Nitrogenase Phylogeny and the Molybdenum Dependence of Nitrogen Fixation in * Methanococcus maripaludis *

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We studied the effects of molybdenum, vanadium, and tungsten on the diazotrophic growth of * Methanococcus maripaludis*. Mo stimulated growth, with a maximal response at 4.0 μM, while V had no effect at any concentration tested. W specifically inhibited diazotrophic growth in the presence of Mo. Coupling the results of our analysis and other known metal requirements with phylogenies derived from *nifD* and *nifK* genes revealed distinct clusters for Mo-, V-, and Fe-dinitrogenases and suggested that most methanogens also have molybdenum-type dinitrogenases.

Nitrogen fixation, the conversion of atmospheric N₂ to ammonia, is carried out by a variety of *Bacteria* and *Archaea* (23). Among the *Bacteria*, it occurs in at least four groups, the *Proteobacteria*, gram-positive bacteria, cyanobacteria, and green sulfur bacteria. Among *Archaea*, nitrogen fixation is found in all three orders of methanogens (10). Despite its wide phylogenetic distribution, the mechanism of nitrogen fixation is conserved. A typical dinitrogenase is an α₂β₂ tetramer encoded by *nifD* and *nifK* and containing an iron-molybdenum cofactor, FeMoCo (22). Mo-independent dinitrogenases (2) have cofactors that coordinate vanadium in place of molybdenum (V-dinitrogenases) or that have neither molybdenum nor vanadium (Fe-dinitrogenases); these are encoded by the *nifD* and *nifK* homologs *vnfD* and *vnfK* and *anfD* and *anfK*, respectively. An additional small subunit (6) found with the Mo-independent dinitrogenases is encoded by *vnfG* or *anfG*. Included in all nitrogenase complexes is dinitrogenase reductase, typically a homodimer in *Bacteria*, encoded by *nifH*, *vnfH*, or *anfH*.

The discovery of nitrogen fixation in methanogens in 1984 redefined the phylogenetic extremity of nitrogen fixation and opened the door to a series of new questions regarding biological nitrogen fixation (1, 12). Many of the properties common to nitrogen fixation in the *Bacteria* hold true in the *Archaea* as well. Lobo and Zinder found the nitrogenase complex from *Methanosarcina barkeri* to resemble those from *Bacteria* in subunit composition (except that dinitrogenase reductase appeared to be a homotetramer), substrate range, and immunological cross-reactivity of dinitrogenase reductase (9). *nif* genes from a variety of methanogens proved to be homologous and similar in organization to those from *Bacteria* (10). Phylogenetic analysis of *nifH*, *nifD*, and *nifK* showed the methanogen genes to be related to certain groups of bacterial genes (discussed below).

One question that has not been resolved is which metals are required for nitrogen fixation in methanogens and might therefore be incorporated into the dinitrogenase cofactor. Lobo and Zinder found that molybdenum but not vanadium stimulated nitrogen fixation in *M. barkeri* 227 (8). In contrast, Scherer reported stimulation by molybdenum and to a lesser extent by vanadium in two strains of *M. barkeri*, 227 and Fusaro (14). The issue is further complicated by the fact that *M. barkeri* 227 has two potentially active nitrogenases, as evidenced by the existence of two sets of *nif* genes that are phylogenetically related to functional *nif* genes of *Bacteria* and other methanogens. Purification of nitrogenase from extracts of *M. barkeri* cells grown in a Mo-supplemented medium yielded one major nitrogenase complex (9). The dinitrogenase reductase was encoded by a *nifH* gene (*nifH2*) related to the corresponding gene of a clostridial molybdenum nitrogenase complex (4). *nifD2* and *nifK2* from the same *M. barkeri* *nif* gene cluster were also related to the clostridial molybdenum dinitrogenase genes. Chien and Zinder (5) have therefore proposed that *M. barkeri* has a major molybdenum-dependent nitrogenase (encoded by the *nif2* set of genes) as well as an alternative nitrogenase (encoded by the *nif1* set of genes) that may be dependent on vanadium or independent of molybdenum and vanadium.

In the nitrogen-fixing methanogen *Methanococcus maripaludis* we found only one set of *nif* genes, and mutagenesis of *nifH* (3) or other *nif* genes (7) by transposon insertion eliminated nitrogen fixation. Here we show that nitrogen fixation in *M. maripaludis* was specifically inhibited when Mo was present in the culture medium, while neither V nor W affected growth of cultures grown in the presence of Mo. We also show that Mo but not V or W inhibits diazotrophic growth of cultures grown in Mo-free media. The results confirm earlier work suggesting that Mo is required for nitrogen fixation in methanogens, while V and W are not.

![FIG. 1. Growth of *M. maripaludis* as a function of Mo concentration. The curve between 0.04 and 4 μM represents the averages of quadruplicate cultures grown for 5 days. All other curves represent the averages of triplicates grown for 6 days in a separate experiment.](image-url)
is entirely dependent on molybdenum. We correlate this observation with nif phylogenies and found that an extended portion of the nifD and nifK trees belong to molybdenum-dependent nitrogenases. From this information we predict the nature of other methanogen nitrogenases.

In order to assess the metal requirements for nitrogen fixation in M. maripaludis, a nitrogen-free medium was prepared as described previously (3). Basal nitrogen-free medium lacked added molybdenum, vanadium, and tungsten. Na₂MoO₄·2H₂O, VCl₃, and Na₂WO₄·2H₂O, as well as NH₄Cl at a final concentration of 10 mM, were added as required to growth tubes as anaerobic solutions sterilized by passage through a 0.2-μm-pore-size filter. The tubes were inoculated with 0.1 ml of a twice-washed culture that had been grown to an optical density (OD) at 660 nm of 0.4 in modified medium 3 (3). The cultures were incubated under an N₂-H₂-CO₂ or Ar-H₂-CO₂ atmosphere as described previously (3). For argon controls, the tubes were additionally flushed with Ar-CO₂ for 10 min before being inoculated to remove all traces of N₂. ODs were measured against a medium blank prepared without sulfide.

Only molybdenum stimulated growth under nitrogen-fixing conditions. Final ODs at 660 nm under N₂ increased over a range of 0.04 to 4 μM Mo, with a half-maximal response near 0.4 μM (Fig. 1). At lower Mo concentrations, ODs remained at the level of control cultures under argon in which no nitrogen was present. Neither additional Mo nor argon affected growth with NH₄⁺. Neither vanadium (over a range of 0.64 to 64 μM) nor tungsten (3 μM) stimulated nitrogen fixation (results not shown). Since tungsten is an inhibitor of Mo-dependent nitrogenase formation, we examined the effect of tungsten on diazotrophic growth. In the presence of 4 μM Mo, W specifically inhibited nitrogen fixation at 1.5 μM and above (Fig. 2). These results indicate that nitrogen fixation in M. maripaludis is carried out entirely by a molybdenum-dependent nitrogenase. In order to attribute molybdenum dependence rigorously to the nitrogenase genes that we have cloned and sequenced, we tested a nifK insertion mutant, Mm31 (7), in our assay for nitrogen fixation. No growth on N₂ was observed.

We next asked whether our finding that the M. maripaludis nitrogenase was molybdenum dependent could shed new light on the phylogenetic distribution of nitrogenases. Previous phylogenetic analyses of nifH, nifD, and nifK had been done (4, 5, 23), and we repeated them, inserting the information for M. maripaludis. nifH (3), nifD, and nifK (7) were cloned from M. maripaludis. FIG. 3. Unrooted distance matrix trees for nifD (A) and nifH (B) amino acid sequences. Abbreviations: Ana7120, Anabaena strain PCC 7120; Anaava, Anabaena variabilis; Azoc, Azotobacter chroococcum; Azovi, Azotobacter vinelandii; Azoh, Azospirillum brasilense; Clopa, C. pasteurianum; Frasp, Frankia species (strain EUN1 for nifD and strain Ar3 for nifH); Klep, Klebsiella pneumoniae; Metar, Methanobrevibacter arboriphilicus; Metiv, Methanococcus ivanovii; Metla, Methanospirillum hungatei; Metma, Methanococcus maripaludis; Mettl, Methanococcus thermolithotrophicus; Metma, Methanococcus maripaludis; Metla, Methanospirillum hungatei; Plebo, Plectonema boryanum; Rhime, Rhizobium meliloti; Rhiph, Rhizobium phaseolicola; Rhipv, Rhizobium strain ANU29 (Parasponia symbiont); Rhoca, Rhodobacter capsulatus; Rhoru, Rhodospirillum rubrum; Synsp, Synechococcus species. The roman numerals in the nifH tree are those of Chien and Zinder (4). nif and anf are genes that have been positively identified as belonging to vanadium- or iron-type nitrogenases (6, 13, 15). Numerical designations follow the published convention where more than one nif gene is present in a species (11, 16–21).

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maripaludis and sequenced on both strands with a combination of subcloning and walking primers by cycle sequencing with the Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). Gels were run by the DNA core facility of the University of Washington’s Molecular Pharmacology Facility, and the results were aligned to form contigs by using SeqApp 1.9, a biosequence editor and analysis application written by D. G. Gilbert. The sequence analysis package of the University of Wisconsin’s Genetics Computer Group was used to align predicted amino acid sequences based on nifH, nifD, and nifK genes. Phylogenetic analysis was performed by distance matrix with Protist and Fitch and by parsimony with Protpars, both from the Phylogeny Inference Package (PHYLIP version 3.572) by Joseph Felsenstein. The distance matrix and parsimony analyses gave similar results.

The results of our phylogenetic analyses agree with previous studies (4, 5) and show that the M. maripaludis genes cluster with certain other methanogen nif genes. The nifD (Fig. 3A) and nifK (not shown) trees show essentially identical patterns and, together with our analysis of metal dependence, establish that an extended portion of the tree belongs to molybdenum dinitrogenases. This model is supported by the known molybdenum extended portion of the tree belonging to molybdenum and, together with our analysis of metal dependence, establish from M. thermolithotrophicus M. maripaludis ium pasteuriannum groupings.

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The metal requirements for nitrogenase activity determined by including that of M. barkeri nifH2 and the pattern of the nifD tree and is found in cluster III with a collection of Mo-dinitrogenase reductase sequences found in C. pasteurianum. In addition, a fourth group of genes from methanogens is present, cluster IV; these genes are related to certain genes involved in bacteriochlorophyll synthesis and probably perform a function other than nitrogen fixation.

In conclusion, the nifH tree (Fig. 3B) shows evidence for the interchangeability of dinitrogen reductases between Mo- and Fe-dinitrogenase subunits forming separate monophyletic groupings. In contrast, the nifH tree (Fig. 3B) shows a monophyletic grouping of bacteria, p. 191–211. In G. Stacey, R. H. Burris, and H. J. Evans (ed.), Biological nitrogen fixation. Chapman and Hall, New York, N.Y.

REFERENCES


