

Nitrogen Fixation In Methanogens: The Archaeal Perspective

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Abstract

The methanogenic Archaea bring a broadened perspective to the field of nitrogen fixation. Biochemical and genetic studies show that nitrogen fixation in Archaea is evolutionarily related to nitrogen fixation in Bacteria and operates by the same fundamental mechanism. At least six *nif* genes present in Bacteria (*nif H, D, K, E, N* and *X*) are also found in the diazotrophic methanogens. Most nitrogenases in methanogens are probably of the molybdenum type. However, differences exist in gene organization and regulation. All six known *nif* genes of methanogens, plus two homologues of the bacterial nitrogen sensor-regulator *glnB*, occur in a single operon in *Methanococcus maripaludis*. *nif* gene transcription in methanogens is regulated by what appears to be a classical prokaryotic repression mechanism. At least one aspect of regulation, post-transcriptional ammonia switch-off, involves novel members of the *glnB* family. Phylogenetic analysis suggests that nitrogen fixation may have originated in a common ancestor of the Bacteria and the Archaea.

Introduction

It has long been recognized that nitrogen fixation occurs in bacteria but not eukaryotes. When Archaea were first discovered to be a prokaryotic domain of life separate from Bacteria (1), it was not known that they too contained nitrogen fixing species. In 1984 S. Zinder and L. Daniels independently discovered diazotrophic growth in two different methanogenic Archaea, *Methanosarcina barkeri* (2) and *Methanococcus thermolithotrophicus* (3). ¹⁵N incorporation from ¹⁵N₂ confirmed that nitrogen fixation occurred in *M. barkeri*, and acetylene reduction confirmed nitrogenase activity in *M. thermolithotrophicus*. This discovery of nitrogen fixation in Archaea raised new questions regarding the diversity of nitrogen fixation at the phylogenetic, biochemical, and genetic levels. A review on nitrogen fixation in methanogens appeared in 1992 (4).

Distribution of Nitrogen Fixation in the Archaea

The well-characterized Archaea consist of the strictly anaerobic methanogens, the extreme halophiles, and the extreme thermophiles (5). Some methanogens are also extreme thermophiles. The Archaea are distributed over two main phylogenetic branches (kingdoms) based on 16S

rRNA sequence comparisons, the Euryarchaeota and the Crenarchaeota (6). The Euryarchaeota contain the methanogens, the halophiles, and some extreme thermophiles, while the Crenarchaeota contain most of the extreme thermophiles. Within the Archaea, nitrogen fixation has been found only in the methanogenic Euryarchaeota. Within the methanogens, however, nitrogen fixation is widespread, extending to all three orders (7) (Table 1). In the *Methanococcales*, diazotrophic growth has been reported for *Methanococcus thermolithotrophicus* (3) and *Methanococcus maripaludis* (8). *M. thermolithotrophicus* is the only organism demonstrated to fix nitrogen at 60°C or above. Neither *Methanococcus jannaschii* (9) nor *Methanococcus voltae* (10) fix nitrogen despite the presence of *nifH* homologues (our unpublished results). In *M. jannaschii* it is clear that other *nif* genes are not present. Within the *Methanomicrobiales*, diazotrophic species include *Methanosarcina barkeri* (2, 11) and *Methanospirillum hungatei* (12). In the *Methanobacteriales*, nitrogen fixation has been demonstrated for *Methanobacterium bryantii* (12). Whether *Methanobacterium thermoautotrophicum* is a diazotrophic species is unclear. There is a report of diazotrophic growth of strain Marburg (13), and both strains Marburg and ΔH have complete or nearly complete *nif* gene clusters compared to *M. maripaludis* (below). On the other hand, recent attempts to grow either strain diazotrophically have not been successful (R. Thauer, S. Zinder, personal communication). In addition, diazotrophic growth, ¹⁵N₂ incorporation, or acetylene reduction has been reported for a number of other species of methanogens (4).

How Different is Nitrogen Fixation in the Archaea?

The discovery of nitrogen fixation in methanogens led to a quest to determine whether it resembled nitrogen fixation in Bacteria. Lobo and Zinder (15) presented evidence that diazotrophic growth in *M. barkeri* was energetically costly and was stimulated by molybdenum. Partial purification of nitrogenase activity revealed that two components were required (16). Subunit analysis suggested the presence of nitrogenase and nitrogenase reductase similar to those in Bacteria, except that the latter appeared to be a

Table 1. Nitrogen Fixing Species in the Archaea

<i>Methanococcales</i>
<i>Methanococcus thermolithotrophicus</i>
<i>Methanococcus maripaludis</i>
<i>Methanomicrobiales</i>
<i>Methanosarcina barkeri</i>
<i>Methanospirillum hungatei</i>
<i>Methanobacteriales</i>
<i>Methanobacterium bryantii</i>

homotetramer rather than a homodimer. The putative nitrogenase reductase cross-reacted with antiserum against nitrogenase reductase of *Rhodospirillum rubrum*. Whole cells, extracts, and purified nitrogenase could reduce acetylene to ethylene, albeit at lower rates than in Bacteria. *nifH* was cloned from *M. thermolithotrophicus* using a bacterial probe. Sequencing revealed *nifD* and part of *nifK* downstream from *nifH* (17). These findings suggested that nitrogen fixation in methanogens was evolutionarily related to nitrogen fixation in Bacteria and operated with the same basic mechanism.

If nitrogen fixation in Archaea fundamentally resembled nitrogen fixation in Bacteria, it seemed possible that Archaea might feature vanadium or iron-only nitrogenases that are present as "alternative" nitrogenases in certain Bacteria. However, this does not appear to be the case. The predominate nitrogenases in methanogens seem to be molybdenum nitrogenases as is the case in Bacteria (below).

Genetics

nif genes in methanogens were first cloned using *nifH* genes of Bacteria as hybridization probes (18). It soon became apparent that *nifH* genes in methanogens were followed by two genes with homology to *glnB* (below), then *nifD* and *nifK* (17, 19). In *M. maripaludis* we used an oligonucleotide designed from a conserved region of *nifH* to clone a fragment from a DNA library (8). We used transposon insertion mutagenesis, a technique that we developed for *M. maripaludis* (8), to show that a region extending 8 kb from the beginning of *nifH* was required for diazotrophic growth (20). Sequencing this region identified the genes *nifH* through *nifX* as shown in Figure 1. These genes are clear homologues of the corresponding genes in Bacteria, further demonstrating that nitrogen fixation in Archaea is fundamentally the same as in Bacteria. Five of these genes have functions in Bacteria that are required for and central to nitrogen fixation. *nifH* encodes nitrogenase reductase, *nifD* and *nifK* encode the α and β subunits of nitrogenase itself, and *nifE* and *nifN* are required for iron-molybdenum cofactor (FeMoCo) synthesis (21). *nifX* and the *glnB* homologues are discussed below.

The *nif* gene organization found in *M. maripaludis* is conserved in other methanogens. In *M. thermoautotrophicum* a cluster with the same eight genes is present, with the sole exception that *nifX* does not seem to be present in one strain (ΔH , (14)), although it is found in another strain of the same species (Marburg, GenBank accession number 1854560). A corresponding gene cluster in *M. barkeri* has been sequenced from *nifH* through most of *nifE* (22), and it is likely that *nifN* and *nifX* lie 3' to *nifE*. In

M. maripaludis, at least, the entire cluster of eight genes belongs to a single operon, since transposons inserted throughout the cluster always had a polar effect, that is, they always eliminated the synthesis of mRNA from genes 3' to the insertion (20). Furthermore, an identifiable promoter sequence was present only in the region upstream of *nifH*. *nif* mRNA observed by Northern blot was usually fragmented into subsets of genes, and this could be attributed to some combination of intergenic termination of transcription and processing of the mRNA.

The organization of the methanogen *nif* gene cluster reveals differences as well as similarities when compared to bacteria. The order, *nifH-nifD-nifK-nifE-nifN-nifX*, is common in bacteria (21). However, the presence of novel homologues of *glnB* (below) between *nifH* and *nifD* is unique to the methanogens. In addition, the presence of the six *nif* genes, *nifH*, *nifD*, *nifK*, *nifE*, *nifN*, and *nifX* in a single operon is unknown in the Bacteria, which tend to separate the first three and the latter three into different operons (21).

Since *nifX* is not required for nitrogen fixation in several Bacteria (21), we checked its function in *M. maripaludis*, taking advantage of genetic methods developed for *Methanococcus* species. An in-frame deletion mutant of *nifX* was not detectably impaired in diazotrophic growth (23). *nifX* has sequence homology to *nifY* and to the C-terminus of *nifB*, and it has been proposed that *nifX* may have a dispensible function in FeMoCo synthesis that is duplicated by *nifB* or *nifY* (21). Whether *M. maripaludis* contains a functional *nifB* or *nifY* gene is unknown. *nifB* homologues (other than *nifX*) have been found in other methanogens (9, 14), but the homology does not extend to the C-terminal end of bacterial *nifB*. Therefore, the dispensibility of *nifX* for nitrogen fixation in methanogens cannot easily be explained by gene duplication.

In contrast to the six *nif* genes of the *nif* operon in methanogens, in *Klebsiella pneumoniae* there are 20 different *nif* genes (21). It is at present unclear whether additional *nif* genes function in diazotrophic methanogens. Neither a complete genome sequence, nor saturation mutagenesis, has yet been performed on a bona fide diazotrophic methanogen. It is possible that in methanogens only the six *nif* genes of the identified *nif* operon are functional homologues of bacterial *nif* genes. If this is the case, functions such as electron transport to the nitrogenase complex, nitrogenase protein maturation and stabilization, and homocitrate synthesis, may be carried out by the products of genes that do not have recognizable homology to bacterial *nif* genes. Genes with similarity to *nifH*, *nifS*, *nifB*, and *nifU* have been found in non-diazotrophic Archaea (9, 24), so the presence of these genes alone does not imply a function in nitrogen fixation.

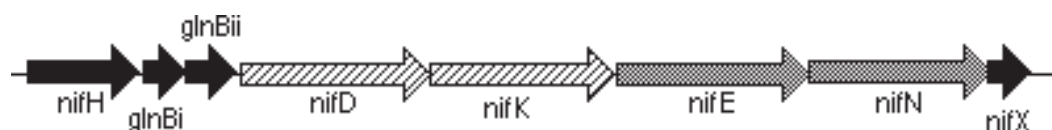


Figure 1. The *nif* gene operon of *Methanococcus maripaludis*.

Metals

A number of workers have used diazotrophic growth assays to determine whether methanogens might contain alternative (vanadium or iron-only) nitrogenases as do some Bacteria. In one study, vanadium as well as molybdenum stimulated *M. barkeri* (25), while in another study only molybdenum was stimulatory (15). The presence of two genes in *M. barkeri* that are phylogenetically related to functional *nifH* genes (19) allows for the possibility that both molybdenum and vanadium nitrogenases are present in this organism, and recently a vanadium nitrogenase has been confirmed (25a). In *M. maripaludis*, molybdenum was clearly required for diazotrophic growth (26). Vanadium failed to stimulate, and tungsten inhibited, so this species may contain a single nitrogenase of the molybdenum type. The presence of molybdenum nitrogenases in other methanogens is corroborated by *nif* gene phylogeny (26), and by the apparent absence from the *nif* gene clusters of a gene corresponding to *vnfG* or *anfG*, which encode the δ subunits of alternative nitrogenases in Bacteria (27). Thus, molybdenum nitrogenases seem to predominate in methanogens, but vanadium nitrogenases may be present as well. Nitrogenases have not been purified sufficiently from methanogens to obtain a definitive metal content.

Regulation

Transcriptional Regulation

In *M. thermolithotrophicus*, mRNAs corresponding to *nifH* and *nifD* were reported to be present when N_2 was the only nitrogen source but absent when ammonia was provided (17). In vitro transcription found no evidence for regulation (28), and it took *in vivo* genetic studies in *M. maripaludis* to identify regulatory features.

The regulation of *nif* transcription in *M. maripaludis* occurs by repression. This mechanism contrasts with the regulation of *nif* transcription in *K. pneumoniae* and other members of the Proteobacteria, which operates by an activation mechanism involving NifA. *M. maripaludis* contains two inverted repeats in the promoter region of the *nif* operon that are similar to repeats previously noted in *M. thermolithotrophicus* (17) (Figure 2). We fused the *nif* promoter region of *M. maripaludis* to *lacZ*, mutagenized the inverted repeats, and showed that the sequence of the first inverted repeat is required for repression in the presence of ammonia (29). Extract from ammonia-grown cells caused a shift in the gel mobility of promoter region

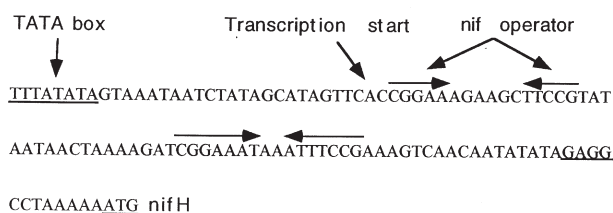


Figure 2. *nif* promoter region of *Methanococcus maripaludis*. The TATA box, ribosome binding site, and translation start are underlined. Inverted repeats are indicated with arrows above.

DNA that depended on the first inverted repeat sequence. Presumably, a repressor protein in the extract bound to the inverted repeat. Therefore, *nif* transcription is regulated by repression and the first inverted repeat is the “*nif* operator”. The second inverted repeat does not appear play a major role in regulation; but it could play a secondary role.

An inverted repeat similar to the *nif* operator of *M. maripaludis*, and matching the consensus GGAAN₆TTCC, is a common feature in the promoter regions of nitrogen-regulated genes in *Methanococcus* and *Methanobacterium* species (23). Genes for nitrogen fixation, ammonia assimilation (glutamine synthetase, *glnA*), ammonia or methylammonia transport (*amtB*), and the regulatory GlnB protein, contain this sequence in their promoter regions. In the case of the *glnA* gene in *M. maripaludis*, we have confirmed by mutagenesis that the inverted repeat sequence functions in repression (30). Thus, sequences that resemble the *nif* operator may mark genes that are coordinately regulated by a common repression mechanism. These genes may therefore belong to a “nitrogen regulon” in at least two genera of methanogens belonging to two different orders. Since *nif* gene transcription in methanogens appears to be regulated directly by this common nitrogen mechanism, it may not have an additional level of regulation specific to nitrogen fixation, as is the case involving NifA in Proteobacteria. However, there does appear to be a post-transcriptional mechanism specific to nitrogen fixation (below).

The repressor protein that binds to the nitrogen operator has not yet been identified, but one may speculate that it has dimeric nature and that each of two identical subunits binds to one half of the inverted repeat on the same face of the helix, as in the paradigm of bacterial repression. The evidence provided by the methanogen *nif* system, that regulation may resemble bacterial repression, is interesting because the basal transcription apparatus in Archaea resembles the eukaryal apparatus (31, 32).

In contrast, there is no evidence for a nitrogen operator sequence in *M. barkeri*. In that species, *nif* transcription may be regulated by a different negative mechanism in which a substance present in ammonia-grown cells inhibits binding of a transcription-associated protein or proteins to the promoter region (33). There is no evidence in this case that the inhibiting substance is itself a DNA-binding protein.

Post-transcriptional Regulation and the Novel *glnB* Homologues of the *nif* Gene Cluster

The *glnB* family of nitrogen sensory-regulatory genes is widespread. New *glnB* homologues have recently been discovered in Bacteria and Archaea, and it is beginning to appear likely that GlnB proteins are involved in all aspects of nitrogen regulation in prokaryotes. Genome sequencing has revealed *glnB* homologues, closely related to bacterial *glnB* genes, in methanogens (9, 14). In addition to these genes, two unique subfamilies of *glnB* homologues reside in the *nif* gene clusters of methanogenic Archaea (see for example Figure 1). Compared to “typical” *glnB* genes of Bacteria and Archaea, and compared to each other, these *nif*-cluster *glnB* genes differ markedly in the T-loop region (Figure 3). In the well-characterized GlnB protein, PII of *Escherichia coli*, the T-loop has been shown to contain the

sites of interaction with other proteins that modulate the regulatory activity of PII or that transmit the nitrogen signal to regulatory targets (34). Therefore, the novel sequences of the regions corresponding to the T-loop indicate that the methanogen *nif*-cluster GlnB proteins are likely to have novel interactions with other proteins.

We have recently determined the function of the *nif*-cluster *glnB* genes of *M. maripaludis*. Any function at the level of *nif* gene transcription was ruled out by the results of insertion mutagenesis across the *nif* cluster (20). (However, a function in transcriptional regulation for "typical" *glnB* genes encoded elsewhere in the genome is still possible). To test for other possible functions of the *nif*-cluster *glnB* genes, we made an in-frame deletion mutation that eliminated most of both genes. We found (23) that the *glnB* mutant was deficient in ammonia switch-off, that is,

in the immediate post-transcriptional inhibition of nitrogen fixation that occurs in some species (35, 36) upon addition of ammonia to nitrogen-fixing cultures. This finding shows that one or both of the *nif*-cluster *glnB* genes acts negatively to regulate nitrogen fixation at a post-transcriptional level. Switch-off also occurs in *Methanosarcina barkeri* (15, 16) and certain Bacteria (*Rhodospirillum rubrum* (35) and *Azospirillum brasilense* (36)), and while an involvement of *glnB* homologues is likely, it has not yet been demonstrated in these cases. It is known that ammonia switch-off in Bacteria is achieved through covalent ADP-ribosylation of nitrogenase reductase, controlled by a specific ADP-ribosyl transferase and a specific glycohydrolase. Similar covalent modification of nitrogenase does not appear to occur in methanogens (16, our unpublished results).

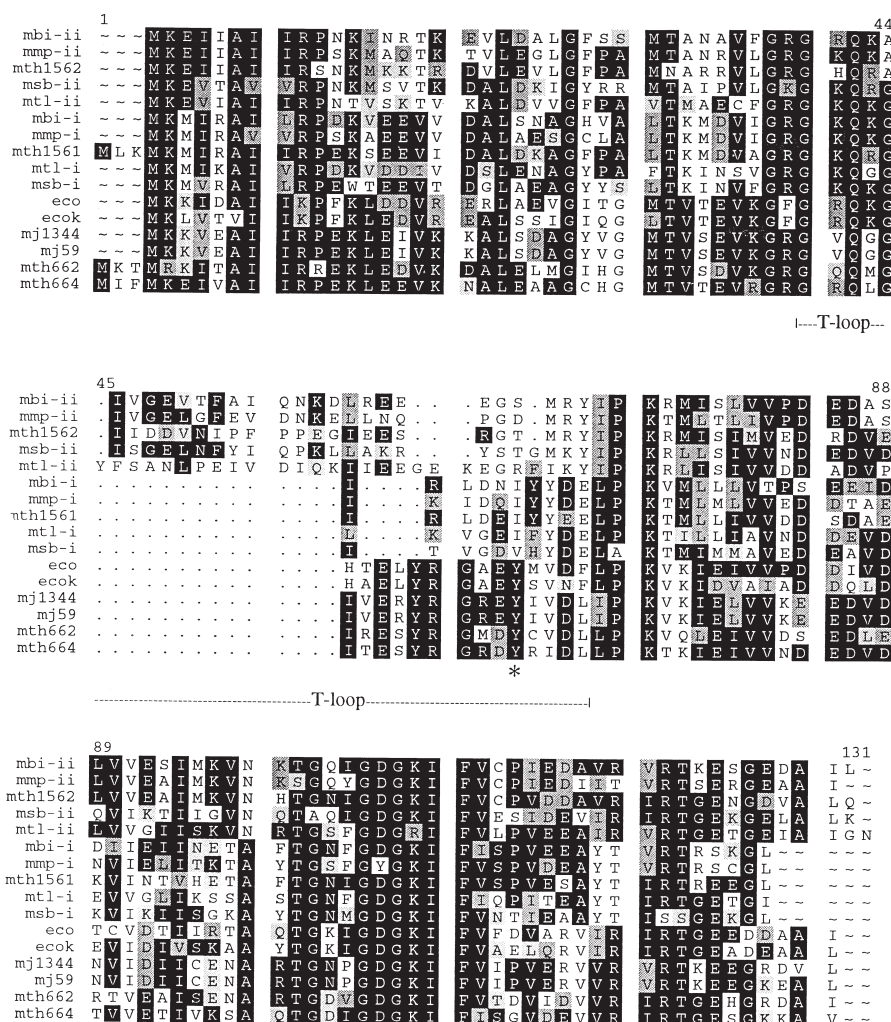


Figure 3. Alignment of *glnB* homologues in Archaea and Bacteria. The top group of five represents the second *glnB* gene in the *nif* operon of methanogens (see for example Figure 1). The next group of five represents the first *glnB* gene in the *nif* operon of methanogens. The last group of six contains the "typical" *glnB* genes of Bacteria and methanogens. Species designations are as follows: mbi, *Methanobacterium ivanovii*; mmp, *Methanococcus maripaludis*; mth, *Methanobacterium thermoautotrophicum* strain DH; msb, *Methanosarcina barkeri*; mtl, *Methanococcus thermolithotrophicus*; eco, *Escherichia coli*; mj, *Methanococcus jannachii*. Arabic numerals indicate gene designations relative to whole genome analyses (9, 14). The predicted T-loop is indicated (39, 40). * indicates the tyrosine residue which is the conserved site of uridylation in Bacteria.

Evolution of Nitrogen Fixation

The availability of *nif* gene sequences from Archaea expands our ability to trace the evolution of nitrogen fixation with phylogenetic methods. Expanding on the results of Chien and Zinder (22), we carried out a phylogenetic analysis of *nifD* and *nifE* (Figure 4). A separate analysis by parsimony gave essentially the same results as the distance matrix analysis. *nifD* and *nifE* are evidently paralogous, that is, related via an ancient gene duplication. Consequently, *nifD* genes provide a root for the *nifE* tree and vice versa. Each tree (*nifD* and *nifE*) contains two main branches. One branch is predominately bacterial, and the other branch contains *nif* genes of Archaea. This observation suggests that nitrogen fixation had its origin in a common ancestor of the two domains. *Anabaena variabilis vnfE* is anomalous, and is distant from both *nifD* and *nifE* of other species (37).

The overall pattern places the *nif* genes of Archaea and Bacteria into separate groups, but there are two exceptions. One exception is that for both *nifE* and *nifD*, *Methanosarcina barkeri* and *Clostridium pasteurianum* are closely related within a separate branch. In the case of *nifE* the *M. barkeri* - *C. pasteurianum* clade groups with the Archaea with 100% bootstrap support. The simplest explanation is that *C. pasteurianum* acquired the *nif* genes from an ancestor of *M. barkeri* by horizontal gene transfer. The *nifD* tree would suggest that the *M. barkeri* - *C. pasteurianum* clade groups with the Bacteria, but this is not supported by bootstrap analysis. The other exception to the Archaea-Bacteria split is that the alternative (vanadium and iron-only) nitrogenase subunit genes *vnfD* and *anfD* (but not *vnfE*) of the Bacteria form a separate branch within the Archaea. *vnfD* and *anfD* could have arisen through an ancient gene duplication that predates the Archaea-Bacteria split. Alternatively, they could have

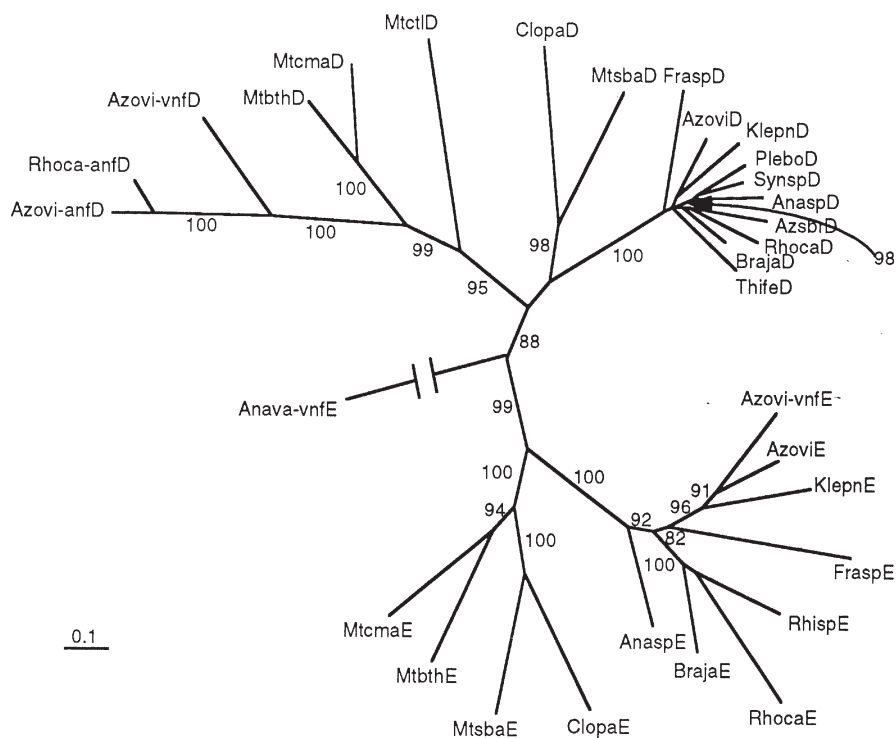


Figure 4. Phylogenetic analysis of *nifD* and *nifE* by distance matrix. Expert analysis kindly provided by A. Rodrigo. The computer program PROTDIST was used to estimate distances between all pairs of aligned amino acid sequences, using the Dayhoff PAM matrix. A phylogenetic tree was reconstructed using the computer program NEIGHBOR, which builds a tree by neighbor-joining (41). Both programs are part of the PHYLIP suite of programs (42). To determine the degree of support for each branch of the neighbor-joining phylogenetic tree, bootstrapping (43) was performed. 1000 pseudoreplicate sequence datasets were generated using SEQBOOT (also part of the PHYLIP suite), and for each pseudoreplicate, pairwise distances were estimated and the phylogenetic tree reconstructed. The number of trees which contained a given branch was counted and expressed as a percentage. Only those branches supported by more than 70% (44, 45) of the bootstrap trees are shown. Alternative nitrogenase genes are designated *vnf* (vanadium type) and *anf* (iron-only type). Species designations are as follows: Anasp, *Anabaena* species; Anava, *Anabaena variabilis*; Azovi, *Azotobacter vinelandii*; Azsbr, *Azospirillum brasilense*; Braja, *Bradyrhizobium japonicum*; Clopa, *Clostridium pasteurianum*; Frasp, *Frankia* species; Klepn, *Klebsiella pneumoniae*; Mtbt, *Methanobacterium thermoautotrophicum* strain Marburg; Mtcma, *Methanococcus maripaludis*; Mtct, *Methanococcus thermolithotrophicus*; Mtsba, *Methanosarcina barkeri*; Plebo, *Plectonema boryanum*; Rhisp, *Rhizobium* species; Rhoca, *Rhodobacter capsulatus*; Synsp, *Synechococcus* species; Thife, *Thiobacillus ferrooxidans*.

evolved in the methanogen line and were passed by horizontal transfer to the Bacteria.

A parsimony analysis of *nifK* and *nifN* (not shown) gave results that were consistent with the conclusions from the *nifD-nifE* tree. Like *nifD* and *nifE*, *nifK* and *nifN* are paralogs. On the *nifK* side of the tree, bootstrap analysis strongly supported the discrete groups, bacterial *nifK*, bacterial *vnfK* and *anfK*, and methanogen *nifK*. *C. pasteurianum* and *M. barkeri nifK* formed a separate clade but with poor bootstrap support. On the *nifN* side, bacterial *nifN* formed a robust group that excluded methanogen *nifN*. In this case, bacterial *vnfN* clustered with *nifN* from the same species.

Similar conclusions can also be drawn from the *nifH* tree. In these analyses (26, 38), there were four major branches. Three branches contained *nifH* genes that function in nitrogen fixation, while the fourth branch contained genes that evidently participate in a process unrelated to nitrogen fixation. Bacterial and archaeal *nifH* genes fell in two separate major branches. *C. pasteurianum* and *M. barkeri nifH* were closely related and formed a third major branch. *anfH* genes of bacteria fell in the archaeal branch. (*vnfH* genes, however, clustered with bacterial *nifH*).

These analyses all support a scenario in which nitrogen fixation had an ancient origin that preceded the divergence of Archaea and Bacteria. In addition, ancient gene duplication or horizontal transfer participated in the evolution of the alternative nitrogenase genes *anfH*, *vnfD*, *anfD*, *vnfK* and *anfK*, which remarkably are equally divergent from typical bacterial *nif* genes as are the methanogen *nif* genes. On the other hand, *vnfH*, *vnfE*, and *vnfN* evolved recently from the corresponding *nif* genes of Bacteria. Finally, horizontal gene transfer apparently occurred between ancestors of *M. barkeri* and *C. pasteurianum*.

Conclusions and Future Directions

Since the discovery of nitrogen fixation in methanogenic Archaea, it has become clear that the process is genetically and evolutionarily related to nitrogen fixation in Bacteria. However, phylogenetic analysis suggests that the relationship is as distant as are the two domains themselves. Given this distance, one expects to find in it the extremities in variations to the fundamental mechanism, making those aspects that are conserved all the more notable. The presence of *nifH*, *D*, *K*, *E*, *N*, and *X*, their gene order, and even whether they are essential for nitrogen fixation, is conserved. However, more work is needed to tell whether additional *nif* genes, less central to the process but nevertheless necessary in Bacteria, also exist in Archaea. The predominance of molybdenum as a metal of the nitrogenase cofactor also seems to extend across domains. Regulation varies across domains but also contains conserved features. The regulation of *nif* gene transcription in some methanogenic species involves repression, but the components of the nitrogen sensory-regulatory apparatus have yet to be identified. *glnB* homologues are present in both domains and may turn out to represent components of the regulatory apparatus in methanogens. We know already that divergent members of the *glnB* family exist in methanogens as well as less

divergent members, and the former function in post-transcriptional regulation of nitrogenase activity. Further work on nitrogen fixation in Archaea is sure to reveal additional variations on familiar paradigms.

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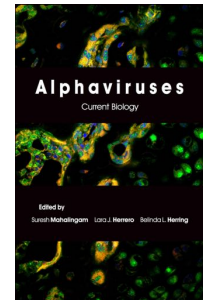
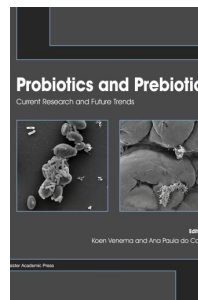
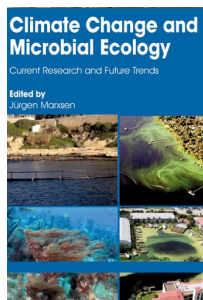
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