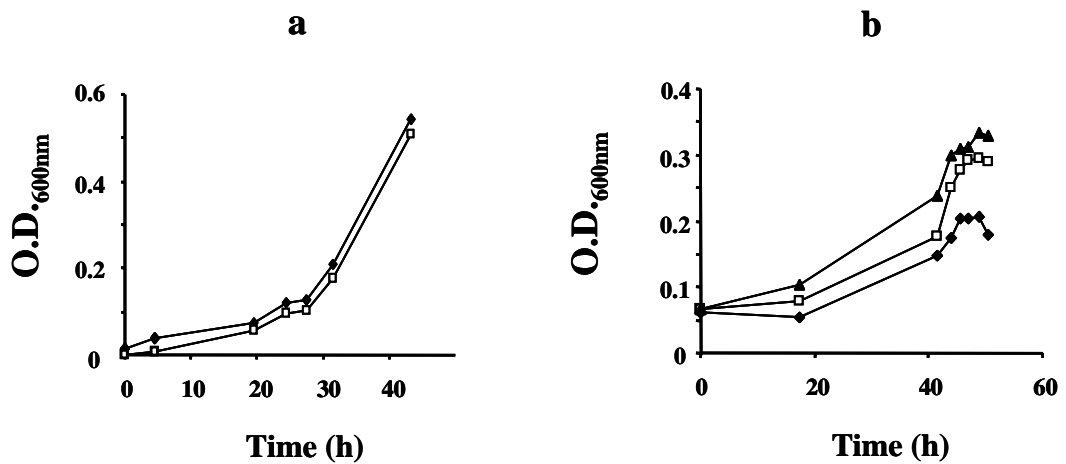
Fig. 6. Growth analysis of *M. mazei** and transformed strains under different nitrogen availabilities. Strains were grown on 150 mM methanol in 5 ml minimal medium under a nitrogen atmosphere containing 20 % CO_2. The medium was supplemented with 10 mM ammonium (**a**) or contained no additional nitrogen source except the molecular nitrogen provided by the gas atmosphere (**b**). *M. mazei** (open squares), *glnK$_1$*-mutant (closed squares) and *M. mazei**/pWM321 (closed triangles).

transcription (RT)-PCR analysis as described in Materials and Methods. The analysis demonstrated that transcription of those exemplarily chosen genes is not affected by the absence of GlnK$_1$ under nitrogen limitation. The respective Ct-values calculated, which correspond to the relative amount of transcripts, did not significantly differ for RNA extracted from wild type and the *glnK$_1$* mutant strain, and were within the range of variation (data not shown). Thus, under nitrogen limitation no differential transcription of those genes involved in nitrogen assimilation (*glnA$_1$*) and nitrogen fixation (*nifH*) could be observed in the *glnK$_1$*-mutant; this was also the case for the paralogue *glnK$_2$*-gene. These findings strongly indicate that the partial growth defect of the *glnK$_1$*-mutant observed under nitrogen limitation is not due to a decrease in transcription of *glnA$_1$* or the *nif*-genes. However, analyzing transcription in the *glnK$_1$* mutant we cannot completely rule out that the gene product of the paralogue *glnK$_2$*-gene, which is in contrast to *glnK$_1$* constitutively transcribed under different nitrogen availabilities, can partially substitute for GlnK$_1$ function in the mutant.

As the GlnK$_1$-protein appears not to be directly involved in transcriptional regulation of genes involved in nitrogen metabolism, it is more likely that GlnK$_1$ affects components of

the nitrogen metabolism directly, which finally results in the observed partial growth defect under nitrogen-limitation. For bacterial PII-like proteins, which have been shown to play ubiquitous roles as sensor and signal transducers in nitrogen control, several such receptor proteins involved in nitrogen metabolism have been described. The activity of those is often modulated by direct protein interaction with the respective PII-like proteins (Arcondeguy et al. 2001). Well studied examples are (i) *E. coli* GlnB, which transduces the nitrogen signal towards the sensor kinase NtrB (Jiang et al. 1998b) and towards the glutamine synthetase (Reitzer 2003), (ii) *E. coli* GlnK, which negatively regulates the activity of ammonium transporter AmtB by protein protein interaction (Coutts et al. 2002; Javelle et al. 2004) and (iii) the *Synechococcus elongatus* PII protein, which forms tight complexes with the N-acetylglutamate kinase under nitrogen excess conditions and thereby enhances its enzyme activity (Heinrich et al. 2004). In contrast to the bacterial PII-like proteins, the knowledge about archaeal PII-like proteins concerning their regulatory functions and even more the understanding of the respective regulatory mechanisms is rather limited. Besides *M. mazei* $GlnK_1$, the only archaeal PII-like proteins functionally examined so far are the gene products of the *glnB*-like genes *nifI$_1$* and *nifI$_2$*, located within the *nif*-gene cluster of *Methanococcus maripaludis* (Kessler et al. 1998). These PII-like proteins are both required for the ammonia switch-off process of nitrogenase activity, apparently by influencing nitrogenase activity directly, as Leigh and coworkers recently demonstrated (Kessler et al. 2001).

Conclusion:

We have demonstrated that using the spontaneous mutant strain *M. mazei** and the modified protocol of the liposome-mediated transformation, *M. mazei* strain Gö1 is now genetically accessible and functional analysis can be performed using genetic approaches. Characterization of the *glnK$_1$*-mutant strain indicated that the archaeal PII-like protein $GlnK_1$ has no direct function in nitrogen dependent transcriptional regulation in *M. mazei*. However, a partial growth phenotype was observed under nitrogen limitation, suggesting that $GlnK_1$ may be involved in post-translational regulation of enzymes required under this growth conditions. As currently no such receptor proteins of $GlnK_1$ are known for *M. mazei*, potential interaction partners have to be identified. In this respect preliminary experiments indicate that $GlnK_1$ is directly interacting with glutamine synthetase (C. Ehlers and R. A. Schmitz, unpublished).

Chapter 4:

Unique mechanistic features of post translational regulation of glutamine synthetase activity in *Methanosarcina mazei* strain Gö1 in response to nitrogen availability

SUMMARY

PII-like signal transduction proteins are found in all three domains of life and have been shown to play key roles in the control of bacterial nitrogen assimilation. This communication reports the first target protein of an archaeal PII-like protein, representing a novel PII-receptor. The $GlnK_1$ protein of the methanogenic archaeon *Methanosarcina mazei* strain Gö1 interacts and forms stable complexes with glutamine synthetase ($GlnA_1$). Complex formation with $GlnK_1$ inhibits the activity of $GlnA_1$. On the other hand, the activity of this enzyme is directly stimulated by the effector molecule 2-oxoglutarate in the absence of $GlnK_1$. Moreover, 2-oxoglutarate antagonized the inhibitory effects of $GlnK_1$ on $GlnA_1$ activity and on $GlnK_1/GlnA_1$ complex formation. On the basis of these findings we hypothesize that the nitrogen sensor $GlnK_1$ allows fine tuning control of the glutamine synthetase activity under changing nitrogen availabilities and propose the following model. (i) Under nitrogen limitation, increasing concentrations of the effector molecule 2-oxoglutarate stimulate maximal $GlnA_1$ activity and prevent the inhibitory complex formation with $GlnK_1$. (ii) Upon a shift to nitrogen sufficiency after a period of nitrogen limitation, glutamine synthetase activity is reduced by decreasing internal 2-oxoglutarate concentrations through diminished direct activation and by the inhibitory protein interaction with $GlnK_1$.

INTRODUCTION

PII-like proteins GlnB and GlnK belong to the family of small signalling proteins identified in all three domains of life (Ninfa and Atkinson 2000; Arcondeguy et al. 2001). They are known to play an important role in sensing and transducing cellular nitrogen signals and

therefore being involved in the regulation of nitrogen metabolism (reviewed by Arcondeguy et al. 2001; Kessler et al. 2001; Ehlers et al. 2002a; Forchhammer 2004). Regulatory mechanisms mediated by PII-like proteins are well studied in bacteria, for which a variety of different receptor proteins have been identified and characterized (Kamberov et al. 1994; Jaggi et al. 1997; de Zamaroczy 1998; Nolden et al. 2001; Coutts et al. 2002; Heinrich et al. 2004; Javelle et al. 2004). In general, bacterial PII-like proteins are covalently modified and de-modified in response to changes in nitrogen availability, however the modification differs: Uridylylation of PII-like proteins has been demonstrated for enteric bacteria (Jiang et al. 1998a; Atkinson and Ninfa 1999), adenylylation for the actinomycetes *Streptomyces coelicolor* and *Corynebacterium glutamicum* (Hesketh et al. 2002; Strösser et al. 2004) and phosphorylation for the cyanobacterium *Synechococcus elongatus* (Forchhammer and Tandeau de Marsac 1995). Exception is the GlnY protein of *Azoarcus* sp. BH72, which exists exclusively in one modification state, in its uridylylated form, independently of the actual nitrogen availability (Martin et al. 2000). Furthermore, in various systems PII-like proteins seem not to be subject to covalent modification, such as in Prochlorophytes (Palinska et al. 2002), *Bacillus subtilis* (Detsch and Stulke 2003), and in plant PII-like proteins (Smith et al. 2004). It has been recently shown that external nitrogen limitation is perceived as internal glutamine limitation in case of *Escherichia coli* (Ikeda et al. 1996; Jiang et al. 1998a), however, in unicellular cyanobacteria the cellular 2-oxoglutarate level is the internal nitrogen signal, which determines the modification state of the PII protein in response to nitrogen availability (Irmler et al. 1997; reviewed by Forchhammer et al. 1999). In addition to covalent modification, allosteric binding of small effector molecules, in particular ATP and 2-oxoglutarate to PII is an important signal input into the PII system, thus allowing the integration of various signals to generate a coherent response. Depending on their modification and ligand binding states, bacterial PII-like proteins modulate the activity of several receptor proteins involved in nitrogen assimilation: (i) *E. coli* GlnB effects the activity of the transcriptional activator NtrC by interacting with the histidine kinase NtrB (Jiang et al. 1998b) and regulates the activity of the adenylyltransferase (ATase), which controls the glutamine synthetase activity (Jiang et al. 1998c; Reitzer 2003). (ii) The second PII-like protein in *E. coli,* GlnK, has been shown to act as a backup system and as a fine control regulator for the GlnB regulatory cascade (Atkinson et al. 2002). In addition, GlnK appears to regulate the activity of the ammonium transporter AmtB by direct protein interaction after a shift to nitrogen sufficiency (Coutts et al. 2002; Javelle et al. 2004). (iii) In nitrogen fixing bacteria, GlnK has been shown to

transduce the internal nitrogen status towards the regulatory proteins NifA or NifL by direct interaction (Liang et al. 1992; Arsene et al. 1996; Arsene et al. 1999; Little et al. 2000; Rudnick et al. 2002; Drepper et al. 2003; Stips et al. 2004). Besides those receptor proteins, a new target protein was recently discovered for the PII protein of *S. elongatus*. Under nitrogen excess conditions, the non-phosphorylated PII-protein forms stable complexes with the key enzyme of the arginine biosynthesis pathway, N-acetylglutamate kinase, and thereby enhances its enzyme activity by an order of magnitude (Heinrich et al. 2004).

In contrast to bacterial PII-like proteins until now only one potential receptor protein has been identified for archaeal PII-like proteins. The gene products of the *glnB*-like genes *nifI$_1$* and *nifI$_2$* located within the *nif* gene operon in *Methanococcus maripaludis* have been shown to be essential for the ammonia switch-off of nitrogenase activity in response to a shift to nitrogen sufficiency (Kessler et al. 2001). Characterizing *nifI* mutant strains demonstrated that both NifI-proteins are essential for modulating nitrogenase activity (Kessler et al. 2001). However, as no biochemical data are available, the regulatory mechanism is still not completely understood. Recently, we characterized the archaeal PII-like protein GlnK$_1$ of *Methanosarcina mazei* strain Gö1 and demonstrated that the archaeal GlnK$_1$ protein structurally differs significantly from bacterial PII-like proteins. Nevertheless, *M. mazei* GlnK$_1$ was able to complement an *E. coli ΔglnK* mutant strain, strongly indicating that the archaeal GlnK protein is involved in nitrogen regulation (Ehlers et al. 2002a). The goal of this work was to elucidate the regulatory function of GlnK$_1$ in nitrogen metabolism of *M. mazei* by identifying potential interacting partners. During our studies, we identified glutamine synthetase (GlnA$_1$) as the first receptor protein of GlnK$_1$ and characterized the effect of complex formation between GlnK$_1$ and GlnA$_1$ on glutamine synthetase activity.

EXPERIMENTAL PROCEDURES

Strains and plasmids. Strains and plasmids used in this study are listed in Table 1. Plasmid DNA was transformed into *E. coli* according to the method of Inoue (Inoue et al. 1990).

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Genotype or description	Source or reference
Strains		
Methanosarcina mazei strain Göl	Wild type	DSM No. 3647
M. mazei* ΔglnK₁⁻	*M. mazei*,* but *glnK₁::pac*	(Ehlers et al. 2004b)
Escherichia coli DH5α BL21-CodonPlus®-RIL	general cloning strain, containing the pRIL plasmid (ileW, leuY, proL)	Stratagene, La Jolla, US
Plasmids		
pET28a	general cloning vector, providing an N-terminal His₆-*tag*	Novagen
pRS196	*M. mazei glnA₁* cloned into pET28a under the control of the T7 promotor coding for His₆-GlnA₁	This work
pRS203	*M. mazei glnK₁* cloned into pET28a under the control of the T7 promotor coding for His₆-GlnK₁	This work

Construction of plasmids. Plasmid pRS203 was constructed as follows. *M. mazei glnK₁*-gene was amplified by PCR using chromosomal *M. mazei* DNA and primers homologous to the *glnK₁* flanking 5' and 3' regions with additional synthetic restriction recognition sites for *Nde*I and *Hind*III, respectively (underlined): glnKNde1-primer (5'-GTGGTC<u>CATATG</u>AAA TACGTAATTGCAATG-3' and glnKHindIII-primer (5'-CGTTTTTACGCTGGAC<u>AAGCTT</u> TCC-3'). The 472 bp PCR fragment was cloned into the *Nde*I and *Hind*III sites of the expression vector pET28a (Novagen), fusing six histidine codons in front of the *glnK₁* start codon (N-terminal His-tag). In order to construct plasmid pRS196 an approx. 1.3 kbp DNA fragment containing the *M. mazei glnA₁*-gene was amplified by PCR using the glnA1 forward primer with an additional *Nde*I restriction site (5'-GGATGGAAT<u>CATATG</u>GTGCAGATG-3') and glnA1 reverse primer (5'-CTGGAGC<u>GGATCC</u>TTCCG-3') with an additional *Bam*H1 site. The obtained PCR product was cloned into the expression vector pET28a restricted with *NdeI* and *BamHI* fusing six histidine codons in front of the *glnA₁* start codon (N-terminal His-tag). The correct insertion and sequence of both genes were confirmed by DNA sequencing of both strands.

Growth conditions. *M. mazei* strains were routinely grown at 37 °C under anaerobic conditions under a N_2/CO_2 (80:20) atmosphere in minimal medium supplemented with 150

mM methanol and 40 mM acetate as described (Deppenmeier et al. 1990; Ehlers et al. 2002a). For nitrogen limited growth, the ammonium was omitted from the media and molecular nitrogen in the gas phase served as sole nitrogen source. For ammonium up-shift experiments, cells were initially grown under nitrogen-limitation until they reached a turbidity of 0.35 - 0.4 at 600 nm (exponential growth phase) and were then supplemented with 15 mM ammonium followed by further incubation at 37 °C for 30 min. Medium for growth of the *M. mazei glnK$_1$*-mutant strain was in generally supplemented with 2.5 µg / ml puromycin (C. Ehlers and R.A. Schmitz, unpublished).

Protein purification. For heterologous expression and purification of *M. mazei* His$_6$-GlnK$_1$ and His$_6$-GlnA$_1$ the respective plasmids pRS203 (pET28a/*glnK$_1$*) and pRS196 (pET28a/*glnA$_1$*) were transformed into *E. coli* BL21-CodonPlus®-RIL (Stratagene, La Jolla), which provides additional tRNAs rare for *E. coli*. 1-liter cultures were grown aerobically in Luria-Bertani (LB) medium at 37 °C and expression of His$_6$-GlnK$_1$ and His$_6$-GlnA$_1$ was induced with 100 µM and 1 µM isopropyl-β-D-thiogalactopyranoside (IPTG), respectively, when cells reached a turbidity of approx. 0.6 at 600 nm. After 2 h induction at 37 °C the cells were harvested and cell extracts were prepared by disruption in buffer A (50 mM NaH$_2$PO$_4$, 300 mM NaCl pH 8.0) supplemented with the protease inhibitor cocktail for bacterial cell extracts (Sigma) using a French pressure cell followed by centrifugation at 20,000 x g for 30 min. *M. mazei* His$_6$-GlnK$_1$ and His$_6$-GlnA$_1$ proteins were purified from the respective supernatant by Ni-affinity chromatography using Ni-NTA agarose (Qiagen) according to the manufactures instructions. His$_6$-GlnK$_1$ was eluted from Ni-NTA agarose in the presence of 250 mM imidazole, dialyzed into 50 mM Tris/HCl (pH 8.0) and stored at -70 °C. Overexpressed His$_6$-GlnA$_1$ mainly accumulated in insoluble inclusion bodies, thus the expression conditions were varied and optimized to achieve maximal solubility of His$_6$-GlnA$_1$ and purification was performed from the remaining amounts of soluble GlnA$_1$ protein present in the supernatant. Prior to affinity chromatography a 30 % ammonium sulphate precipitation was performed leaving His$_6$-GlnA$_1$ in the soluble fraction, which was applied to the Ni-NTA-agarose after dialysis. His$_6$-GlnA$_1$ protein was finally eluted in the presence of 100 mM imidazole, which was subsequently removed by dialysis into 50 mM Tris-HCl (pH 8.0). Samples of each purification step were analyzed by 12.5 % SDS-PAGE according to Laemmli (Laemmli 1970) and protein concentrations were determined via the method of Bradford (Bradford 1976) with the BioRad protein assay using bovine serum albumin as standard.

Complex analysis by affinity co-chromatography. 1-l culture of *M. mazei* wild type and *glnK₁* mutant strain were grown under nitrogen limitation to a turbidity of 0.3 at 600 nm. The cultures were split into two equal parts, one of which was further incubated for 30 min (N_2); the other parts were shifted to growth in the presence of 10 mM ammonium for 30 min. After harvesting, the respective cells were resuspended in 50 mM Tris/HCl (pH 6.9) and cell extracts were prepared in the presence of the protease inhibitor cocktail for bacterial cell extracts (Sigma) using a French pressure cell. 1 mg purified His_6-GlnK₁ and 0.5 mg His_6-GlnA₁, respectively, were bound to 500 µl Ni-NTA agarose for 1 h at 4 °C. The matrix was subsequently split, 250 µl each was filled into an empty column and 15 mg protein of crude extracts (N_2 or NH_4^+-shift) were applied. After washing with 2 x 8 ml buffer A supplemented with 20 mM imidazole, immobilized His_6-GlnK₁ and His_6-GlnA₁ and potentially interacting proteins were eluted with 5 x 100 µl in the presence of 250 mM (His_6-GlnK₁) and 100 mM imidazole (His_6-GlnA₁). Elution fractions were analyzed by 12.5 % SDS-PAGE and silver staining or by Western blot analysis.

Complex analysis by gel filtration. 80 µg GlnA₁ (1.8 µmol GlnA₁) and 40 µg GlnK₁ (2.8 µmol GlnK₁) were incubated in a total volume of 50 µl for 5 min at RT before loading on an analytic Bio-Sil® Sec 250-5 column (BioRad Laboratories), which was equilibrated with 50 mM NaH_2PO_4 buffer pH 8.0 containing 300 mM NaCl. Protein was eluted from the column using a flow rate of 1.0 ml/min and 0.25 ml fractions were collected. Protein gel filtration standards from BioRad were used as marker proteins (thyroglobulin, IgG, myoglobin, ovalbumin, Vitamin B12). When analyzing the effect of 2-oxoglutarate and ATP on complex formation, the effector molecules were added each to a final concentration of 1 mM followed by 5 min incubation at RT prior to gel filtration analysis. Peak fractions containing the oligomeric GlnA₁ protein were assayed for the presence of GlnK₁ by Western blot analysis using specific antibodies directed against GlnK₁.

Western blot analysis. Purified GlnK₁ protein and purified His_6-GlnA₁ protein were used to generate polyclonal rabbit antibodies directed against GlnK₁ and GlnA₁ (Goetek Göttingen, Germany). Proteins from the respective elution fractions were separated on denaturing polyacrylamide gels and transferred to nitrocellulose membranes (BioTrace®NT, Pall Life Science) (Sambrook 1989). Membranes were exposed to specific polyclonal rabbit antisera directed against GlnK₁ and GlnA₁. Protein bands were detected with secondary antibodies directed against rabbit immunoglobulinG coupled to horseradish

peroxidase (BioRad Laboratories) and visualized using the ECL plus system (Amersham/Pharmacia) with a PhosphoImager (Storm, Molecular Dynamics) using the ImageQuant v1.2 software.

Determination of N-terminal amino acid sequences. Elution fractions of $GlnK_1$ obtained from co-elution experiments were separated by 12.5 % SDS-PAGE and transferred onto a PVDF membrane (Immobilon-P from Millipore Cooperation Eschborn, Germany). The amino acid sequences of the co-eluting proteins were determined at the Max Planck Institute for experimental medicine by Dr. B. Schmidt using a Procise491 Protein Sequencer from Applied Biosystems (USA).

Determination of glutamine synthetase activity. Glutamine synthetase (GS) activity was determined by using the coupled optical test assay described by Shapiro (Shapiro and Stadtman 1970), which couples the consumption of ATP by the conversion of ammonium and glutamate to glutamine catalyzed by glutamine synthetase to the oxidation of NADH by lactate dehydrogenase. The test assay was performed as follows: 30 µl NADH (14 mM), 60 µl ATP (60 mM), water and sample were added to 400 µl of freshly prepared assay buffer (0.125 M MOPS pH 7.0, 0.225 M KCl, 0.125 M $MgCl_2$, 0.075 M sodium glutamate pH 7.0, 0.125 M NH_4Cl) to give a final volume of 980 µl. When the absorbance at 340 nm was constant, 10 µl of the enzyme mixture consistent of pyruvate kinase and lactate dehydrogenase (Roche, Mannheim) was added and after additional 30 s the reaction was finally started with 10 µl 0.1 M phosphenolpyruvate (PEP). The absorbance was monitored over a time course of 600 s. Assays in the presence of different concentrations of 2-oxoglutarate and purified His_6-$GlnK_1$ were either performed with or without preincubating $GlnA_1$ with the respective supplement for 5 min at RT before the reaction was started with PEP. When cell extracts were analyzed the absorbance at 340 nm was monitored until no further decrease in absorbance was detectable before adding pyruvate kinase and lactate dehydrogenase to exclude unspecific NADH oxidation by dehydrogenases present in the cell extracts.

RESULTS

The goal of this work was to gain a deeper insight into the regulatory function of $GlnK_1$ in nitrogen metabolism in *M. mazei*. In order to address the question, whether the archaeal

PII-like protein interacts with other proteins involved in nitrogen metabolism, we examined potential complex formation between $GlnK_1$ and cell extract proteins using different approaches.

Identification of potential interacting proteins by pull-down experiments.

In order to identify potential receptor proteins that directly interact with $GlnK_1$ we studied complex formation between $GlnK_1$ N-terminally fused to a His_6-tag (His_6-$GlnK_1$) and cell extract proteins by pull-down experiments using affinity chromatography on Ni-NTA agarose for detecting complexes. His_6-$GlnK_1$ was heterologously expressed from pRS203 in *E. coli,* and purified to an apparent homogeneity of 98 % by Ni-NTA affinity chromatography as described in Experimental Procedures. 1 mg purified His_6-$GlnK_1$ protein was bound to Ni-NTA agarose (0.5 ml) and 30 mg cell extract protein of *M. mazei*
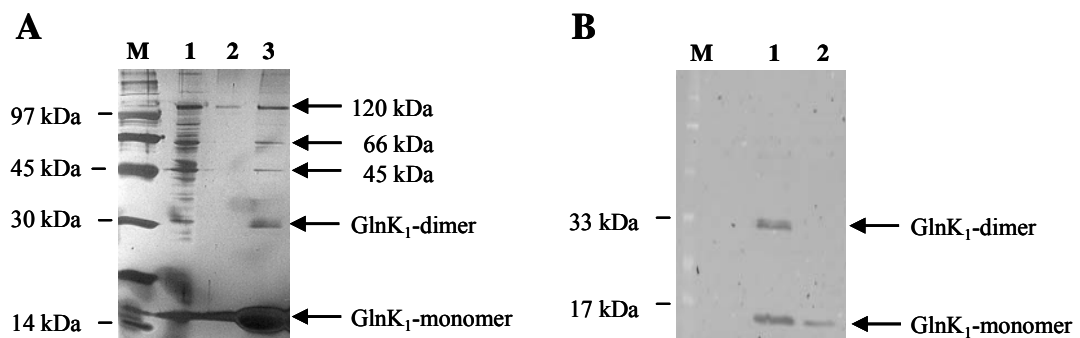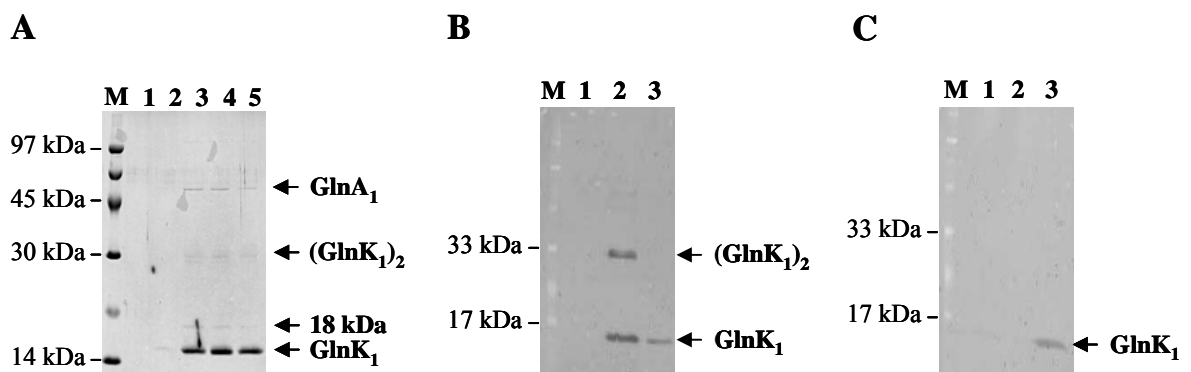

Fig. 1. Co-chromatography of $GlnK_1$ with $GlnA_1$ present in *M. mazei* cell extracts. 1 mg purified His_6-$GlnK_1$ protein was immobilized to 0.5 ml Ni-NTA-agarose and 30 mg cell extract protein of ammonium-shifted *M. mazei* cells were applied to the Ni-NTA column. After washing the matrix with buffer A containing 20 mM imidazole, His_6-$GlnK_1$ and potentially interacting proteins were eluted from the column in the presence of 250 mM imidazole in 0.1 ml fractions (see Experimental Procedures). The respective wash and elution fractions were analyzed by denaturing 12.5 % PAGE **(A)** and Western blotting **(B)** using antibodies directed against $GlnK_1$. **(A)** Silver stained SDS-gel: M, low-molecular-weight marker (Amersham/Pharmacia); lanes 1 and 2, wash fractions 1 and 2, respectively; lane 3, elution fraction 1. **(B)** Western blot analysis of the wash and elution fractions using antibodies directed against $GlnK_1$. Protein detection was performed using the ECLplus system (Amersham-Pharmacia) and the PhosphoImager (Molecular Dynamics) as described in Experimental Procedures. M, prestained high-molecular-weight marker (New England Biolabs); lane 1, elution fraction 1; lane 2, purified $GlnK_1$ (0.1 µg).

cells, which were grown under nitrogen limitation (N_2) or shifted to nitrogen sufficiency for 30 min (NH_4^+-shift), were applied to the immobilized His_6-$GlnK_1$. After extensively washing the chromatography material in order to remove all cell extract proteins, which bound unspecifically to $GlnK_1$ or the Ni-NTA agarose, His_6-$GlnK_1$ and potential specifically interacting cell extract proteins were eluted in the presence of imidazole. The respective elution fractions were analyzed by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) und subsequent silver staining. When nitrogen-limited cell extracts were applied to immobilized $GlnK_1$, no distinct protein bands in addition to $GlnK_1$ were detected in significant amounts. However, when cell extracts of ammonium-exposed cells were applied, four additional protein bands corresponding to an approximate molecular mass of 30 kDa, 45 kDa, 62 kDa and 120 kDa were detected in the elution fractions besides the dominant protein band of $GlnK_1$ (Fig. 1A). The protein band of approx. 120 kDa was also detected in comparable amounts in control experiments, in which cell extract of ammonium-shifted cells was passed through unloaded Ni-NTA agarose;
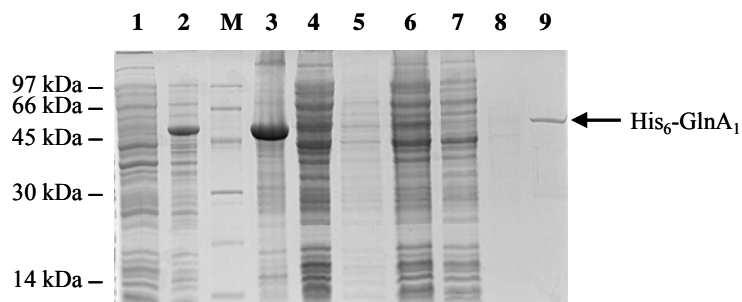
Fig. 2. Expression and purification of $GlnA_1$ by Ni-NTA affinity chromatography. The *M. mazei* His_6-$GlnA_1$ was heterologously expressed in *E. coli* BL21 (DE3/pRIL) containing pRS196. Protein induction was achieved by the addition of 1 μM IPTG followed by further incubation for 2 h at 37 °C. After cell breakage the cell free extract was brought to 30 % ammonium sulphate saturation and the obtained supernatant was further purified by Ni-NTA affinity chromatography (Qiagen). Aliquots of the purification steps were separated by denaturing 12.5 % PAGE and proteins were visualized by Coomassie staining. Lanes 1 and 2, cell extracts before and after induction, respectively; M, low-molecular-weight-marker (Amersham/Pharmacia); lanes 3 and 4, resuspended pellet and supernatant of 20.000 x g centrifugation after cell disruption; lanes 5 and 6, resuspended pellet and supernatant of the 30 % ammonium sulphate precipitation; lane 7, flow-trough of Ni-NTA agarose; lane 8, wash fraction; lane 9, elution fraction containing purified His_6-$GlnA_1$.

indicating that the protein binds to the agarose material in an unspecific way. Western blot analysis using antibodies directed against $GlnK_1$ revealed that the co-eluting protein band with an approx. molecular mass of 30 kDa represents dimeric $GlnK_1$-protein, which was not completely denatured (Fig. 1B). The remaining co-eluted protein bands with molecular masses of 45 and 62 kDa were further analyzed by N-terminal sequencing as described in Experimental Procedures. We did not succeed in identifying the 62 kDa protein, as a mixture of at least 2-3 overlapping sequences were obtained; however, a distinct amino acid sequence was obtained for the 45 kDa protein. Based on the N-terminal amino acid sequence, the 45 kDa protein was identified by genome-wide protein sequence analysis to be the gene product of the *M. mazei* open reading frame MM0964. This open reading frame encodes for a homologous protein of a bacterial glutamine synthetase and thus was designated *glnA₁* (Deppenmeier et al. 2002). In order to exclude that the $GlnA_1$ protein


Fig. 3. Reverse co-chromatography analysis of immobilized $GlnA_1$ and cell extract proteins. 0.5 mg purified His_6-$GlnA_1$ protein was immobilized to 0.5 ml Ni-NTA agarose and 30 mg protein of ammonium-shifted *M. mazei* cell extract was applied to the column. Following the washing steps, His_6-$GlnA_1$ and potentially interacting cell extract proteins were eluted from the affinity material and analyzed by SDS-PAGE and Western blotting. (**A**) SDS-PAGE analysis of the respective co-elution fractions after silver staining. M, low-molecular-weight-marker; lanes 1 and 2, wash fractions; lanes 3 − 5, elution fractions 1 - 3. (**B**) Western blot analysis of wash and elution fractions using antibodies directed against *M. mazei* $GlnK_1$. M, prestained high-molecular-weight marker (New England Biolabs); lane 1, wash fraction; lane 2, combined elution fractions 1 - 3; lane 3, purified His_6-$GlnK_1$-protein (0.1 μg). (**C**) Western blot analysis of the control chromatography with cell extracts passed through unloaded Ni-NTA-agarose using antibodies directed against $GlnK_1$ (negative control). M, prestained high-molecular-weight marker; lane 1; wash fraction; lane 2, combined elution fractions 1-3; lane 3, purified His_6-$GlnK_1$ protein (0.1 μg).

binds unspecifically to the Ni-NTA matrix, control experiments with *M. mazei* cell extracts from nitrogen limited or ammonium-shifted cells and unloaded Ni-NTA agarose were performed. SDS-PAGE analysis followed by silver staining or Western blot analysis using antibodies directed against $GlnA_1$ clearly demonstrated that $GlnA_1$ was not detectable in these elution fractions.

In order to verify the complex formation between $GlnK_1$ and $GlnA_1$ a complementary pull-down experiment was performed. His_6-$GlnA_1$ was heterologously expressed from pRS196 in *E. coli* and purified to an apparent homogeneity of 99 % by fractionated ammonium precipitation followed by Ni-NTA affinity chromatography as described in Experimental Procedures (see Fig. 2). The purified fraction was loaded on Ni-NTA columns and exposed to *M. mazei* cell extracts. His_6-$GlnA_1$ co-eluted from the affinity column together with three additional proteins present in cell extracts of ammonium-shifted cells with approx. molecular masses of 14, 18 and 30 kDa (Fig. 3A). Western blot analysis using antibodies directed against $GlnK_1$ confirmed that the prominent 14 and the faint 30 kDa protein bands detected in the silver stain represent monomeric and dimeric $GlnK_1$, respectively (Fig. 3B). Control experiments in which *M. mazei* cell extracts were passed through non-loaded Ni-NTA columns ruled out unspecific binding of $GlnK_1$ (Fig. 3C), further confirming complex formation between $GlnK_1$ and $GlnA_1$. Identification of the 18 kDa protein did not succeed due to insufficient amount of protein; the minute amounts of this protein suggest that it might be a contamination.

Interaction studies of $GlnK_1$ and $GlnA_1$ by gel filtration analysis.

To confirm the observed protein-protein interaction, complex formation between $GlnK_1$ and $GlnA_1$ was studied by gel filtration analysis using independently expressed and purified proteins both fused to a *His*-tag. When applied separately to the BioSil gel filtration column, purified His_6-$GlnA_1$ (80 μg) eluted as a single peak corresponding to a molecular mass of approximately 550 kDA as determined in comparison with standard protein markers. As monomeric His_6-$GlnA_1$ shows a molecular mass of 42 kDa, the native protein eluting from the gel filtration column appears to be in a higher oligomeric structure consisting of 12 subunits (dodecamer) (Fig. 4A). In comparison, purified His_6-$GlnK_1$ (40 μg) eluted as a single peak, corresponding to a molecular mass of 42 kDa representing trimeric $GlnK_1$, which is consistent with recent findings obtained by native PAGE analysis of *M. mazei* $GlnK_1$ in cell extracts (Ehlers et al. 2002a). In order to analyze complex formation between $GlnK_1$ and $GlnA_1$, 40 μg His_6-$GlnK_1$ was preincubated together with 80
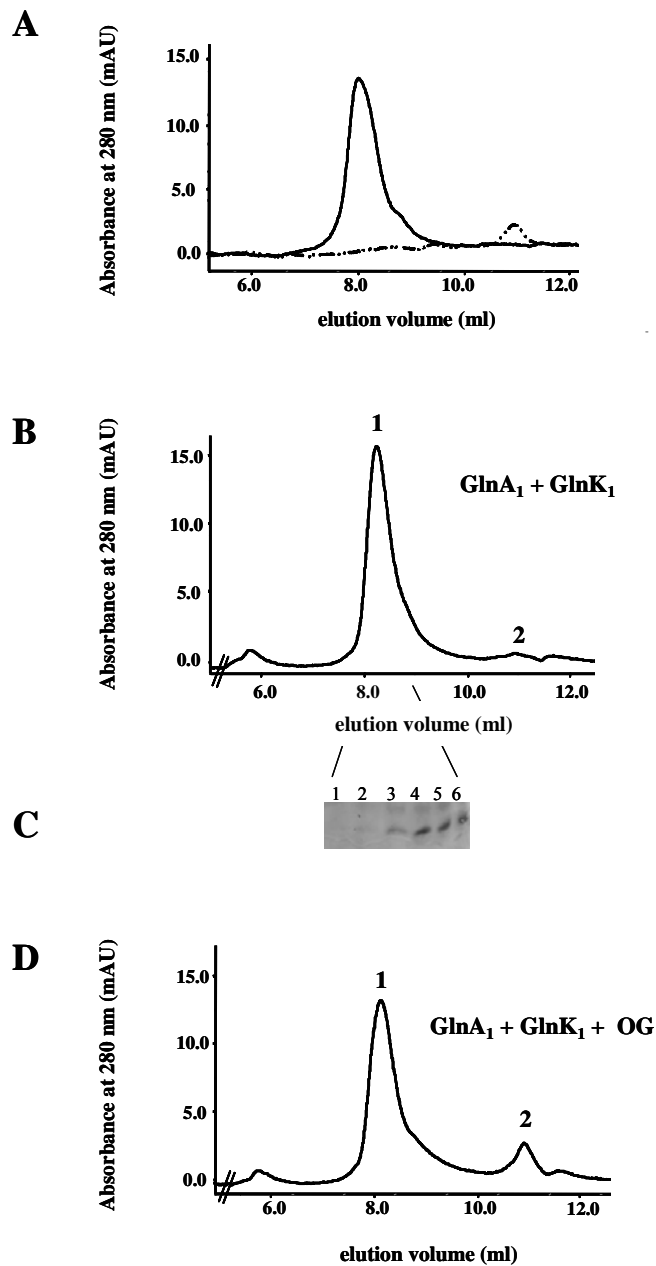
Fig. 4. Gel filtration analysis of complex formation between purified *M. mazei* His$_6$-GlnA$_1$ and His$_6$-GlnK$_1$ proteins. Gel filtration analysis was performed on a Bio-Sil$^®$ Sec column (BioRad) using 50 mM NaH$_2$PO$_4$, 300 mM NaCl pH 8.0 as buffer system and a flow rate of 1.0 ml/min. Proteins were detected by monitoring the absorbance at 280 nm. (**A**) 80 μg purified His$_6$-GlnA$_1$ (solid line) and 40 μg purified His$_6$-GlnK$_1$ (dots) were applied in separate runs. Calibration of the column was performed using the gel filtration mass standard (BioRad Laboratories) containing thyroglobulin (670 kDa), IgG (150 kDa), myoglobulin (44 kDa), ovalbumin (17 kDa), and vitamin B12 (1.35 kDa). (**B**) 80 μg purified His$_6$-GlnA$_1$ and 40 μg His$_6$-GlnK$_1$ proteins were preincubated in a total volume of 50 μl for 5 min at RT prior to the application. Peak numbers mark the elution

peak of the dodecameric $GlnA_1$ **(1)** and of trimeric $GlnK_1$ **(2)**. **(C)** 20 μl aliquots of the peak fractions containing the dodecameric $GlnA_1$ protein were analyzed for the presence of $GlnK_1$ by Western blotting using antibodies directed against $GlnK_1$; lanes 1 - 6, elution fractions 10 – 15. **(D)** Gel filtration analysis of complex formation between $GlnA_1$ and $GlnK_1$ in the presence of 1 mM 2-oxoglutarate. 80 μg His_6-$GlnA_1$ and 40 μg His_6-$GlnK_1$ were incubated for 5 min in the presence of 1 mM 2-oxoglutarate in a total volume of 50 μl at RT prior to the application. Peak numbers mark the elution peak of the dodecameric $GlnA_1$ **(1)** and of trimeric $GlnK_1$ **(2)**.

μg His_6-$GlnA_1$ for 5 min at RT prior the application to the gel filtration column. The respective elution profile after gel filtration showed that the absorption peak representing unbound trimeric His_6-$GlnK_1$ was clearly reduced in comparison to the gel filtration experiment analyzing the same amount of His_6-$GlnK_1$ in the absence of $GlnA_1$ as depicted in Fig. 4A/B. Integration of the peak areas confirmed that in case of preincubation with $GlnA_1$, the His_6-$GlnK_1$ peak was reduced to approximately 30 %. No significant change of the elution volume of the His_6-$GlnA_1$-elution peak was detectable, as an increase of the molecular mass from 550 kDa to approximately 600 kDa upon complex formation with trimeric $GlnK_1$ is not resolved by the gel filtration column used here. Thus, the fractions of the $GlnA_1$ elution peak were analyzed by Western blotting using antibodies directed against $GlnK_1$, which clearly demonstrated the presence of significant amounts of $GlnK_1$ in the peak fractions of dodecameric $GlnA_1$ (Fig. 4C). The finding that $GlnK_1$ co-elutes with $GlnA_1$ from the gel filtration column strongly indicates that dodecameric $GlnA_1$ forms stable complexes with $GlnK_1$ and confirms the protein-protein interaction between $GlnK_1$ and $GlnA_1$ demonstrated in the pull-down experiments.

For bacteria, it has been demonstrated that the small effector molecules 2-oxoglutarate and ATP can influence binding and interaction of PII-like proteins with interaction partners (Jiang et al. 1998c; Xu et al. 1998; Little et al. 2002; Ruppert et al. 2002). Therefore, we investigated the influence of 2-oxoglutarate and ATP on $GlnA_1$/$GlnK_1$ complex formation by gel filtration. Complex formation between $GlnK_1$ and $GlnA_1$ was carried out in the presence of either 1 mM 2-oxoglutarate, 1 mM ATP or both for 5 min at RT followed by gel filtration as described above. The elution profiles obtained revealed that the presence of 2-oxoglutarate resulted in a significant increase of the protein peak representing unbound trimeric $GlnK_1$ (3-fold increase in peak area) compared to complex formation in the absence of 2-oxoglutarate (Fig. 4B/D). This strongly indicates that in the presence of 2-oxoglutarate significant less or no $GlnA_1$/$GlnK_1$-complexes were formed. In contrast, an effect of ATP on complex formation was not detected; simultaneous ATP presence
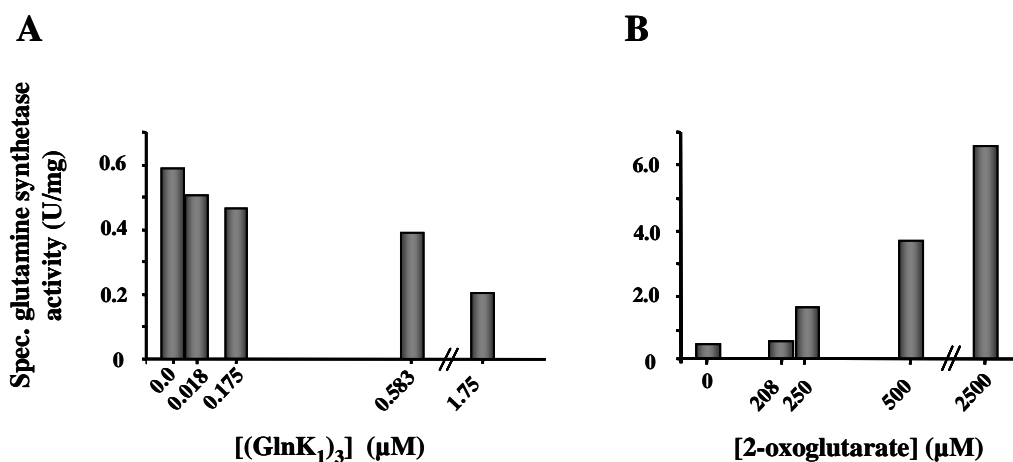
Fig. 5. Glutamine synthetase activity of purified GlnA₁. Glutamine synthetase activity of purified heterologously expressed His₆-GlnA₁ was determined at RT using the coupled optical enzyme assay described in Experimental Procedures in the presence of varying amounts of GlnK₁ and 2-oxoglutarate. (**A**) Effects of GlnK₁ on glutamine synthetase activity: 40 μg purified His₆-GlnA₁ was preincubated with increasing amounts of purified His₆-GlnK₁ for 5 min at RT prior to activity analysis. (**B**) Effects of 2-oxoglutarate on glutamine synthetase activity. Increasing concentrations of 2-oxoglutarate (final concentrations of 0.208 – 2.5 mM) were added to 40 μg purified His₆-GlnA₁ and preincubated for 5 min at RT prior to activity analysis.

however, did not diminish the inhibitory effect of 2-oxoglutarate on complex formation (data not shown).

GlnK₁ affects glutamine synthetase activity of purified GlnA₁.

Glutamine synthetase activity of purified heterologously expressed His₆-GlnA₁ was analyzed immediately after purification to avoid storage of the enzyme preparation (see below). The specific glutamine synthetase activity of several independently synthesized and purified His₆-GlnA₁ preparations was determined to be in the range between 0.4 – 0.6 U/mg using the coupled optical test assay developed by Shapiro (Shapiro and Stadtman 1970) as described in Experimental Procedures. Upon storage at either 4 °C, -20 °C or -70 °C glutamine synthetase activity of purified His₆-GlnA₁ dramatically decreased, indicating that the purified protein is rather instable. This is consistent with results obtained by gel filtration analysis of purified GlnA₁, revealing that the native structure of GlnA₁ is a dodecamer, which tends to rapidly dissociate into its monomers (data not shown). As

GlnA$_1$ monomers are apparently inactive this dissociation into monomers is probably the cause of the instability of GlnA enzyme activity.

To analyze, whether complex formation with GlnK$_1$ affects GlnA$_1$ activity, glutamine synthetase activity of 40 μg GlnA$_1$ was determined in the presence of different amounts of purified His$_6$-GlnK$_1$ (0.73–73.5 μg). Analyzing at least four independent enzyme purifications of GlnA$_1$ indicated that the presence of increasing GlnK$_1$ amounts in the test assay resulted in a significant decrease of GlnA$_1$ activity as depicted in Fig. 5A. In the presence of 1.75 μM trimeric GlnK$_1$, glutamine synthetase activity (0.9 μM GlnA$_1$) decreased to approx. 30 % of the activity in the absence of GlnK$_1$. However, this inhibition of GlnA$_1$ activity by GlnK$_1$ was only detectable, when GlnA$_1$ was preincubated for at least 5 min with GlnK$_1$ before the assay was started, indicating that direct interaction and / or complex formation between the two proteins requires a certain period of time.
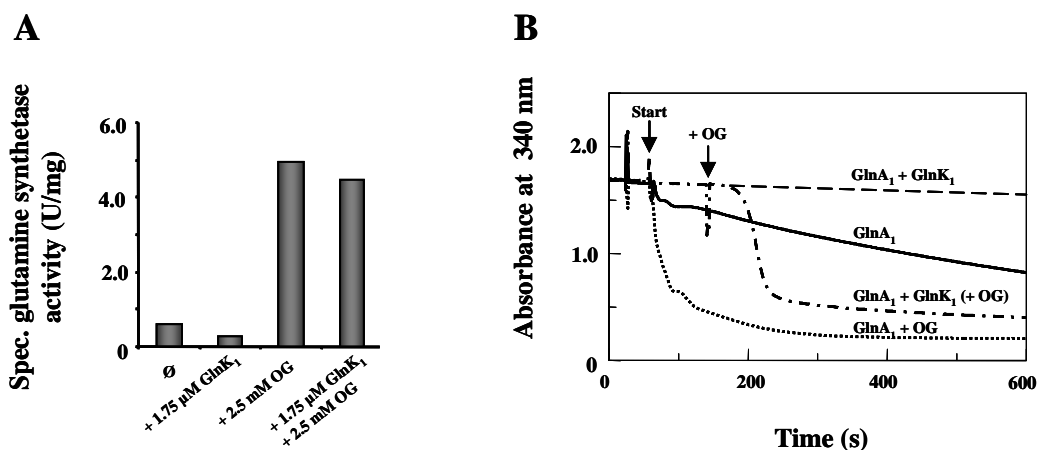
Fig. 6. Modulation of GlnA$_1$ activity by GlnK$_1$ and 2-oxoglutarate. Glutamine synthetase activity assays were performed as described in Fig. 5. Enzyme activity of 100 μg purified His$_6$-GlnA$_1$ was determined after preincubation in the presence of 1.75 μM GlnK$_1$, 0.5 mM 2-oxoglutarate and in the presence of both effectors. (**A**) Glutamine synthetase activities calculated from the time course measurements, depicted in **B**. Ø, GlnA$_1$ activity in the absence of effectors. (**B**) Original time course measurement data monitoring glutamine synthetase activity by the decrease in absorbance at 340 nm. Line, GlnA$_1$ in the absence of effectors (GlnA$_1$); dots, GlnA$_1$ preincubated with 0.5 mM 2-oxoglutarate for 5 min at RT (GlnA$_1$ + OG); small lines, GlnA$_1$ preincubated with GlnK$_1$ (GlnA$_1$ + GlnK$_1$); dots and lines, GlnA$_1$ preincubated for 5 min at RT with GlnK$_1$ but additionally supplemented with 0.5 mM 2-oxoglutarate after 100 s (GlnA$_1$ + GlnK$_1$(+ OG)).

2-oxoglutarate stimulates glutamine synthetase activity and antagonizes inhibitory effects of GlnK₁.

As we observed a significant influence of 2-oxoglutarate on complex formation with $GlnK_1$ we investigated, whether 2-oxoglutarate affects glutamine synthetase activity of $GlnA_1$ as well. Purified His_6-$GlnA_1$ was preincubated for 5 min at RT in the presence of increasing 2-oxoglutarate concentrations (200 µM to 2.5 mM) followed by determination of glutamine synthetase activity. Unexpectedly, the presence of 2-oxoglutarate resulted in a significant increase of glutamine synthetase activity (Fig. 5B). Up to 16-fold induction of glutamine synthetase activity of $GlnA_1$ was obtained, when incubated in the presence of 2.5 mM 2-oxoglutarate. When $GlnA_1$ (100 µg, equals 2.25 µM) was preincubated for 5 min in the presence of both, the positive effector molecule 2-oxoglutarate (2.5 mM) and its inhibitor $GlnK_1$ (1.75 µM trimeric $GlnK_1$), glutamine synthetase activity was in the same range as the activity observed for $GlnA_1$ preincubated exclusively with 2-oxoglutarate (4.9 U/mg vers. 4.5 U/mg) (Fig. 6 A). This suggests that the effector 2-oxoglutarate is able to abolish the inhibitory effects of $GlnK_1$. 2-oxoglutarate could antagonize inhibition by $GlnK_1$ even after GlnK/GlnA complex formation has already occurred. As shown by the experiment depicted in Fig. 6 B, preincubation of $GlnA_1$ in the presence of $GlnK_1$ inhibits glutamine synthetase activity ($GlnA_1$ + $GlnK_1$). However, only a few seconds after the addition of 2-oxoglutarate to the $GlnA_1$/$GlnK_1$ mixture, glutamine synthetase activity rapidly increased as visualized by the decrease in absorbance due to NADH oxidation ($GlnA_1$ + $GlnK_1$ (+OG)). This suggests that 2-oxoglutarate appears to have a dominant positive effect on glutamine synthetase activity over $GlnK_1$ inhibition. Together with the results obtained for $GlnA_1$/$GlnK_1$ complex formation by gel filtration analysis these findings demonstrate that 2-oxoglutarate negatively affects complex formation between $GlnA_1$ and $GlnK_1$ and thus prevents inhibition of glutamine synthetase activity of $GlnA_1$.

GlnK₁ inhibits glutamine synthetase activity present in *M. mazei* cells.

In order to investigate, whether the $GlnK_1$ effect can also be shown for glutamine synthetase activity of $GlnA_1$ synthesized in the native background, the influence of $GlnK_1$ on $GlnA_1$ activity in cell-free extracts was studied. Cell extracts of *M. mazei* cells grown under nitrogen limitation were prepared in the presence or absence of 50 mM 2-oxoglutarate. Determination of glutamine synthetase activity demonstrated that an

inhibitory effect of additional purified His_6-$GlnK_1$ was only obtained, when the cell extracts were prepared in the absence of 2-oxoglutarate. In this case glutamine synthetase activity of the nitrogen limited cell extract was reduced from 25 mU/mg to 10 mU/mg upon the presence of $GlnK_1$ (1.75 µM trimeric $GlnK_1$), which is consistent with the findings observed with purified $GlnA_1$. However, when 2-oxoglutarate was present during cell breakage, no change in enzyme activity was detectable in the presence of $GlnK_1$, again demonstrating the positive dominant effect of 2-oxoglutarate over $GlnK_1$ inhibition.

Characterization of $GlnA_1$ in a *$glnK_1$*-mutant *M. mazei* strain.

In *M. mazei* a second GlnK protein, $GlnK_2$, is constitutively expressed independently of the nitrogen source (C. Ehlers and R.A. Schmitz, unpublished). In order to analyze, whether $GlnK_2$ also affects $GlnA_1$, we studied (i) potential complex formation between $GlnA_1$ and $GlnK_2$ and (ii) glutamine synthetase activity in a *$glnK_1$* mutant strain previously constructed (C. Ehlers and R.A. Schmitz, unpublished). Pull-down experiments with immobilized $GlnA_1$ protein using cell extracts of ammonium-shifted *$glnK_1$*-mutant cells demonstrated that no additional protein was detectable in the elution fractions by Coomassie or silver staining (data not shown). This finding, which was confirmed in several independent experiments, indicates that $GlnA_1$ appears not to interact with $GlnK_2$. We cannot completely rule out that very low amounts of $GlnK_2$ are present in the $GlnA_1$ elution fractions, which are not detectable by silver staining. However, the apparent specific activity of $GlnA_1$ determined in the cell extract of the *$glnK_1$* mutant strain was comparable to the one determined in the cell extract of the wild type strain, demonstrating that $GlnK_2$ appears not to interact with $GlnA_1$ in the absence of $GlnK_1$.

DISCUSSION

We proposed that the archaeal PII-like protein $GlnK_1$ of *M. mazei* is involved in nitrogen regulation (Ehlers et al. 2002a). We now identified the first receptor protein of the archaeal GlnK protein, glutamine synthetase ($GlnA_1$), and show that interaction with $GlnK_1$ directly affects enzyme activity of $GlnA_1$, the key enzyme of ammonium assimilation.

**GlnK$_1$ inhibits GlnA$_1$ activity by direct protein interaction.**

As ammonium assimilation via the glutamine synthetase / glutamate synthase (GS/GOGAT) pathway is one of the major intersections in central metabolism, the synthesis and activity of glutamine synthetase (GS) is strictly controlled by nitrogen availability in all organisms (Reitzer 2003). Three major families of GS are described to date (Brown et al. 1994), however until now, in methanogenic archaea exclusively glutamine synthetases of the GSI-α subdivision were identified including GlnA$_1$ of *M. mazei* (Bhatnagar et al. 1986; Possot et al. 1989; Cohen-Kupiec et al. 1999; Deppenmeier et al. 2002). As they do not exhibit a potential adenylylation site nor have homologues of adenylyltransferase been found in archaeal genomes, it is unlikely that the activity of those archaeal glutamine synthetases is regulated by adenylylation in response to nitrogen (Brown et al. 1994; Smith et al. 1997; Cohen-Kupiec et al. 1999; Deppenmeier et al. 2002). Thus, regulation of glutamine synthetase activity in methanogenic archaea in response to changes in nitrogen availability was not known.

We now obtained conclusive experimental evidence that the archaeal GlnK$_1$ protein interacts and forms stable complexes with the glutamine synthetase GlnA$_1$ in *M. mazei* thereby negatively affecting its enzyme activity. (i) Pull-down experiments demonstrated that purified *M. mazei* His$_6$-GlnK$_1$ directly interacts with GlnA$_1$ in cell extracts, which was confirmed by the reverse pull-down experiment with immobilized His$_6$-GlnA$_1$ (Figs. 1 and 3). (ii) Complex analysis by gel filtration with independently purified proteins revealed that GlnK$_1$ co-eluted with dodecameric GlnA$_1$, when preincubated with the GlnA$_1$-protein (Fig. 4B, C). (iii) Incubation of purified GlnA$_1$ with GlnK$_1$ significantly reduces glutamine synthetase activity (Fig. 5A). This inhibitory effect of GlnK$_1$ was further confirmed in cell extracts. Taking into account that GlnK$_1$ is only synthesized under nitrogen limitation (Ehlers et al. 2002a), we hypothesize that GlnK$_1$ inhibits glutamine synthetase activity of GlnA$_1$ by direct protein interaction in response to a shift to nitrogen sufficiency after a period of nitrogen limitation. This proposed mechanism for modulating glutamine synthetase activity in *M. mazei* in response to an ammonium-upshift differs significantly from what is known for regulation of glutamine synthetases in bacteria. In most of the proteobacteria, after an ammonium up-shift, PII-like proteins activate the adenylyltransferase (GlnE), which then mediates the nitrogen signal to the glutamine synthetase by inactivating the enzyme via covalent modification (reviewed in (Jiang et al. 1998c; Reitzer 2003). The finding that in *M. mazei* GlnK$_1$ interacts and effects glutamine synthetase directly, demonstrates that the small ubiquitous distributed PII-like sensory

proteins evolved quite differently in various lines of descent taking over distinct functions in controlling nitrogen metabolism by protein-protein interactions with various receptor proteins, depending on the different metabolic requirements. In this respect, N-acetylglutamate kinase, the key enzyme of the arginine biosynthetic pathway, which is activated in response to nitrogen sufficiency by direct interaction with the non-modified PII protein in *Synechococcus elongates* (Heinrich et al. 2004), is another recent example for a newly identified receiver protein of a PII-like protein.

Glutamine synthetase activity of *M. mazei* GlnA$_1$ is directly affected by 2-oxoglutarate.

Analyzing glutamine synthetase activity of purified heterologously expressed GlnA$_1$ demonstrated that purified *M. mazei* GlnA$_1$ showed a low specific activity compared to other archaeal glutamine synthetases (approx. 0.4 – 0.6 U/mg) (Bhatnagar et al. 1986; Cohen-Kupiec et al. 1999), which might be due to the fact that GlnA$_1$ is not correctly folded or assembled to its active conformation, when expressed in an *E. coli* background. This is further supported by gel filtration analysis of purified GlnA$_1$, which clearly demonstrated that the active archaeal glutamine synthetase consists of 12 subunits (Fig. 4 A), however, this oligomeric structure (dodecamer) appeared not to be very stable. Over a period of 12 h incubation on ice the protein dissociated almost completely into monomers, which resulted in a dramatic decrease in enzyme activity. Most interestingly, we obtained conclusive evidence, that glutamine synthetase activity of purified GlnA$_1$ was highly dependent on the presence of 2-oxoglutarate, which is known to be the major indicator of nitrogen status in cyanobacteria (Irmler et al. 1997; Heinrich et al. 2004). The specific activity of GlnA$_1$ was induced up to 16-fold, when 2-oxoglutarate was present in the test assay at a final concentration of 2.5 mM. The minimal concentration, which showed an effect on the glutamine synthetase activity, was determined to be 250 µM 2-oxoglutarate (Fig. 5B). This finding is consistent with the expected physiological concentration range of 2-oxoglutarate at different nitrogen availabilities, as internal 2-oxoglutarate concentrations of approx. 100 µM under nitrogen sufficiency and of approx. 1.0 mM under nitrogen limitation have been determined for *E. coli* (Senior 1975). A positive effect of 2-oxoglutarate was also observed for glutamine synthetase present in cell extracts of *M. mazei* cells grown under nitrogen limitation, confirming the regulatory role of the internal 2-oxoglutarate-pool, which is highly diluted upon cell disruption. The induction of glutamine synthetase activity by 2-oxoglutarate in *M. mazei* is to our knowledge the first
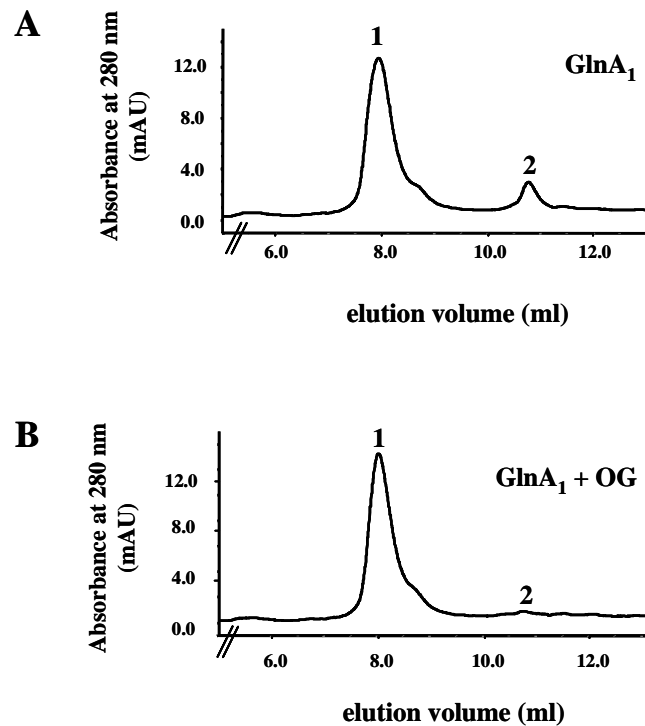
Fig. 7. Gel filtration analysis of purified *M. mazei* GlnA₁ in the presence and absence of 2-oxoglutarate. Potential structural changes of purified His₆-GlnA₁ protein in dependence of 1 mM 2-oxoglutarate were assayed by gel filtration analysis as described in Experimental Procedures. 80 µg purified His₆-GlnA₁ was applied to the column either in the absence of an effector (**A**) or after preincubation with 1 mM 2-oxoglutarate for 5 min at RT (**B**). (**1**), dodecameric GlnA₁; (**2**), monomeric GlnA₁.

example for 2-oxoglutarate directly modulating glutamine synthetase activity. Taking together these findings indicate that *M. mazei* perceives external nitrogen limitation by changes in the internal 2-oxoglutarate pool, which is further supported by the finding that the TCA cycle is incomplete and no gene encoding for a 2-oxoglutarate dehydrogenase has been identified in *M. mazei* (Deppenmeier et al. 2002). This is in contrast to several proteobacteria and *Bacillus subtilis*, for which it has been shown that external nitrogen limitation is perceived as internal glutamine limitation (Ikeda et al. 1996; Hu et al. 1999; Yakunin et al. 1999; Schmitz 2000). To date a central role of 2-oxoglutarate for the perception of changes in nitrogen availabilities has been exclusively demonstrated for cyanobacteria e.g. *Synechococcus elongatus* and *Synechocystis* sp. PCC6803 (Forchhammer et al. 1999; Muro-Pastor et al. 2001). In these organisms, the TCA cycle is

also incomplete and synthesis of 2-oxoglutarate, as in methanogens, has merely anabolic functions as precursor for the synthesis of glutamate via the GS/GOGAT pathway. It is thus tempting to speculate that the similar metabolic organization with respect to nitrogen assimilation has led to similar regulatory solutions. Further it should be kept in mind that *M. mazei* has been shown to contain a high number of bacterial-like genes closely related to genes of cyanobacteria apparently acquired by lateral gene transfer (Deppenmeier et al. 2002).

2-oxoglutarate antagonizes inhibitory effects of $GlnK_1$ on $GlnA_1$ activity.

Since 2-oxoglutarate directly affected glutamine synthetase activity it is very likely that $GlnA_1$ binds this effector molecule. Preliminary experiments indicate that the presence of 2-oxoglutarate stabilizes the dodecameric conformation of purified $GlnA_1$ (Fig. 7). This suggests that binding of 2-oxoglutarate to $GlnA_1$ induces a conformational change in the protein structure which could affect complex formation with $GlnK_1$. Whether 2-oxoglutarate binds in addition to $GlnK_1$, as suggested by 2-oxoglutarate binding to its bacterial PII homologues, remains to be investigated. Regulation of a glutamine synthetase activity by 2-oxoglutarate and a PII-like protein is novel. The only other example, in which glutamine synthetase activity is regulated by direct protein-protein interaction is known from a cyanobacterium. Glutamine synthetase activity in *Synechocystis* sp. PC6803 is inhibited upon increasing ammonium concentrations by protein-protein interaction with two small inactivating factors (IF3 and IF7) (Garcia-Dominguez et al. 1999; Garcia-Dominguez et al. 2000). The IF encoding genes *gifA* and *gifB* are only transcribed under nitrogen excess conditions and are repressed under nitrogen-limiting conditins by the global transcription factor NtcA, which itself responds to 2-oxoglutarate (Vazquez-Bermudez et al. 2002).

Hypothetical model for post-translational regulation of glutamine synthetase in response to nitrogen availability in *M. mazei*.

On the basis of our findings we propose the following working model (Fig. 8): *M. mazei* perceives external nitrogen limitation by sensing the internal 2-oxoglutarate pool, which increases due to reduced consumption by the ammonium-dependent GS/GOGAT way. Under those conditions, 2-oxoglutarate by binding to $GlnA_1$ induces a conformational change, which enhances $GlnA_1$ activity and prevents inhibitory complex formation with $GlnK_1$. After a shift to nitrogen sufficiency, however, the internal 2-oxoglutarate level
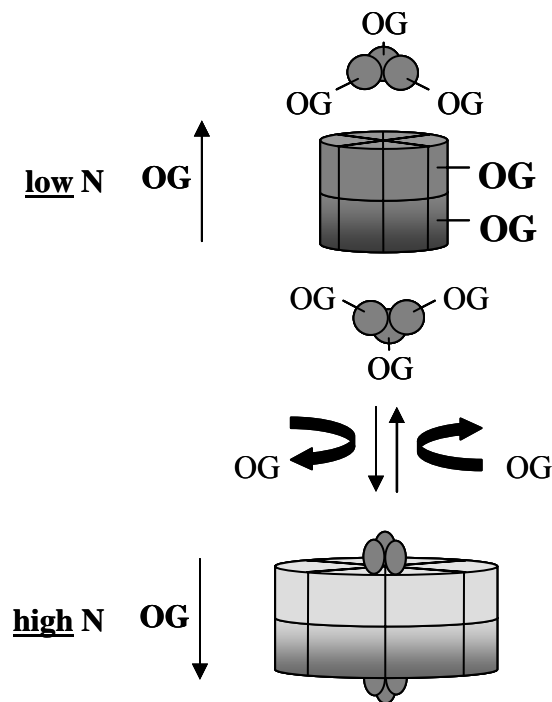
Fig. 8. Hypothetical model for modulation of glutamine synthetase activity in *M. mazei* strain Gö1 in response to different nitrogen availabilities.

decreases and, therefore, glutamine synthetase activity is reduced and is further inhibited by direct interaction with $GlnK_1$. At the current experimental status we do not know, whether $GlnK_1$ additionally is controlled by the 2-oxoglutarate levels. Due to the dodecameric structure of $GlnA_1$ and the trimeric structure of $GlnK_1$ it is tempting to speculate that one $GlnK_1$-trimer interacts with one hexameric-ring of $GlnA_1$. When cells grow under nitrogen excess conditions for prolonged periods of time, $GlnK_1$ levels strongly decrease and glutamine synthetase may solely be regulated by 2-oxoglutarate. Thus, the regulatory network in *M. mazei* allows for an efficient glutamine synthetase down-regulation in cells that previously accumulated $GlnK_1$ due to ammonium limited conditions.

CONCLUSIONS

Our goal is to analyze the nitrogen metabolism in *M. mazei* strain Gö1 with the main focus on the characterization of the PII-like protein $GlnK_1$. Members of these small sensor proteins are known to play a crucial role in transferring the internal nitrogen status to enzymes involved in nitrogen assimilation and thereby regulating the nitrogen metabolism. However, long time, for archaeal PII-like proteins neither biochemical data nor functional analyses were available. In *M. mazei* four genes encoding for PII-like proteins were identified: Two genes are located within the *nif*-gene cluster and are designated as *$nifI_1$* and *$nifI_2$* (Ehlers et al. 2002b). Today, a function of NifI proteins has only been assigned for the respective proteins in *Methanococcus maripaludis*. Here, it has been shown that both NifI proteins are essential for nitrogenase switch-off following an ammonium-upshift (Kessler and Leigh 1999; Kessler et al. 2001). In *M. mazei* the function of these proteins still has to be analyzed, though it seems likely that they fulfil a similar function due to the close relation between *M. mazei* and *Mc. maripaludis*. The other two genes encoding for PII-like proteins in *M. mazei* belong to the group of *glnK*-genes as they are organized together with a gene encoding for an ammonium transporter (*amtB*) in one operon. The respective operons were designated as *$glnK_1amtB_1$* and *$amtB_2glnK_2$* (Ehlers et al. 2002a).

The focus of this thesis was the functional characterization of the $GlnK_1$ protein. We showed that in several aspects the *M. mazei* $GlnK_1$ protein is very similar to bacterial GlnK proteins indicating the highly conserved nature of these small regulatory proteins. On the other hand the archaeal GlnK-like protein has many unique features that differ from all GlnK proteins described today, which will be discussed in the following in more detail.

Transcriptional analysis and complementation studies of *M. mazei $glnK_1$*.

M. mazei $glnK_1$ is only transcribed under nitrogen limitation, as it is known for all bacterial *glnK*-genes investigated demonstrating that it is under nitrogen control itself (Ehlers et al. 2002a). However, the regulatory mechanism that controls transcription of *$glnK_1$* is not known for *M. mazei*. Sequence analysis revealed that in *M. mazei* no NtrB/C dependent regulation exists as it is known from enteric bacteria, hence a different regulatory mechanism has to be postulated. As in general, transcriptional regulation e.g. in response to environmental conditions, remains unresolved in archaea, several open question about activation and repression of transcription still need to be answered. Complementation experiments of *M. mazei $glnK_1$* using an *E. coli glnK*-mutant strain clearly demonstrated

that GlnK$_1$ is able to substitute for the bacterial GlnK protein when growing on arginine as sole nitrogen source (Ehlers et al. 2002a), indicating that the archaeal GlnK$_1$ protein is involved in regulation of nitrogen metabolism in *M. mazei*.

Structural analysis of *M. mazei* GlnK$_1$.

We demonstrated by *in vitro* uridylylation assays and *in vivo* up-shift experiments that *M. mazei* GlnK$_1$ is apparently not modified upon changes in nitrogen availability (Ehlers et al. 2002a). In this respect, the archaeal GlnK protein differs significantly from most bacterial PII-like proteins that are modified by uridylylation, adenylylation or phosphorylation upon a shift to nitrogen sufficiency (Atkinson et al. 1994; Forchhammer and Tandeau de Marsac 1995; Jiang et al. 1998a; Hesketh et al. 2002; Strösser et al. 2004). Thus, the question rises, how the nitrogen status is sensed and signaled in *M. mazei*. One possible mechanism might be based on different conformational forms of GlnK$_1$ in response to changes in nitrogen availabilities.

Due to the high conservation of PII-like proteins heterotrimer formation have been demonstrated *in vivo* and *in vitro* between different PII-like proteins of one species and even between PII-like proteins of different species. It was shown e.g. that *Synechococcus* PII and *E. coli* GlnB can form heterotrimers *in vivo,* although these are functionally inactive (Forchhammer et al. 1999). However, by *in vitro* and *in vivo* experiments for heterotrimerization analysis between *M. mazei* GlnK$_1$ and *K. pneumoniae* GlnK we obtained conclusive evidence that no heterotrimer formation between these proteins occurs (Ehlers et al. 2002a). This indicates that in regard to their overall structure both PII-like proteins differ significantly. This difference in structure is also reflected by crystallization studies of *M. mazei* GlnK$_1$. Though the structure of *E. coli* GlnK is solved all attempts to crystallize this highly soluble archaeal PII-like protein failed so far; again indicating differences in the core protein's crystal structure that influence the interaction of GlnK$_1$ monomers and might be important for crystal package (Ehlers, Andrade, Einsle, Schmitz unpublished).

Localization of PII-proteins.

In *E. coli* and *C. glutamicum* a change in localization of GlnK was observed under different nitrogen availabilities (Coutts et al. 2002; Javelle et al. 2004; Strösser et al. 2004). Under nitrogen limitation the main part of the protein is located in the cytoplasm, whereas upon a change to nitrogen sufficiency GlnK is sequestered to the membrane apparently in

an AmtB-dependent manner. Binding of GlnK to the ammonium transporter AmtB is favoured to be the regulatory mechanism for inactivating the ammonium transporter and titrating GlnK out of the cytoplasm under ammonium-upshift conditions. In contrast, in *M. mazei* we did not observe any changes in the localization of $GlnK_1$ under changing nitrogen availabilities. Both under N-limitation and after an ammonium-upshift approximately 20 % of $GlnK_1$ was found to be unspecifically associated to the membrane; indicating no dependency of its localization on nitrogen supplementation (Ehlers and Schmitz, unpublished). Therefore it seems unlikely that in *M. mazei* $GlnK_1$ fulfils a similar function in regulating the ammonium transporter as proposed for *E. coli* and *C. glutamicum*. Furthermore, we can exclude a regulatory mechanism that depends on different localizations of $GlnK_1$ in response to different nitrogen availabilities.

Phenotypic characterization of a *M. mazei glnK₁*-mutant strain.

In order to assign a function to *M. mazei* $GlnK_1$, a chromosomal deletion mutant was constructed using the optimized liposome-mediated transformation protocol specifically developed for *M. mazei* strain Gö1 (Ehlers et al. 2004a). By characterizing this *glnK₁*-mutant no growth phenotype was obtained under nitrogen excess, which was expected as the *glnK₁*-gene is not transcribed under those conditions. Under nitrogen limitation, however, a partial reduced growth rate was observed in the mutant strain compared to the wild type (Ehlers et al. 2004a). This is an indication that $GlnK_1$ is not essential for nitrogen fixation in *M. mazei*, though it is apparently required for maximal growth under nitrogen-limited conditions. A regulatory function of $GlnK_1$ in transcriptional activation or repression of genes involved in nitrogen metabolism and nitrogen fixation was further excluded by reverse transcription (RT) PCR analysis of the mutant versus wild type investigating selected genes of the nitrogen regulon (Ehlers et al. 2004a). For those genes no effect of $GlnK_1$ on transcription was detectable under nitrogen limitation. However, this finding has to be confirmed in a genome-wide scale using the microarray chip technology.

**Identification of *M. mazei* GlnK₁ interaction partners.**

PII-like proteins are known to interact with several different receptor proteins and thereby modulate the activity of enzymes involved in nitrogen metabolism. The *E. coli* GlnB protein e.g. interacts with the sensor kinase NtrB of the general nitrogen two-component system NtrB/C and regulates thereby transcription of nitrogen regulated genes (Jiang et al.

1998b). Furthermore GlnB affects the adenylyltransferase, which regulates the glutamine synthetase activity by covalently modifying the enzyme under high nitrogen availabilities (Jiang et al. 1998c; Reitzer 2003). By pull-down experiments the first interaction partner of an archaeal PII-like protein was identified during this thesis. The *M. mazei* $GlnK_1$ protein forms stable complexes with the glutamine synthetase $GlnA_1$ (Ehlers et al. 2004b). This complex formation is inhibited by the presence of 2-oxoglutarate as revealed by gel filtration experiments. We further obtained conclusive evidence that 2-oxoglutarate (i) induces disintegration of $GlnA_1$-$GlnK_1$ complexes and (ii) stabilizes oligomerization of $GlnA_1$ to higher oligomeric structures, apparently dodecamers. The 2-oxoglutarate effect is also observed in assays determining the glutamine synthetase (GS) activity of $GlnA_1$. Here, the specific activity of purified $GlnA_1$ is increased up to 16-fold in the presence of 2-oxoglutarate demonstrating that $GlnA_1$ is apparently active in its dodecameric form (Ehlers et al. 2004b). Preincubation of $GlnA_1$ together with $GlnK_1$ results in a decrease in enzyme activity up to 70 % indicating that complex formation between these two proteins apparently inhibits $GlnA_1$ activity. These effects were confirmed in *M. mazei* cell extracts by adding purified $GlnK_1$ and 2-oxoglutarate. Based on these findings, it is attractive to speculate that 2-oxoglutarate acts as the signal of a low nitrogen status in *M. mazei*. This differs significantly from signal sensing in proteobacteria, where a high glutamate/glutamine ratio serves as signal of nitrogen limitation (Ikeda et al. 1996; Yakunin and Hallenbeck 1998; Schmitz 2000). However, one example for 2-oxoglutarate concentration reflecting the internal nitrogen status is found in the cyanobacterium *Synechocystis* sp. PCC6803 (Muro-Pastor et al. 2001). It is postulated that due to the lack of the 2-oxoglutarate dehydrogenase within the TCA cycle under conditions of nitrogen limitation 2-oxoglutarate is accumulated being the signal for a low internal nitrogen status in *Synechocystis*. Genome analysis revealed that in *M. mazei* the TCA cycle also is incomplete and lacks the 2-oxoglutarate dehydrogenase, which further supports our hypothesise that in *M. mazei* 2-oxoglutarate is the internal signal for nitrogen limited conditions.

Current working model

Based on our findings, we postulate the following working model for the regulation of GlnA$_1$ activity by 2-oxoglutarate and GlnK$_1$ (see also Fig. 1): *M. mazei* perceives external nitrogen limitation by sensing the internal 2-oxoglutarate pool, which increases as the 2-oxoglutarate consuming glutamate dehydrogenase is repressed under nitrogen limitation. Under those conditions, 2-oxoglutarate induces a conformational change by directly binding to GlnA$_1$, which enhances GlnA$_1$ activity and prevents inhibitory complex formation with GlnK$_1$. After a shift to nitrogen sufficiency, however, the internal 2-oxoglutarate level decreases resulting in reduction of glutamine synthetase activity and
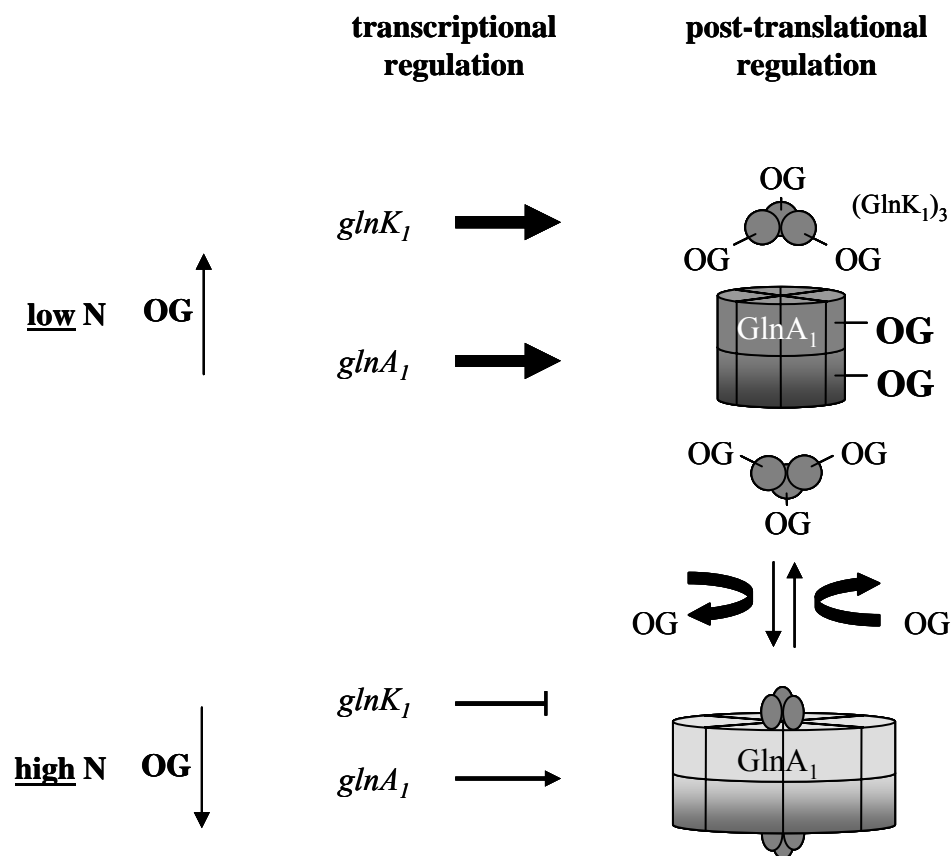
Fig. 1. Hypothetical model for modulation of glutamine synthetase activity in *M. mazei* strain Gö1 in response to different nitrogen availabilities by 2-oxoglutarate and GlnK$_1$.

inhibition by direct interaction with $GlnK_1$. Due to the dodecameric structure of $GlnA_1$ and the trimeric structure of $GlnK_1$ it is tempting to speculate that one $GlnK_1$-trimer interacts with one hexameric-ring of $GlnA_1$. When cells grow under nitrogen excess conditions for prolonged periods of time, $GlnK_1$ levels strongly decrease due to repressed transcription and $GlnA_1$ appears solely be regulated by 2-oxoglutarate. Thus, the regulatory network in *M. mazei* allows for an efficient glutamine synthetase down-regulation in cells that previously accumulated $GlnK_1$ due to ammonium limited conditions. At the current experimental status we do not know, whether $GlnK_1$ is additionally controlled by the 2-oxoglutarate levels.

This model of regulating GS activity of $GlnA_1$ in *M. mazei* by protein-protein-interactions (see Fig. 1) differs significantly from the mechanism well known for enteric bacteria, where dependent on the nitrogen availability GS activity is modulated by increasing and decreasing the adenylylation state of the enzyme at a conserved Tyr residue (Tyr397) (Reitzer 2003). As $GlnA_1$ of *M. mazei* does not show a potential adenylylation site and no genes encoding for proteins with similarities to an adenylyltransferase were identified in the genome, this kind of regulatory mechanism was not expected for *M. mazei.* Furthermore, no enzymes responsible for ADP-ribosylation in the switch-off process of GS as described for *Rhodospirillum rubrum* (Woehle et al. 1990) were identified in the *M. mazei* genome, excluding this kind of regulation, too. However, the regulation of a GlnA protein by protein-protein-interaction has been recently described for *Synechocystis* sp. PC6803 (Garcia-Dominguez et al. 1999; Garcia-Dominguez et al. 2000). Here, at increasing ammonium concentrations the GS is not modified by adenylylation but two small inactivating factors (IF3 and IF7) bind and thereby inhibit the GS activity (Garcia-Dominguez et al. 1999). Homologous proteins of these IF encoding genes *gifA* and *gifB* are not present in *M. mazei*. However, *gifA* and *gifB* are only transcribed under nitrogen limitation (Garcia-Dominguez et al. 2000) as it is the case for the *glnK₁* gene in *M. mazei* (Ehlers et al. 2002a). Genome sequence analysis of *M. mazei* revealed that 18 % of the genes are highly similar to cyanobacterial genes, apparently acquired by horizontal gene transfer. Therefore it is attractive to speculate that some aspects of nitrogen regulation e.g. sensing the internal 2-oxoglutarate pool might be closer related to the cyanobacterial metabolism than one would expect for archaea.

Further studies

- In our current working model we postulate that 2-oxoglutarate binds to $GlnA_1$. However, we cannot exclude that the effector molecule not only binds to the glutamine synthetase. Therefore it is necessary to perform binding assays to determine the affinity of 2-oxoglutarate to $GlnA_1$ and $GlnK_1$. This can be done (i) by isothermal titration or (ii) by Plasmon Resonance Spectroscopy using a BiaCore machine, which allows further investigation of the 2-oxoglutarate effect on $GlnA_1$/$GlnK_1$ complex formation. To get a deeper insight into the function of 2-oxoglutarate as signal for nitrogen limitation the amino acid pools of *M. mazei* have to be determined under different nitrogen availabilties.

- In bacteria it is known that the glutamine synthetase is not only regulated by post-translational modification, but also on transcriptional level by activating different transcriptional start points under different nitrogen availabilities. It is interesting to analyze whether this is also the case for *M. mazei*; furthermore this would help to get a better understanding of the archaeal transcription machinery.

- Besides the *glnA₁* gene a second gene encoding for a potential glutamine synthetase (*glnA₂*) was identified in the *M. mazei* genome. Interestingly, homologous *glnA₂* genes are present in several methanogenic archaea, however no function of the corresponding enzymes has been determined today. Analysis of the *M. mazei* $GlnA_2$ protein concerning GS activity as well as a chromosomal *glnA₂* mutant should reveal the function of this protein in *M. mazei*.

- To analyze potential effects of the chromosomal *glnK₁* deletion on both *M. mazei* transcription and translation patterns (i) microarray experiments using RNA derived from wild type and the *glnK₁*-mutant strain and (ii) 2D-gel analysis of *M. mazei* *glnK₁*-mutant and wild type cells extracts can be performed.

- We started to solve the structure of the *M. mazei* $GlnK_1$ protein using crystallization approaches, which will be continued. By comparing the structures of the archaeal GlnK protein with bacterial GlnB and GlnK proteins it may be possible to explain functional differences of the proteins and to predict functions of further PII-like proteins.

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Curriculum vitae

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