Nitrate reduction and the nitrogen cycle in archaea

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The nitrogen cycle (N-cycle) in the biosphere, mainly driven by prokaryotes, involves different reductive or oxidative reactions used either for assimilatory purposes or in respiratory processes for energy conservation. As the N-cycle has important agricultural and environmental implications, bacterial nitrogen metabolism has become a major research topic in recent years. Archaea are able to perform different reductive pathways of the N-cycle, including both assimilatory processes, such as nitrate assimilation and N2 fixation, and dissimilatory reactions, such as nitrate respiration and denitrification. However, nitrogen metabolism is much less known in archaea than in bacteria. The availability of the complete genome sequences of several members of the eury- and crenarchaeota has enabled new approaches to the understanding of archaeal physiology and biochemistry, including metabolic reactions involving nitrogen compounds. Comparative studies reveal that significant differences exist in the structure and regulation of some enzymes involved in nitrogen metabolism in archaea, giving rise to important conclusions and new perspectives regarding the evolution, function and physiological relevance of the different N-cycle processes. This review discusses the advances that have been made in understanding nitrate reduction and other aspects of the inorganic nitrogen metabolism in archaea.

Introduction

As nitrogen is an essential element for living organisms, the availability of a suitable nitrogen source often limits primary productivity in both natural environments and agriculture. Nitrogen can be found in several oxidation states, from −3 in the most oxidized compound (nitrate) to +5 in the most reduced form (ammonium), but in biological compounds it is almost exclusively present in the fully reduced state. The global biogeochemical nitrogen cycle (N-cycle) allows the interconversions of nitrogen compounds to maintain a relatively small amount of fixed or combined nitrogen in a continuous exchange with atmospheric dinitrogen (N2), a huge nitrogen reservoir. The N-cycle includes both reductive and oxidative processes, in which prokaryotes play a predominant role (Fig. 1). Assimilatory pathways, such as N2 fixation and nitrate assimilation, generate ammonium that is further incorporated into cell material, mainly by the glutamine synthetase–glutamate synthase (GS–GOGAT) route. However, whereas N2 fixation is only driven by free-living or symbiotic diazotrophic prokaryotes, assimilatory nitrate reduction is carried out by bacteria, fungi, algae and higher plants, and almost all living organisms are able to incorporate ammonium into carbon skeletons. On the other hand, some prokaryotes can obtain metabolic energy by redox processes involving nitrogen compounds, such as nitrification and denitrification. Autotrophic nitrification is a two-step process consisting of the oxidative conversion of ammonia to nitrite via hydroxylamine, carried out by ammonia-oxidizing bacteria, and the further oxidation of nitrite to nitrate, performed by nitrite-oxidizing chemolithoautotrophic bacteria. Denitrification is a respiratory process whereby nitrate is successively reduced to nitrite, NO, N2O and N2, which takes place predominantly under anaerobic conditions in many facultative bacteria (Berks et al., 1995; Zumft, 1997; Moreno-Vivián & Ferguson, 1998; Moreno-Vivián et al., 1999; Richardson & Watmough, 1999). Some heterotrophic bacteria are able to combine nitrification and aerobic denitrification by oxidizing ammonia to nitrite, which is later reduced to N2 (de Boer & Kowalchuk, 2001). Ammonification is the dissimilatory reduction of nitrate to ammonia that does not serve the purpose of nitrogen autotrophy, although this term normally describes the release of ammonia from organic nitrogen compounds (Zumft, 1997). Finally, anaerobic ammonium oxidation (anammox) is a reaction that produces N2 by reducing nitrite and oxidizing ammonium. This process, performed by bacterial communities of planctomycetes, seems to be of ecological importance in marine environments (Dalsgaard et al., 2003; Kuypers et al., 2003).
Assimilatory and respiratory nitrate reduction in archaea

Many archaea are able to reduce nitrate by assimilatory or respiratory pathways, dissimilatory nitrate reduction being much more frequent than nitrate assimilation (Martínez-Espinosa et al., 2001b). In addition, genes encoding putative nitrate transporters, nitrate reductases and nitrite reductases have been found in the sequenced genomes of members of both crenarchaeota and euryarchaeota (Table 1). However, in most cases, either functional or sequence data, but not both, are available. Therefore, future research should be directed to characterizing at the physiological and biochemical levels the products of the genes identified by these genome sequencing projects and their regulation. This will be of special evolutionary interest because significant differences between the bacterial and archaeal nitrate-reducing systems are emerging.

Phylogenetic relationships of the nitrate reductases

Three different nitrate-reducing systems (Nas, Nar and Nap) have been described in bacteria (Berks et al., 1995; Moreno-Vivián et al., 1999; Richardson, 2000; Richardson et al., 2001). Assimilatory nitrate reductases (Nas) are usually cytoplasmic enzymes which are repressed by ammonium and use either NADH or ferredoxin as physiological electron donor. The NADH-Nas enzymes of Klebsiella and Rhodobacter capsulatus are dimers composed of a small FAD-containing diaphorase and a large catalytic subunit with one Fe–S centre and a molybdo-bis-molybdopterin guanine dinucleotide (Mo–bisMGD) cofactor, whereas cyanobacterial ferredoxin-dependent enzymes are monomers (Moreno-Vivián et al., 1999; Richardson et al., 2001). Membrane-bound nitrate reductases (Nar) are involved in anaerobic nitrate respiration and denitrification, being negatively regulated by O₂ and unaffected by ammonium. Nar enzymes are composed of a catalytic subunit with MGD cofactor (NarG) and an electron-transfer subunit with four iron–sulfur centres (NarH). This complex is bound to a membrane bihaem b quinol-oxidizing component (NarL). Recently, the crystal structure of Nar has been determined at high resolution, revealing that NarG protein has an aspartate residue acting as the oxidizing component (NarI). Recently, the crystal structure of Nar has been determined at high resolution, revealing that NarG protein has an aspartate residue acting as the

Fig. 1. The biological N-cycle. The different nitrogen compounds are arranged according to their oxidation states and the main reductive or oxidative pathways are indicated by arrows. Conversion of hydroxylamine to nitrous oxide by heterotrophic nitrification, and production of N₂ from ammonium and nitrite by anaerobic ammonium oxidation (anammox), are not indicated. The enzymes involved in the different pathways are: 1–2, glutamate synthetase–glutamate synthase (GS–GOGAT); 3, glutamate dehydrogenase (GDH); 4, nitrogenase; 5, assimilatory nitrite reductase (Nas); 6, assimilatory nitrite reductase (sirhaem-Nir); 7, dissimilatory and respiratory nitrite reductases (Nap and Nar); 8, respiratory nitrite reductases (Cu-Nir and cd-1-Nir); 9, nitric oxide reductase (Nor); 10, nitrous oxide reductase (Nos); 11, ammonia monooxygenase; 12, hydroxylamine oxidase; 13, nitrite oxidase.
Table 1. Presence of putative nitrate transporters and enzymes involved in nitrate reduction and denitrification in archaea

Evidence for the presence of the different proteins is derived from gene bank sequences and genome sequence analysis (genetic) conducted on websites http://gib.genes.nig.ac.jp and http://www.genome.ad.jp, from physiological and biochemical studies (functional) or both (genetic and functional). The accession numbers of the corresponding genes are indicated. ND indicates that the protein or enzyme has not been detected by genetic or functional analysis. ? indicates that no clear assignation is deduced only on the basis of sequence similarities. For details and references, see the text.

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<th>Nitrate transporter</th>
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Mo-ligand for the Mo-bisMGD cofactor, and also includes an unusual iron–sulfur cluster, previously undetected (Bertero et al., 2003; Jormakka et al., 2004). Finally, the periplasmic Nap systems of many Gram-negative bacteria participate in different processes, like redox balancing, scavenging nitrate in nitrate-limited environments, and aerobic or anaerobic denitrification (Potter et al., 2001; Gavira et al., 2002; Ellington et al., 2003). Usually, Nap enzymes are heterodimers with a catalytic subunit (NapA) and a cytochrome c (NapB), which receives electrons from NapC, a membrane cytochrome c (Berks et al., 1995; Reyes et al., 1996, 1998). However, the enzyme of Desulfovibrio desulfuricans is a single NapA subunit, for which the crystal structure has been solved (Dias et al., 1999).

Preliminary sequence comparisons of Nar, Nas and Nap indicated that these enzymes represent different phylogenetic groups (Reyes et al., 1996). A recent study of the sequences of eukaryotic and prokaryotic nitrate reductases revealed the existence of three different clades, corresponding to the eukaryotic nitrate reductase, the prokaryotic respiratory Nar, and the prokaryotic Nas/Nap enzymes, with the Nap from Desulfovibrio desulfuricans providing the evolutionary link between Nas and Nap subclades (Stolz & Basu, 2002). It is worth noting from this study that the deepest branch of the Nar clade is the euryarchaeote Haloarcula marismortui, and that the Nar enzymes of the thermophilic bacterium Thermus thermophilus and the crenarchaeote Aeropyrum pernix are closely related. Phylogenetic trees based on NarH sequences also correlate well with the 16S rDNA tree, making the archaea Aeropyrum pernix and Pyrobaculum aerophylum the outgroup and closely joining these archaeal sequences to the T. thermophilus bacterial subtree (Petri & Imhoff, 2000; Philippot, 2002). However, the NarG-based phylogeny is not entirely consistent with the 16S rDNA-based taxonomy (Che`neby et al., 2003; Gregory et al., 2003). As the nar genes were present before the phylogenetic divergence of bacteria and archaea, it can be assumed that respiratory nitrate reductase played a key role in energy metabolism during pre-oxic times (Petri & Imhoff, 2000). In addition, there is evidence that Nap and Nar have probably been acquired in some bacteria by horizontal gene transfer (Stolz & Basu, 2002). This seems to be the case for the nar gene cluster of T. thermophilus (Ramirez-Arcos et al., 1998b) and for the plasmid-encoded nap genes of Rhodobacter sphaeroides (Castillo et al., 1996). Results of NarG-based trees are also consistent with the possibility of lateral gene transfer (Gregory et al., 2003). Evidence of recent lateral gene transfer among hyperthermophilic archaea has been published (DiRuggiero et al., 2000), but the relative importance of this mechanism of genetic exchange among archaea, and also from and to bacteria, should be determined in future studies.

Table 1. cont.

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<thead>
<tr>
<th>Archaea</th>
<th>Nitrate transporter</th>
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Nitrate reductase cofactors

Genome sequences of several archaea contain MGD cofactor biosynthesis genes, and archaeal nitrate reductases seem to be molybdoenzymes. However, tungsten and molybdenum have similar chemistry and it is known that hyperthermophilic archaea possess W-containing enzymes, such as aldehyde ferredoxin oxidoreductase, with the basic structure of the pterin cofactor shared in the Mo- and W-enzymes (Hille, 2002). Recently, Mo-free nitrate reductases with vanadium and haem c as cofactors have been isolated from vanadate-reducing, iron-reducing and sulfur-oxidizing bacteria (Antipov et al., 1998, 2003; Murillo et al., 1999). In addition, active W-substituted molybdoenzymes have been described in mesophilic bacteria (Buc et al., 1999; Gates et al., 2003). Thus, tungstate can function at the active site of the Paracoccus pantotrophus NapA, although the enzyme shows lower affinity for nitrate (Gates et al., 2003). Although nitrate reductases and other Mo-enzymes are usually inactivated by tungstate, this inhibition is not relevant in vivo since bacteria exhibits high-affinity Mo-uptake systems for extracting traces (micromolar or nanomolar) of Mo from the media, and because Mo is usually present in natural environments at higher concentrations than W. However, Mo(IV) forms a highly insoluble sulfide (MoS₂), while V (as VO²⁺ or V³⁺) and W (as WO₄²⁻ or WS₄²⁻) form relatively soluble salts, and therefore there are chemical reasons to believe that both W and V were more available than Mo in the primitive strongly reducing sulfide-rich environments (Williams & Fraústo da Silva, 2002). In addition, some extreme environments such as hot springs and deep-sea hydrothermal vents usually contain high levels of tungsten, and in fact an obligate W requirement for the anaerobic growth of the hyperthermophile Pyrobaculum aerophilum has been reported (Afshar et al., 1998). The respiratory nitrate reductase of this archaeon is a Mo-enzyme, but its activity is not abolished at high W concentrations, in contrast to bacterial Nar systems. Moreover, the presence of tungstate in the culture medium stimulates the anaerobic growth with nitrate (Völk et al., 1993). The ability to respire nitrate in these conditions has been interpreted as an adaptation of the archaeon to the W-rich hyperthermophilic environment (Afshar et al., 1998, 2001). Clearly, further biochemical studies will be necessary to determine the possible involvement of alternative metal cofactors in archaeal nitrate reduction, but the possibility that V or W, rather than Mo, may be included in some archaeal nitrate reductase cofactors cannot be excluded.

Nitrate transport systems

Two types of nitrate transporters are involved in bacterial assimilatory nitrate reduction: the ATP-dependent ABC transporters composed of an integral membrane subunit, a cytoplasmic ATP-binding component and a periplasmic substrate-binding protein; and the monomeric NarK-type transporters belonging to the major facilitator superfamily, which depend on proton-motive force. However, only transport systems of the NarK type are involved in bacterial respiratory nitrate reduction (Moir & Wood, 2001). Within the bacterial NarK-like transporters there are two distinct subgroups. Recent studies in the bacterium Paracoccus pantotrophus suggest that NarK1 is a proton : nitrate symporter that allows initiation of nitrate respiration, whereas NarK2 is a nitrate : nitrite antiporter required for maintenance of a steady-state rate (Wood et al., 2002). Genome sequencing projects reveal that ABC-type transporters are widespread in archaea (Table 1). In particular, putative ABC-type nitrate transporters are present in the crenarchaeote Pyrobaculum aerophilum (Fitz-Gibbon et al., 2002) and the euryarchaeota Archaeoglobus fulgidus (Klenk et al., 1997), Methanocaldococcus jannaschii (Bult et al., 1996), Methanopyrus kandleri (Slesarev et al., 2002), Thermoplasma acidophilum (Ruepp et al., 2000) and Thermoplasma volcanium (Kawashima et al., 2000). However, substrate specificity for these putative transporters has been assigned solely on the basis of sequence similarities. A functional characterization of archaeal nitrate transporter genes has been only performed in Haloferax volcanii, where three ABC-type transporters are essential for nitrate respiration (Wanner & Soppa, 1999). The type of transport system used for nitrate uptake has a serious impact on the bioenergetics of the process because an ATP-driven nitrate uptake system is equivalent to consuming about three protons and this energetic cost makes its involvement in dissipatory processes unlikely (Moir & Wood, 2001). For this reason, as mentioned above, bacterial nitrate assimilation usually requires ATP-dependent ABC nitrate transporters whereas nitrate respiration is associated with proton-motive-force-driven NarK transporters (Moreno-Vivian et al., 1999, 2001), in even the extreme thermophilic bacterium Thermus thermophilus (Ramirez et al., 2000). It is worth noting that most archaea with putative ABC nitrate transporters seem to contain respiratory nitrate reductases rather than assimilatory Nas enzymes. However, as discussed below, nitrate uptake may not be required for respiratory nitrate reduction in archaea, in contrast to bacteria, and the putative ATP-dependent nitrate transporters may indeed be involved in nitrate assimilation.

Assimilatory nitrate reduction in archaea

Most nitrate-reducing archaea use nitrate as alternative electron acceptor in anaerobic respiration, but recently, assimilatory nitrate and nitrite reductases have been purified from Haloferax mediterranei (Martinez-Espinosa et al., 2001a, b). This extreme halophilic archaean grows aerobically with nitrate as the sole nitrogen source, but it can also denitrify because it contains both Nas and Nar enzymes. Assimilatory nitrate reductase is a dimer of 105 and 50 kDa subunits and uses ferredoxin as reductant, but not NAD(P)H (Martinez-Espinosa et al., 2001b). This could indicate a different structure for archaeal Nas because bacterial ferredoxin-Nas enzymes are usually monomeric, whereas NADH-dependent Nas enzymes are heterodimers (Moreno-Vivian et al., 1999). The assimilatory enzyme of H. mediterranei has a Km for nitrate of 0.95 mM and an
optimum temperature of 80 °C at 3·1 M NaCl, but 60 °C at 1·3 M NaCl. Activity is induced by nitrate and repressed by ammonium, as described for the bacterial Nas (Martinez-Espinoza et al., 2001b). The assimilatory nitrite reductase of H. mediterranei has also been purified. It is a 66 kDa monomer which uses ferredoxin as electron donor, has a $K_m$ for nitrite of 8·6 mM, and shows maximal activity at 60 °C with 3·3 M NaCl. Like the bacterial assimilatory nitrite reductases, the H. mediterranei enzyme contains sirohaem and Fe–S centres (Martinez-Espinoza et al., 2001a). The 21 kDa ferredoxin from this halophilic archaeon has been purified and characterized, and its physiological role as the electron donor for both assimilatory nitrate and nitrite reductases has been confirmed (Martinez-Espinoza et al., 2003).

Analysis of the published archaeal genome sequences suggests the presence of putative assimilatory nitrate/nitrite reductases (Table 1). Thus Methanobacterium thermautotrophicus has a putative nitrate reductase (MTH1567 gene product) homologous to bacterial Nas (Smith et al., 1997). Thermoplasma strains contain nitrate reductase related proteins (T. acidophilum Ta0136 and T. volcanicum VN0215 gene products), which are similar to each other and to the eukaryotic assimilatory NADPH-nitrate reductases (Kawashima et al., 2000; Ruepp et al., 2000), although these putative proteins are too small (about 200 residues) compared to the nia-encoded eukaryotic nitrate reductases. The eukaryotic nitrate reductases are quite distinct from any of the bacterial Nas, Nar or Nap enzymes. They share no sequence similarity and do not bind the Mo-bieMGD cofactor, but instead have the so-called molybdopterin cofactor (Mo-co), in addition to FAD and cytochrome $b_{557}$ (Moreno-Vivian et al., 1999; Campbell, 2001). Therefore, the assignation of a role in nitrate reduction to the Thermoplasma small nitrate-reductase-related proteins will be of special evolutionary interest and should be explored in future studies.

The Pyrobaculum aerophilum genome contains a gene ($nirA$) encoding a putative assimilatory ferredoxin-dependent sirohaem nitrite reductase (PAE2577), in addition to putative genes encoding a respiratory cytochrome $cd_{1}$-nitrite reductase (Fitz-Gibbon et al., 2002). Putative nirA homologous genes are also present in the genomes of Pyrococcus furiosus, Pyrococcus horikoshii (PF1197 and PH0827, respectively; Maeder et al., 1999), Aeropyrum pernix (APE1326; Kawarabayasi et al., 1999), Archaeoglobus fulgidus (AF0164; Klenk et al., 1997), Methanocaldococcus jannaschii (MJ0551; Bult et al., 1996), and Methanopyrus kandleri (MK0801; Slesarev et al., 2002). However, biochemical and functional studies are necessary to confirm the assimilatory nature of these putative nitrate and nitrite reductases and to establish if these archaeal enzymes are structurally different from the bacterial assimilatory enzymes.

**Respiratory nitrate reduction in archaea**

The ability to use nitrate as a terminal electron acceptor in energy metabolism is found in a variety of halophilic and hyperthermophilic archaea; many of them can perform the entire denitrification process (see below). Respiratory Nar enzymes have been purified from several denitrifying halophilic euryarchaeota, including three Haloferax species and Haloarcula marismortui (Table 1). A salt-requiring Nar has been purified from Haloferax mediterranei, with an activity maximum at 89 °C in 3·2 M NaCl and $K_m$ for nitrate values in the range from 2·5 to 6·7 mM depending on salt concentration (Alvarez-Ossorio et al., 1992). The membrane-bound Nar of Haloferax denitrificans is composed of two subunits of 116 and 60 kDa, with a $K_m$ for nitrate of 0·2 mM. Curiously, the enzyme is stable in the absence of salt, and activity decreases with increasing NaCl concentrations (Hochstein & Lang, 1991). Haloferax volcanii contains a membrane-bound trimeric Nar, with 100, 61 and 31 kDa subunits. Activity is salt-independent, with a temperature optimum of 80 °C and a $K_m$ for nitrate of 0·36 mM (Bickel-Sandkötter & Ufer, 1995). The Haloarcula marismortui Nar has a $K_m$ for nitrate of 80 mM with 2·0 M NaCl, and activity is enhanced by salt. The enzyme was first described as a homotetramer of a 63 kDa polypeptide (Yoshimatsu et al., 2000), but further characterization revealed that it is a heterodimer with 117 and 47 kDa subunits, the 63 kDa band being an incomplete denaturation state of the enzyme (Yoshimatsu et al., 2002). It has been proposed that the H. marismortui Nar is a new archaeal type of membrane-bound nitrate reductase (Yoshimatsu et al., 2002). First, the enzyme is composed of two subunits, which are homologous to the NarGH bacterial subunits, but lacks the NarI membrane-associated protein. Second, the H. marismortui NarGH complex has significant sequence and structure similarity to the dissimilatory selenate reductase from Thauera selenatis, although it does not reduce selenate. Recently, the similarity of Nar to selenate reductases has also been indicated on the basis that both enzymes have Asp ligands to the Mo atom (Jormakka et al., 2004). In contrast, the N-terminal region of NarG includes a typical twin-arginine signal peptide for protein translocation across the membrane by the Tat export pathway (Berks et al., 2000) and the enzyme has activity in situ with both membrane-permeable benzyl viologen and membrane-impermeable methyl viologen, suggesting that the catalytic site is located in the outside of the membrane, as described for the T. selenatis selenate reductase (Yoshimatsu et al., 2002). In contrast, bacterial NarG faces the cytoplasmic side of the membrane and nitrate reduction takes places in the cytoplasm (Berks et al., 1995; Moreno-Vivian et al., 1999). Finally, although the H. marismortui NarGH complex does not contain any quinol-oxidizing cytochrome $b$ subunit, a gene encoding a homologue to SerC, the cytochrome $b$ subunit of selenate reductase, is found downstream of the narGH genes and together with a $naf$-homologous gene, which probably encodes a nitrate-reductase-specific chaperone for MGD cofactor assembly.

A gene cluster encoding a putative respiratory Nar has also been identified in Archaeoglobus fulgidus (Klenk et al., 1997; Table 1). The AF0176 gene encodes a protein with 42 %
similarity to *Escherichia coli* NarG, although this *A. fulgidus* NarG protein is smaller (80 kDa) and contains a twin-arginine signal peptide, suggesting that it is translocated across the membrane like the *H. marismortui* NarG protein is smaller (80 kDa) and contains a twin-arginine signal peptide, suggesting that it is translocated across the membrane like the *H. marismortui* and probably other archaeal NarG proteins. The AF0175 product is predicted to bind four Fe–S clusters, whereas the AF0174 gene encodes an integral membrane protein, although no haem groups are bound to this protein. Finally, a NarJ homologue is also found in the gene cluster (Klenk et al., 1997; Richardson et al., 2001). In addition, genes encoding an ABC-type nitrate transporter (AF0638–AF0640), a putative nirA gene encoding the cytoplasmic sirohaem nitrite reductase (AF0164) and other putative iron–sulfur proteins clustered to narI-like genes (AF0499–AF0501 and AF0543–AF0547) are also present in the *A. fulgidus* genome. Some hyperthermophilic archaea are also able to respire nitrate. Thus, *Aeropyrum pernix* has a putative *nar* cluster (Kawarabayasi et al., 1999) and *Pyrobaculum aerophilum* uses nitrate as respiratory electron acceptor (Völkl et al., 1993). The purified Nar of this archaeon shows very high specific activity, with a *K*<sub>m</sub> for nitrate of 58 μM and an optimal temperature of 95 °C, and is active in the presence of high tungstate concentrations. Interestingly, the *P. aerophilum* Nar is a heterotrimer, with subunits of 130, 52 and 32 kDa, and contains a b-type cytochrome similar to the *Thauera selenatis* SerC, in addition to molybdenum cofactor and iron–sulfur cluster (Afshar et al., 2001).

Bacterial and archaeal Nar systems also differ in the organization of the *nar* genes (Philippot, 2002). The main *narGHJI* operon organization is conserved in all bacteria, including *Thermus thermophilus* (Ramirez-Arcos et al., 1998a), although this organism has an extra *narC* gene in the operon, and *Paracoccus pantotrophus* includes the *narK* gene in the *narKGHIJ* cluster (Wood et al., 2001). However, archaeal *nar* genes do not conserve this organization. Thus, in *Pyrobaculum aerophilum* a putative *narJ* gene is located upstream from the *narGH* genes, in the opposite direction. A gene encoding a cytochrome b is present downstream from *narH*, although its role as Nar subunit is conjectural, and another putative *narJ* homologue is located elsewhere in the genome (Fitz-Gibbon et al., 2002). In *Halobacterium marismortui*, two small ORFs and a *narJ* homologue are located downstream from the *narGH* genes, and putative genes encoding an iron–sulfur protein and a cytochrome *b* are located upstream from *narGH* (Yoshimatsu et al., 2001). In *Aeropyrum pernix*, no *narJ*–like gene has been found and *narGH* genes are followed by a putative cytochrome *b* gene and *narJ*, which is located about 2 kb downstream from *narH* (Kawarabayasi et al., 1999). Therefore, based on subunit composition, subcellular location of the active site and *nar* gene organization, it can be concluded that archaeal Nars are a new type of enzyme with the active site facing the outside and anchored to the membrane by a SerC-like cytochrome *b*, or stabilized by the lipid environment in the membrane as described for the *P. aerophilum* Nar (Afshar et al., 2001). This system could be an ancient respiratory nitrate reductase, although the nitrite formed could also be assimilated (Richardson et al., 2001).

![Fig. 2. Schematic representation of the denitrification pathway in bacteria. The periplasmic nitrate reductase (NAP), the membrane-bound nitrate reductase (NAR), the periplasmic nitrite reductases (Cu-NIR or cd<sub>1</sub>-NIR), the membrane-bound nitric oxide reductase (NOR) and the periplasmic nitrous oxide reductase (NOS) are represented without indicating their subunit composition and cofactors. Note that not all represented components are present in a single organism (i.e. NAP and NAR, Cu-NIR and cd<sub>1</sub>-NIR). cyt bc<sub>1</sub>, proton-pumping cytochrome bc<sub>1</sub> complex; cyt c, NapC membrane-bound tetrahaem cytochrome c; cyt c<sub>550</sub>, cytochrome c<sub>550</sub>; MQH<sub>2</sub>/MQ, menaquinol/maenaquinone pool.](http://mic.sgmjournals.org)
and 3 schematize the different organization of the bacterial and archaeal respiratory nitrate-reducing systems, and the other enzymes involved in the whole denitrification pathway (see below).

The possible outside location of the catalytic site of NarG in the archaeal respiratory nitrate reductase has important bioenergetic implications. First, bacterial nitrate respiration involves the generation of a proton-motive force by a redox-loop mechanism, with two protons consumed in the nitrate reduction reaction in the cytoplasm and two protons released from quinol oxidation on the periplasmic side of the membrane (Berks et al., 1995; Jormakka et al., 2003), although periplasmic nitrate reduction by the Nap enzyme and other reductases involved in the denitrification pathway are coupled to proton-pumping protein complexes (Fig. 2). However, the possible outside location of the nitrate reduction site in archaeal Nar would imply the coupling of this process to a proton-translocating complex, although the identity of this complex is presently unknown (Fig. 3). The genome sequence of Pyrobaculum aerophilum reveals that this organism apparently lacks a cytochrome bc₁ complex (Fitz-Gibbon et al., 2002), but the occurrence of membrane-bound bc complexes and different proton-pumping NADH dehydrogenases and hydrogenases has been described in archaea (Castresana & Moreira, 1999; Deppenmeier et al., 1999; Schütz et al., 2000; Sapra et al., 2003). In addition, an active nitrate-uptake system would not be required for respiratory nitrate reduction in archaea, thus increasing the energetic yield of the nitrate reduction process. This could also explain the apparent absence of NirK-type nitrate transporters in archaea and suggests that, as indicated above, the putative ATP-dependent ABC-type nitrate transporters found in several archaeal species would be probably involved in nitrate assimilation rather than in nitrate respiration.

Denitrification in archaea

Denitrification can be considered as the modular assembly of four partly independent respiratory processes: nitrate, nitrite, nitric oxide and nitrous oxide reduction (Zumft, 1997; Figs 2 and 3). All denitrifiers isolated so far are also able to respires oxygen, indicating a close evolutionary relationship between the two processes, and denitrification has been considered the ancestor of aerobic respiration (Saraste & Castresana, 1994). Therefore, knowledge of denitrification in archaea is of special interest from an evolutionary point of view. Denitrification has been described for several halophilic archaea, such as Haloferax and Haloarcula strains (Tomlinson et al., 1986; Inatomi & Hochstein, 1996; Ichiki et al., 2001), and extreme thermophilic archaea, such as Ferroglobus placidus (Vorholt et al., 1997) and Pyrobaculum aerophilum (Völk et al., 1993; de Vries & Schröder, 2002). As the archaeal respiratory nitrate reductases that catalyse the first step of denitrification have been described above, we will focus now on the nitrite and N-oxide respiration processes in archaea.

Nitrite respiration

In bacteria, there are two different and unrelated NO-producing respiratory nitrite reductases: the homotrimeric copper-containing enzyme encoded by nirK, and the homodimeric cytochrome cd₁-nitrite reductase encoded by nirS. The crystal structures of both enzymes, which are located in the periplasm, are known and have been reviewed in detail elsewhere (Zumft, 1997; Ferguson,
Nitrogen cycle in archaea

1998; Richardson & Watmough, 1999). Azurin and pseudoazurin are the electron donors to Cu-Nir, whereas cytochrome $b_{535}$ is the reductant for $c_{dl}$-Nir (Zumft, 1997).

Denitrifying archaea also contain either Cu-Nir or $c_{dl}$-Nir. Haloarcula marismortui has a nirK gene encoding a Cu-Nir. EPR analysis reveals the presence of type I and type II Cu centres in the purified enzyme, which has optimal activity at 2 M NaCl. A 46 kDa precursor of this protein with an N-terminal signal peptide for translocation across the plasma membrane has been detected by SDS-PAGE (Ichiki et al., 2001). Although the physiological electron donor is unknown, the archaeal halocyanin, a blue Cu-protein present in some archaea (Scharf & Engelhard, 1993), could be the putative reductant. Protein sequence analysis of the H. marismortui nirK gene product reveals a close relationship with the Neisseria gonorrhoeae Cu-Nir, thus suggesting lateral gene transfer of the nirK gene (Ichiki et al., 2001; Philippot, 2002). Interestingly, the Haloferax denitrificans nirK gene sequence is also available (CAD89521, Table 1), and is highly similar to both H. marismortui and N. gonorrhoeae Cu-Nir. However, antibodies against bacterial Cu-Nir does not recognize the H. denitrificans enzyme, suggesting possible structural differences (Inatomi & Hochstein, 1996).

The existence of a nirS homologous gene in the genome of the hyperthermophile Pyrobaculum aerophilum suggests the presence of a $c_{dl}$-nitrite reductase (Table 1). Recently, it has been reported that this archaeon has a membrane-bound respiratory nitrite reductase which uses menaquinol as the physiological electron donor, thus having different subcellular location and reductant than bacterial $c_{dl}$-Nir (Figs 2 and 3). It is worth noting that, in contrast to denitrifying bacteria, all four reductases involved in denitrification in P. aerophilum are membrane-bound enzymes which use menaquinol as electron donor (de Vries & Schröder, 2002). The dependence of the menaquinol pool and the membrane confinement of all these enzymes in archaeal denitrification could be related to the relatively small volume between the S-layer and the cytoplasmic membrane, in comparison with the larger periplasmic space in bacteria, or could reflect the ancient origin of this pathway. The membrane location of all denitrification enzymes in P. aerophilum could also explain the absence in this archaeon of soluble components of the denitrification pathway, such as c-type cytochromes or cupredoxins (Lubben & Morand, 1994; de Vries & Schröder, 2002; see Figs 2 and 3).

N-oxide respiration

Nitric oxide, the product of the respiratory nitrite reductases, is a toxic compound that is reduced to N$_2$O by nitric oxide reductases (Nor) immediately after it has been generated. Several enzymes with Nor activity have been described. In fungal denitrification, NO reduction to N$_2$O is catalysed by a soluble monomeric enzyme of the cytochrome P-450 family (Shoun & Tamimoto, 1991; Nakahara et al., 1993). In most denitrifying bacteria, Nor is a membrane complex of a 17 kDa cytochrome c (encoded by norC) and a 38 kDa cytochrome b with 12 transmembrane regions (encoded by norB). This enzyme, known as cNor, receives electrons from cytochrome c. The denitrifying bacterium Ralstonia eutropha and several non-denitrifying pathogenic bacteria such as Neisseria species possess a monomeric Nor with 14 transmembrane regions, which is called qNor because it is a quinol-oxidizing enzyme. This protein, encoded by norZ, is similar to the norB gene product but contains an N-terminal extension absent in NorB, with a quinone-binding site. In Gram-positive bacteria, a menaquinol Nor with an additional small Cu$_A$-containing subunit has been described as qCu$_A$Nor (Henriks et al., 2000; de Vries & Schröder, 2002; Philippot, 2002).

Genetic and biochemical evidence indicates that the archaeon Pyrobaculum aerophilum has a qNor-type NO reductase (Table 1). Putative nir (nitrite reductase) and nor (nitric oxide reductase) genes are located 3-3 kb upstream from the nar (nitrate reductase) genes (Völkl et al., 1993; Fitz-Gibbon et al., 2002). The purified Nor is a single membrane subunit with two modified haem groups and one non-haem iron, and uses menaquinol as electron donor (de Vries & Schröder, 2002). The genome of Methanosarcina mazei also contains a NO reductase gene (MM2988; Deppenmeier et al., 2002) and Sulfolobus solfataricus includes a putative gene encoding a haem NO reductase, but it is truncated by a transposase separating the N-terminal (SSO1573) and C-terminal (SSO1571) domains (She et al., 2001). Sequenced genomes of other archaea do not include nor homologous genes (Table 1), although the presence of NO reductase has been reported in some denitrifying halophilic archaea that have not yet been sequenced. The presence of qNor in some archaea is of evolutionary interest because qNor could be the ancestor not only of the cNor, by acquiring haem c-binding residues in the N-terminal region and separating this domain as a small cytochrome c subunit, but also of the whole superfamily of haem-copper oxidases and aerobic respiratory enzymes. The capacity for N-oxide respiration appeared early in evolution and qNor may have arisen to detoxify NO; this could still be the role of the enzyme in non-denitrifying bacteria. After the appearance in the atmosphere of oxygen generated photosynthetically, these enzymes could have replaced non-haem iron by copper and evolved to generate the proton-pumping cytochrome oxidases (Saraste, 1994; Saraste & Castresana, 1994; Richardson, 2000; de Vries & Schröder, 2002).

On the other hand, the E. coli flavinb redoxygen has been recently described as a novel type of prokaryotic NO reductase. The purified protein has a turnover number similar to that of canonical bacterial Nor and also has oxygen reductase activity, although it shows lower affinity for O$_2$ than for NO (Gomes et al., 2002). Flavinb redoxyins are A-type flavoproteins which are present in bacteria and
some archaea, such as *Pyrococcus furiosus*, *Archaeoglobus fulgidus*, *Methanocaldovococcus jannaschii* and *Methanothermobacter thermautotrophicus*. Therefore, it has been proposed that this protein is a widespread class of NO reductase, which could detoxify NO or oxygen, allowing versatility in response to environmental conditions (Gomes et al., 2002). However, the physiological relevance of this protein should be determined in future studies.

The last step of denitrification, the reduction of N$_2$O to N$_2$, is of great environmental importance because it closes the N-cycle. Although N$_2$O is less toxic than NO or nitrite, and bacteria could manage without converting N$_2$O into N$_2$, most denitrifying bacteria contain nitrous oxide reductases (Nos) encoded by the nosZ gene. These enzymes are periplasmic multicopper homodimers that receive electrons from cytochrome c or pseudoazurin (Zumft, 1997). In each 65 kDa monomer, the copper ions are organized in two centres, a di-copper cluster CuA resembling that of cytochrome oxidase, and a CuZ cluster in which four Cu atoms are ligated by seven His residues. A bridging inorganic sulfur in the CuZ centre has also been identified (Brown et al., 2000; Rasmussen et al., 2000). In some archaea, such as *Halococula marisnortii* and *Ferroglobus placidus*, the predominant gas species produced by denitrification is N$_2$O (Werber & Mevarech, 1978; Vorholt et al., 1997), although other archaea such as *Halofexis denitrificans* and *Pyrobaculum aerophilum* are able to reduce N$_2$O to N$_2$, having a complete denitrification pathway (Tomlinson et al., 1986; Völdl et al., 1993; de Vries & Schröder, 2002). However, in *P. aerophilum*, N$_2$O formed by qNor accumulates because its reduction to N$_2$ by the nitrous oxide reductase is very slow. In contrast to the periplasmic bacterial Nos, the *P. aerophilum* enzyme is located in the membrane and uses menaquinol as electron donor, like bacterial Nos, the *P. aerophilum* enzyme is located in the membrane and uses menaquinol as electron donor, like the other three reductases of the denitrification pathway in this hyperthermophile (de Vries & Schröder, 2002; Fig. 3). N$_2$O reduction has not been investigated in other species of archaea and putative nos genes are not present in the sequenced archaeal genomes (Table 1), although two long hypothetical quinol oxidases (ST2395 and ST0105) with the motif of the Nos binuclear copper centre are found in *Sulfolobus tokodaii* (Kawarabayasi et al., 2001).

### N$_2$ fixation in archaea

N$_2$ is the most abundant gas in Earth’s atmosphere, but it is highly stable and unreactive and is only used as nitrogen source by some prokaryotic organisms. Biological N$_2$ fixation is catalysed by nitrogenase, an O$_2$-sensitive enzyme complex which generates two molecules of NH$_3$ by reduction of N$_2$. Nitrogenase can also reduce protons to H$_2$ and several substrates with double or triple bonds, such as acetylene and cyanide (Halbleib & Ludden, 2000). Nitrogenase consists of a molybdenum–iron protein (dinitrogenase), which receives electrons from an iron protein known as dinitrogenase reductase. MoFe protein is an $\alpha_2\beta_2$ tetramer encoded by the *nifD* and *nifK* genes, and Fe protein is a homodimer of the *nifH* gene product (Howard & Rees, 1996). Several *nif* genes are also involved in cofactor biosynthesis, electron transfer and regulation. Alternative nitrogenases have been found in several bacteria, with V or Fe substituting for Mo. These enzymes have lower catalytic efficiency than Mo-nitrogenases and are encoded by *nif* homologous genes, termed *vnf* (V-nitrogenase) or *anf* (Fe-nitrogenase). Alternative nitrogenases include an additional small subunit $\delta$, encoded by *vnfG* or *anfG* (Eady, 1996; Rees, 2002). N$_2$ fixation is highly regulated since it is an energy-expensive process requiring large amounts of reducing power and ATP. Ammonium causes a reversible short-term inhibition of enzyme activity, called switch-off/on (Zumft & Castillo, 1978), and a long-term effect at the transcriptional level. Ammonium switch-off occurs by ADP-ribosylation of NifH by DraT/DraG, an ADP-ribosyltransferase/glycohydrolase system. Expression of *nif* genes requires NifA, a transcriptional activator under the control of Ntr, a two-component regulatory system of bacterial nitrogen metabolism (Ludden, 1994; Merrick & Edwards, 1995; Halbleib & Ludden, 2000).

N$_2$ fixation in archaea, first described in *Methanosarcina barkeri* (Murray & Zinder, 1984) and *Methanococcus thermolithotrophicus* (Belay et al., 1984), is exclusive of, but widespread within, methanogenic euryarchaeota (Table 2). However, neither *Methanococcus voltae* nor *Methanocaldovococcus jannaschii* grows diazotrophically, despite the presence of *nifH*-like genes in their genomes (Leigh, 2000). Mo-nitrogenase predominates in diazotrophic methanogens, which contain at least the *nifHDKEX* genes (Lobo & Zinder, 1990; Chien & Zinder, 1996; Kessler et al., 1998; Leigh, 2000). *nifHDK* are the structural genes for nitrogenase, whereas *nifEX* code for FeMo-cofactor biosynthesis proteins (Moreno-Vivián et al., 1989). The diazotrophic growth of *Methanococcus maripaludis* shows Mo dependence, indicating that N$_2$ fixation is catalysed by a Mo-nitrogenase (Kessler et al., 1997), although *Methanosarcina* species contain alternative nitrogenases (Table 2). *M. barkeri* has two clusters of nitrogenase genes, the *nif2* gene cluster encoding the Mo-nitrogenase and the *nif1* (*vnf*) cluster encoding an alternative V-nitrogenase, which also includes a *vnfG* gene (Chien et al., 2000). Like the *nif* regions of other methanogens, both gene clusters contain two *glnB* genes encoding nitrogen signal transduction proteins (Chien et al., 2000). The *M. acetivorans* genome includes *nif*, *vnf* and *anf* genes, being unique among archaea in having all three nitrogenase systems (Galagan et al., 2002).

There are significant differences in gene organization and regulation between bacterial and archaeal nitrogenase systems. First, all *nif* genes and the *glnB* genes are co-transcribed in a single unit (*nifHglnBglnBII*nifD*KENX*), this being a gene organization unique to archaea (Kessler et al., 1998; Leigh, 2000). Transcription of *nif* genes occurs from typical archaeal promoters, probably reflecting the differences between archaeal and bacterial transcription apparatus, and is regulated by repression, whereas an activation mechanism involving NifA operates in bacteria. As stated
Table 2. Presence of genes encoding ammonium transporters and proteins involved in N\textsubscript{2} fixation and ammonium assimilation in the completely sequenced genomes of archaea

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Genome sequence analysis was conducted on websites http://gib.genes.nig.ac.jp and http://www.genome.ad.jp and the accession numbers of the corresponding genes in the different genomes are indicated. ND indicates that putative genes encoding these proteins or enzymes have not been detected. For details and references, see the text.
<table>
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<th>Nitrogenases</th>
<th>N₂-fixation-related proteins</th>
<th>Glutamine synthetase</th>
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Table 2. cont.
below, a repressor NrpR found only in euryarchaeota regulates nif and glnA (glutamine synthetase) gene expression (Lie & Leigh, 2003). Finally, post-transcriptional regulation by ammonium (switch-off) also occurs in archaea (Lobo & Zinder, 1990; Kessler & Leigh, 1999; Kessler et al., 2001), but does not involve ADP-ribosylation or covalent modification of NifH and requires two members of the GlnB family, termed Nif1 and Nif2 (Kessler et al., 2001).

Phylogenetic analysis suggests that N2 fixation may have originated in a common ancestor of bacteria and archaea, before branching of these domains and prior to the appearance of oxygen in the atmosphere (Fani et al., 2000; Leigh 2000; Berman-Frank et al., 2003). Comparative analysis of the amino acid sequences of the NifDK and NifEN proteins suggests a two-step in-tandem duplication of an ancestor gene, giving rise first to a bicistronic operon, which later underwent a paralogous duplication leading to the nifDK and nifEN genes before the divergence of archaea and bacteria (Fani et al., 2000). The function of this ancient enzyme might have depended on the composition of the early atmosphere, being either a nitrogenase for N2 fixation in a neutral anoxic atmosphere containing N2 but not NH3, or alternatively, a cyanide-detoxifying enzyme in a strongly reducing atmosphere, which could change its specificity to N2, another triple-bond substrate, after progressive exhaustion of combined nitrogen, thus enabling survival of ancient micro-organisms in N-deficient environments before the appearance of photosynthetic O2 (Fani et al., 2000; Berman-Frank et al., 2003). After the divergence of archaea and bacteria, many organisms lost nif genes due to the weakening of selection pressure and the high energetic cost of N2 fixation, although lateral gene transfer of nif genes might have also occurred (Fani et al., 2000). However, there are no apparent reasons to explain the lost of diazotrophy in all archaea except methanogens. Earth’s earliest organisms may have been similar to the thermophiles found in deep-sea hydrothermal vents, but currently the most thermophilic micro-organism known, Methanococcus thermolithotrophicus, is able to fix N2 at 64 °C (Mehta et al., 2003). This makes especially interesting a recent paper describing the first evidence of potential nitrogen fixers in diffuse hydrothermal vent fluids and deep-sea water, and reporting that all of the nifH genes from these samples are most closely related to M. thermolithotrophicus (Mehta et al., 2003).

**Transport and assimilation of ammonium in archaea**

Glutamine synthetase–glutamate synthase cycle (GS–GOGAT) and glutamate dehydrogenase (GDH) are the major pathways for ammonium assimilation. The GS–GOGAT route requires ATP but has a high affinity for ammonium, whereas GDH does not consume ATP but is less effective in cells growing in N-limited conditions. As these enzymes are essential in ammonia assimilation and amino acid metabolism, they are present in all three domains of life. Movement of ammonium across biological membranes is also an important process, and ammonium transporters (Amt) are found in bacteria, archaea and eukarya.

**Ammonium transport**

N2 fixation and nitrate assimilation generate ammonium for incorporation into carbon skeletons inside the cells. However, when ammonium is available in the environment, organisms usually take up this ion by a specific transport system, inhibiting and/or repressing the reductive assimilatory pathways of the N-cycle. Although NH3 diffuses through biological membranes, and this could represent a significant process in organisms growing in alkaline environments, ammonium transporters are found in both prokaryotic and eukaryotic organisms. Amt proteins are a family of ammonium and methylammonium transporters, which are integral membrane proteins of 45–50 kDa with 10–12 transmembrane helices (Thomas et al., 2000). It is widely accepted that Amt proteins mediate an active NH4+ uniport which depends on proton-motive force, although it has been also proposed that Amt proteins facilitate diffusion of NH3 across the plasma membrane bidirectionally (Soupene et al., 2002). The E. coli AmtB protein is essential for transport of ammonium and is able to complement a Saccharomyces cerevisiae mutant (Blakey et al., 2002). Putative ammonium transporters (AmtB) are also present in most archaeal genomes (Table 2). In bacteria and archaea, the amtB gene is invariably linked to the regulatory glnK gene, which encodes a GlnB-like protein (P10 protein). Both GlnK and AmtB proteins interact for regulation of the transport activity in response to nitrogen availability (Thomas et al., 2000; Coutts et al., 2002). In E. coli, AmtB is inactivated by formation of a membrane-bound complex with GlnK, a process regulated by uridylylation/deuridylylation of GlnK in response to the nitrogen status of the cells, and the AmtB activity is also required for GlnK deuridylylation. Therefore, AmtB could be considered as an ammonium sensor, and transport is also an integral part of the signal transduction cascade (Javelle et al., 2004).

**The glutamine synthetase–glutamate synthase cycle**

Glutamine synthetase (GS) is a ubiquitous enzyme with a central role in nitrogen metabolism. Three types of GS catalyse the ATP-dependent synthesis of glutamine from glutamate and ammonia. GSI, found in most prokaryotes, is a dodecameric protein composed of identical 50–56 kDa subunits arranged in two hexagonal rings. GSII is mainly present in eukaryotes and has an octameric structure, with identical subunits of 45–50 kDa. GSIII is only found in a few bacterial species, such as Bacteroides fragilis, and has a hexameric structure with identical subunits of about 83 kDa (Pesole et al., 1995; DiRuggiero & Robb, 1996; Eisenberg et al., 2000).

Putative glnA genes encoding GSI are present in archaeal...
Sulfolobus acidocaldarius glnA is more related to GSI-

Microbiology and GSI-(Yin et al. 1998). The genomes of most archaea show very high glutamate dehydrogenase activity, depending on the archaeal species. Most hyperthermophilic archaea have no detectable GS activity (Kabayashi et al., 1993). However, the crenarchaeote Sulfolobus solfataricus GS is not placed in either the GSI-α or the GSI-β division (Brown et al., 1994), although the Sulfolobus acidocaldarius glnA is more related to GSI-β, despite its enzyme properties being more similar to GSI-α (Yin et al., 1998). Most GSI-β enzymes are regulated by adenylylation/deadenylylation, but GSI-α enzymes are not controlled by this reversible mechanism. Purified GSs from Pyrococcus sp., S. acidocaldarius and Methanobacterium ivanovi are dodecamers of identical subunits that are not regulated by adenylylation (Bhatnagar et al., 1986; Rahman et al., 1997; Yin et al., 1998). Therefore, branching of GSI-α and GSI-β could precede the separation of archaea and bacteria.

The relevance of GS in ammonium assimilation varies depending on the archaeal species. Most hyperthermophilic archaea show very high glutamate dehydrogenase activity, suggesting that this enzyme is the major pathway for ammonium assimilation. In fact, Thermococcus profundus has no detectable GS activity (Kabayashi et al., 1995) and the GS of Pyrococcus sp. has low biosynthetic activity, suggesting that the reaction is biased towards glutamate production. Therefore, hyperthermophilic archaea seem not to use GS predominantly for ammonium assimilation. In contrast, the inability to obtain a glnA null mutant in Methanococcus maripaludis suggests that this archaeon cannot transport glutamine and that GS is an essential enzyme, even in the presence of alternative nitrogen sources (Cohen-Kupiec et al., 1999).

Glutamate synthase (glutamine: 2-oxoglutarate amidotransferase, GOGAT) is an iron–sulfur flavoprotein that catalyses the transfer of the amide group of glutamine to 2-oxoglutarate, yielding two molecules of glutamate. In bacteria, GOGAT is usually an NADPH-dependent enzyme arranged in an $\alpha_4\beta_4$ structure, with large $\alpha$ subunits of about 135 kDa and small $\beta$ subunits of approximately 53 kDa (Trotta et al., 1974). The genomes of most archaea contain putative genes encoding either the large or the small GOGAT subunit (Table 2). The gltA gene encoding a putative small GOGAT subunit in the hyperthermophile Pyrococcus sp. has been cloned. The purified GltA protein is a tetramer with identical subunits of 53 kDa. This GOGAT uses NADPH and has both glutamine- and ammonium-dependent activities, showing different temperature optima, 80°C for the glutamine reaction and 90°C for the ammonium-dependent reaction (Jongsareejit et al., 1997). The amino acid sequence of this GOGAT includes two Cys clusters, and adenylate- and FAD-binding domains, which are conserved in the small subunits of bacterial GOGAT. It is worth noting that Pyrococcus sp. lacks the large subunit gene and GOGAT is encoded by the gltA gene alone. In contrast, genomes of other archaea include only the gltB gene encoding the large subunit (Table 2). Methanocaldococcus jannaschii has only a gltB gene encoding a 55 kDa protein, which includes features of the bacterial large subunit and probably has GOGAT activity (Bult et al., 1996). It has been proposed that both M. jannaschii GOGAT (GltB) and Pyrococcus sp. GOGAT (GltA) represent ancestral forms of the large and small subunits, respectively, which are functional without their subunit counterpart (Jongsareejit et al., 1997). On the other hand, in eukaryotes, GOGAT is a monomeric enzyme homologous to either the large bacterial subunit, as the maize ferredoxin-GOGAT, or to both large and small bacterial subunits, as the alfalfa NADH-GOGAT (Jongsareejit et al., 1997).

Glutamate dehydrogenase

Glutamate dehydrogenase (GDH) catalyses both the oxidative deamination of glutamate and the reductive incorporation of ammonium into 2-oxoglutarate. There are three types of GDH based on coenzyme specificity. Dual NAD(P)-GDHs are found in animals and higher plants, but NADP-GDHs are mostly involved in ammonia assimilation in bacteria, fungi and algae. Both NADP-dependent and dual NAD(P)-GDHs are hexamers composed of 48–55 kDa identical subunits. On the other hand, NAD-GDHs consist of either four or six identical subunits and usually have a catabolic role, being involved in the oxidative deamination of glutamate (Smith et al., 1975).

Most hyperthermophilic archaea studied to date express high levels of GDH, and it is assumed that GDH plays a major role in ammonium assimilation in these extremophiles (DiRuggiero & Robb, 1996). The enzymes from Pyrococcus furiosus, Thermococcus litoralis, Thermococcus profundus and Sulfolobus solfataricus are hexameric, as in mesophilic bacteria, and show a high temperature optimum for activity. However, the coenzyme specificity of GDH from these hyperthermophiles resembles that of the eukaryotes rather than bacteria, since the enzyme uses both NADH and NADPH (DiRuggiero & Robb, 1996). The three-dimensional structure of the P. furiosus NAD(P)-GDH has been determined at 2–2 Å resolution (Yip et al., 1995). The gdhA genes encoding GDH have been cloned or identified in the genome of several archaea (Table 2). The P. furiosus gdhA gene is transcribed from a typical archaeal promoter, but it has been expressed in E. coli, yielding a recombinant GDH fully active in vitro at 85°C (DiRuggiero & Robb, 1995). Sequencing of the Pyrococcus horikoshii genome revealed the presence of a gdhA gene homologous to the GDH-encoding genes of P. furiosus and T. litoralis. Recently, this gdhA gene has been cloned and a recombinant His6-tagged GDH was overexpressed in E. coli and purified. The enzyme is a hexamer of 290 kDa (subunit
mass 48 kDa), requires NAD(P)H and has a temperature optimum of 90 °C (Wang et al., 2003). This *P. horikoshii* GDH shows a lower $K_m$ value for glutamate than for ammonium, suggesting that the reaction is biased toward oxidative deamination of glutamate. NADP is the preferred coenzyme for this reaction. The enzyme subunit contains many charged residues that at high temperature arrange networks of ion pairs for proper folding and maintaining the native conformation of the enzyme (Wang et al., 2003). The GDH enzyme of *Aeropyrum pernix* has also been characterized (Bhuiya et al., 2000; Helianti et al., 2001, 2002). The enzyme also has a hexameric structure with a molecular mass of about 285 kDa, and is specific for NADP. This GDH is thermostable, and the N-terminal length seems to be important for its thermostability (Helianti et al., 2002). The *A. pernix* enzyme has biphasic kinetics for glutamate, but monophasic kinetics for NAD at 50–90 °C, and it has also been crystallized and analysed by X-ray diffraction (Bhuiya et al., 2002a, b).

Protein sequence analysis indicates that the enzymes from *Pyrococcus furiosus* and *Pyrococcus horikoshii* are more closely related to the mesophilic bacterial GDH than to the thermophilic archaeon *Sulfolobus solfataricus* or to the halophilic archaeon *Halobacterium salinarum* (DiRuggiero & Robb, 1996), suggesting that lateral gene transfer between bacterial and archaeal *gdhA* genes may have occurred during evolution. Some organisms have two or more GDHs, which differ in coenzyme specificity. This is the case for *H. salinarum*, which has both NADP- and NAD-dependent enzymes (Bonete et al., 1987; Hayden et al., 2002). The NAD-enzyme of *H. salinarum* has been characterized and is subject to activation by amino acids and inhibition by TCA cycle intermediates such as fumarate, oxalacetate, succinate and malate (Bonete et al., 1996). The *Halofexa mediterranei* GDH has been also purified and is an NADP-specific enzyme composed of six identical monomers of 55 kDa (Ferrer et al., 1996). In addition, a putative *gdhA* gene is present in the *Halobacterium sp.* genome (Ng et al., 2000; Table 2). It is also remarkable that the amino acid sequence of the grapevine (*Vitis vinifera*) GDH is more similar to the archaeal GDH than to those of eukaryotes. A stress-related function of plant GDH has been invoked to explain this resemblance and the thermostability of the enzyme (Syntichaki et al., 1996).

**Regulation of nitrogen metabolism in archaea**

Denitrification and other anaerobic respiratory processes are alternative metabolic pathways developed by facultative micro-organisms to obtain energy for growth under anoxic conditions, and therefore they are usually repressed by oxygen (Moreno-Vivián & Ferguson, 1998). Regulation of anaerobic metabolism in *E. coli* and control of denitrification genes in *Pseudomonas* strains and other denitrifying bacteria are well studied (Zumft, 1997), but the mechanisms controlling nitrate respiration and denitrification in archaea are still not established. In addition, database searches reveal that homologues of different potential regulators involved in bacterial nitrate reduction, such as FNR, NNR, NorR, NarR, NarXL, ResDE and RegAB, are not present in the sequenced archaeal genomes, thus suggesting that completely novel regulatory systems could operate in archaea, probably as a consequence of the fundamental differences in the transcription machinery between bacteria and archaea. However, although most archaeal helix–turn–helix-containing transcription factors belong to archaea-specific families, it has recently been described that the molybdopterin-responsive transcription factor ModE is present in some archaea. Moreover, the upstream ModE DNA-binding sequence seems to be conserved between bacteria and archaea (Studholme & Pau, 2003). These results indicate that Mo also regulates the expression of the genes required for archaeal molybdoproteins, and suggest either an ancient common origin for the ModE regulon, or more likely, horizontal gene transfer probably between green sulfur bacteria and methanogenic archaea (Studholme & Pau, 2003).

On the other hand, ammonium is usually the preferred nitrogen source in bacteria because it is easily assimilated with low energy cost. As other inorganic nitrogen sources, like nitrate, nitrite or N$_2$, must be reduced before assimilation, inorganic nitrogen assimilation is often repressed by ammonium.

Glutamine synthetase (GS) is highly regulated at both transcriptional and post-translational levels. In bacteria, GS is reversibly modified by adenylylation in response to the cellular nitrogen status, basically the glutamine/2-oxoglutarate ratio. An adenylyltransferase catalyses the adenylylation and deadenylylation of GS in response to the P$_H$ protein, which in turn can be uridylylated or deuridylylated by a uridylyltransferase. When glutamine levels are high, deuridylylated P$_H$ stimulates GS deadenylylation for activation when 2-oxoglutarate levels are high. P$_H$ is also involved in transcriptional regulation of several nitrogen metabolism genes in response to the nitrogen status, acting on the general Ntr regulatory system or affecting specific regulators, such as the NifA protein that activates *nif* gene expression. P$_H$ is encoded by the *glnA* gene, and *glnB* homologues are known in a variety of bacteria and archaea, and have been recently identified in higher plants, indicating that the GlnB family plays a central role in nitrogen regulatory processes in both prokaryotes and eukaryotes (Ninfa & Atkinson, 2000; Arcondéguy et al., 2001). However, P$_H$ proteins are not ubiquitous and several bacterial and archaeal genomes do not encode *glnB* genes. This is the case for *Aeropyrum pernix*, *Pyrococcus horikoshii* and *Pyrococcus abyssi* (Arcondéguy et al., 2001). In addition, as mentioned above, archaeal GSs are not regulated by adenylylation, and control of GS activity in response to P$_H$ proteins could be via a different mechanism than that in most bacteria. Many organisms contain a second P$_H$ gene called *glnK*, which is located upstream from the *ammtB* gene encoding an ammonium transporter in most prokaryotes,
including archaea, where this gene pair is found in multiple copies. This conserved gene organization indicates that the two genes were associated before the divergence of bacteria and archaea, and suggests that the GlnK regulatory protein and the ammonium transporter interact directly, probably for regulation of ammonium transport by GlnK (Thomas et al., 2000). It has recently been demonstrated that GlnK binds to AmtB in the membrane and acts as a negative regulator of the transport activity of AmtB. This binding is dependent on GlnK uridylylation and is modulated according to the cellular nitrogen status, being maximal at high nitrogen (Coutts et al., 2002; Javelle et al., 2004). Therefore, this regulatory mechanism could be conserved throughout bacteria and archaea. Some archaeal genomes contain other glnB-like genes in addition to glnB and glnK. Thus, two different GlnB homologues are encoded in the nif gene clusters of diazotrophic methanogens (Chien & Zinder, 1996; Kessler & Leigh, 1999; Kessler et al., 2001). In Methanococcus maripaludis, these glnB-like genes (termed nifL1 and nifL2) are both required for ammonia switch-off of nitrogenase, probably acting through a novel reversible mechanism which does not require ADP-ribosylation of NifH protein and does not affect transcription of nif genes or stability of the NifH protein (Kessler et al., 2001).

Transcription in archaea resembles the eukaryotic process more than the bacterial, and could be considered as a simplified version of the eukaryotic transcription complex (Bell & Jackson, 2001). In addition, although bacteria and archaea contain homologous regulatory proteins, the possibility that some regulators could be present only in archaea should not be excluded. In Methanosarcina barkeri, a putative TATA box is located in the nifHDK2 promoter region that is transcribed like typical archaeal genes. Expression of nitrogenase structural genes is repressed by ammonium (Lobo & Zinder, 1990; Chien & Zinder, 1996) and ammonium-grown cells contain a putative protein that inhibits binding of transcription initiation proteins at the TATA box promoter region (Chien et al., 1998). In Methanococcus maripaludis, expression of nif (nitrogenase) and glnA (glutamine synthetase) operons are repressed by ammonium (Kessler et al., 1998; Cohen-Kupiec et al., 1999) and a palindromic consensus sequence has been identified as a nitrogen operator for binding of a putative repressor which negatively regulates the nif and glnA operons in response to ammonia (Kessler & Leigh, 1999). Very recently, this nitrogen repressor has been identified in M. maripaludis and called NrpR (Lie & Leigh, 2003). NrpR is a tetramer of 60 kDa subunits with a putative N-terminal winged helix–turn–helix motif, which represents a new type of regulator exclusive to euryarchaeota (Lie & Leigh, 2003). C/N balance could regulate binding of the NrpR repressor to the nif promoter, because 2-oxoglutarate decreases the binding of NrpR to the nitrogen operator sequence. Therefore, archaea could use a different mechanism than bacteria for the control of nitrogen gene expression in response to the C/N balance (Lie & Leigh, 2003).

Concluding remarks

Archaea can sustain the N-cycle in the extreme environments where they live because they are able to drive both dissimilatory and assimilatory processes of the cycle. In spite of this, archaeal inorganic nitrogen metabolism is much less known than bacterial nitrogen metabolism. Emerging data from recent functional studies and genome sequencing projects reveal important differences in the structure and regulation of enzymes and transport systems involved in the denitrification and nitrogen assimilation pathways, with interesting evolutionary implications. In particular, different regulatory mechanisms may operate in the control of nitrogen assimilation in archaea. Of special interest is whether or not transcriptional regulation in archaea is unique or possesses similarities to bacterial or eukaryotic regulation. Major goals of future studies will be to characterize at the molecular level the regulatory mechanisms acting on nitrogen metabolism in archaea, especially those for nitrate assimilation and denitrification. Functional genomic approaches should be also performed in order to identify the proteins and enzymes involved in the N-cycle pathways in the different archaea and their biochemical properties.

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Nitrogen cycle in archaea


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