

## Analysis of Genes Encoding an Alternative Nitrogenase in the Archaeon *Methanosarcina barkeri* 227

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*Methanosarcina barkeri* 227 possesses two clusters of genes potentially encoding nitrogenases. We have previously demonstrated that one cluster, called *nif2*, is expressed under molybdenum (Mo)-sufficient conditions, and the deduced amino acid sequences for nitrogenase structural genes in that cluster most closely resemble those for the Mo nitrogenase of the gram-positive eubacterium *Clostridium pasteurianum*. The previously cloned *nifH1* from *M. barkeri* shows phylogenetic relationships with genes encoding components of eubacterial Mo-independent eubacterial alternative nitrogenases and other methanogen nitrogenases. In this study, we cloned and sequenced *nifD1* and part of *nifK1* from *M. barkeri* 227. The deduced amino acid sequence encoded by *nifD1* from *M. barkeri* showed great similarity with *vnfD* gene products from vanadium (V) nitrogenases, with an 80% identity at the amino acid level with the *vnfD* gene product from *Anabaena variabilis*. Moreover, there was a small open reading frame located between *nifD1* and *nifK1* with clear homology to *vnfG*, a hallmark of eubacterial alternative nitrogenases. Stimulation of diazotrophic growth of *M. barkeri* 227 by V in the absence of Mo was demonstrated. The unusual complement of *nif* genes in *M. barkeri* 227, with one cluster resembling that from a gram-positive eubacterium and the other resembling a eubacterial V nitrogenase gene cluster, suggests horizontal genetic transfer of those genes.

The nitrogenase enzyme complex typically found in eubacteria contains two components, component 2, which contains a single iron-sulfur center and is encoded by *nifH*, and component 1, which contains an iron-sulfur center and an iron-molybdenum cofactor and is encoded by *nifD* and *nifK*. There also exist alternative nitrogenases that contain vanadium instead of molybdenum in their component 1 and are encoded by *vnf* genes or that contain only iron in their component 1 and are encoded by *anf* genes (3). Component 1 of alternative nitrogenases also contains a third subunit type encoded by *vnfG* or *anfG*. This subunit has been found in all alternative nitrogenases studied and appears to play a role in cofactor processing and function (6, 29). The *vnfG/anfG* genes are situated between the *vnfD/anfD* and the *vnfK/anfK* genes, and in the case of *Anabaena variabilis* (28), the *vnfD* and *vnfG* genes form a single open reading frame (ORF).

Sibold and colleagues (25) demonstrated that the methanogenic archaeon *Methanosarcina barkeri* 227 possesses two sets of *nifH* genes (designated *nifH1* and *nifH2*), and our studies have expanded upon this finding. Phylogenetic analysis of predicted gene products (Fig. 1) shows that *nifH1* from *M. barkeri* clusters with several other methanogen *nifH* genes as well as *anfH* genes from several eubacteria in a clade we have termed cluster II (7). The *nifH2* gene product from *M. barkeri* clusters with part of a Mo nitrogenase from *Clostridium pasteurianum* and those of other anaerobes, such as *Desulfovibrio gigas*, in a clade called cluster III. Most common eubacterial nitrogenase *nifH* gene products are in cluster I. Not shown in Fig. 1 are more distantly related *nifH* homologues found in methanoar-

chaea which apparently serve purposes other than nitrogen fixation (7, 18, 21) and *nifH* homologues in eubacteria which play a role in chlorophyll synthesis (5).

We cloned and sequenced *nifD2* and *nifK2* (7) and *nifE2* and part of *nifN2* (8) from *M. barkeri* 227 and demonstrated that all of these genes clustered most closely with their corresponding *nif* homologues in *C. pasteurianum*. Moreover, we demonstrated that the *nif2* genes were expressed during growth in typical Mo-sufficient medium (7, 8). We had previously demonstrated that Mo stimulated diazotrophic growth in *M. barkeri* 227 (16), so it appears likely that the *nif2* gene cluster contains genes involved in the synthesis of a Mo nitrogenase.

The role of the *M. barkeri nifH1* gene described by Sibold et al. (25) (GenBank accession no. X56072) is not clear. Although the eubacterial members of cluster II are part of *anf*-type alternative nitrogenase genes, evidence has been obtained that at least in the cases of *Methanococcus thermolithotrophicus* (17) and *Methanococcus maripaludis* (15) these genes encode proteins that are components of Mo nitrogenases. One possibility is that the *nif1* genes encode an alternative nitrogenase, possibly of the vanadium (V) type. In our initial attempts to detect stimulation of diazotrophic growth by V (16), inconclusive results were obtained. Scherer (23) showed stimulation of diazotrophic growth by either Mo or V in *M. barkeri* Fusaro and mentioned that similar but less convincing results could be obtained with strain 227.

To obtain a better understanding of the function of the *nif1* genes in *M. barkeri*, we used PCR to clone and sequence the remaining part of *nifD1* from *M. barkeri*, only 94 nucleotides of which had been sequenced by Sibold et al. (25), as well as part of *nifK1*. In this publication, we describe these genes and their phylogenetic similarity to *vnf*-type alternative nitrogenases, as well as a homologue of *vnfG/anfG* found between *nifD1* and *nifK1* in *M. barkeri* 227. We also describe experiments demonstrating that V stimulated diazotrophic growth in *M. barkeri* 227.

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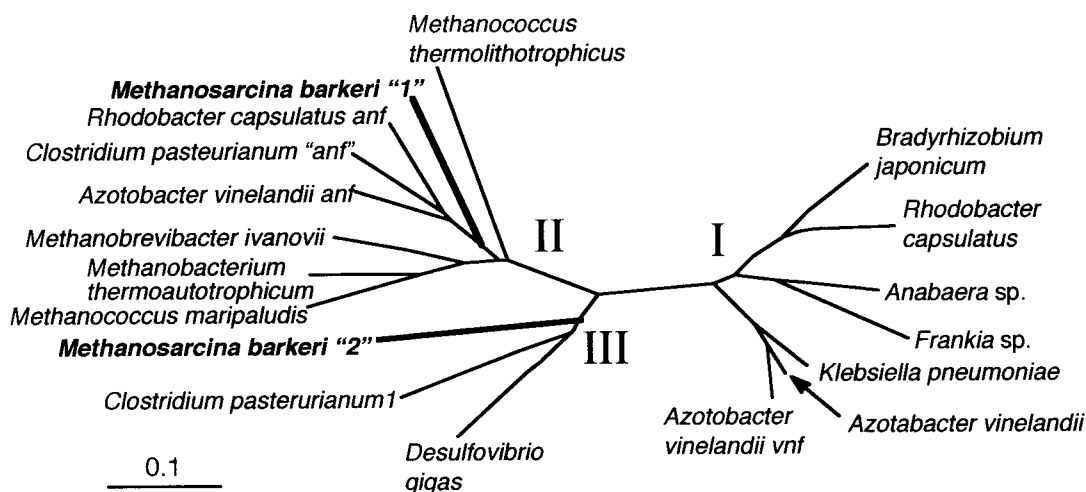


FIG. 1. Unrooted phylogenetic tree for *nifH* gene products prepared using the neighbor-joining method as described in Materials and Methods. Sequences in clusters II and III are more highly represented than are those in cluster I, and the two *nifH* sequences from *M. barkeri* 227 are in boldface.

#### MATERIALS AND METHODS

**Cloning and sequencing studies.** *M. barkeri* 227 (ATCC 43241, DSM 1538, and OCM 35) was obtained from our own culture collection, and DNA was extracted and purified from it as described previously (7). Two primers were designed for the amplification of the *nifDK* segment. The forward primer (5'-GAAGAAGGAGAAGACAC-3') was designed from the published sequence (25) of the *M. barkeri nifD1* gene. The degenerate, reverse primer [5'(AG)CA(AGTC)CC(TC)G(AGTC)CC(AGTC)CC(AG)TG-3'] was designed from the alignment of 12 different *nifK* sequences. A Hybaid OMN-E thermal cycler (Labnet, Woodbridge, N.J.) was used for the PCRs. The solutions were subjected to 32 cycles of PCR (denaturing, 1 min at 92°C; annealing, 40 s at 50°C; and extension, 1 min at 72°C). Ligation was performed overnight using an Invitrogen (San Diego, Calif.) TA cloning kit and pCR2.0 plasmid and subsequent transformation of the plasmid into competent *Escherichia coli* cells. DNA sequencing was performed at the Cornell Biotechnology Institute using an ABI 373A automatic sequencing apparatus and vector-specific and internal primers.

Phylogenetic analysis was performed on amino acid sequences aligned using Clustal X software (12) by using the PHYLIP 3.5 package (10), specifically the PROTDIST, PROTPARS, NEIGHBOR, and FITCH programs as previously described (7). Phylogenetic trees were generated using TreeView software (20). Bootstrap analyses were performed on 100 replicates using the SEQBOOT, NEIGHBOR, and CONSENSE programs.

**Media and conditions for examining effect of vanadium on growth.** *M. barkeri* 227 (ATCC 43241, DSM 1538, and OCM 35) cultures were grown in molybdenum-free minimal salts medium based on that of Scherer (23), which contained the following (in millimolar): imidazole, 50; NaH<sub>2</sub>PO<sub>4</sub>, 0.5; Na<sub>2</sub>HPO<sub>4</sub>, 0.5; MgCl<sub>2</sub>, 1.7; CaCl<sub>2</sub>, 2; KCl, 5.4; NaCl, 34; and trace elements consisting of (in micromolar) H<sub>3</sub>BO<sub>3</sub>, 1.45; CoCl<sub>2</sub>, 0.25; NaSeO<sub>3</sub>, 0.1; ZnCl<sub>2</sub>, 0.1; MnCl<sub>2</sub>, 0.045; NiCl<sub>2</sub>, 0.025; and CuCl<sub>2</sub>, 0.017. All glassware used in molybdenum starvation experiments was prepared by washing twice with 1 M H<sub>2</sub>SO<sub>4</sub> followed by copious rinsing in MilliQ water (Millipore Corp., Bedford, Mass.). Moreover, all chemicals used in the molybdenum starvation experiments were of the highest grade obtainable. V was added in the form of vanadyl sulfate (Gold Label; Aldrich, Milwaukee, Wis.) containing less than 1 ppm of molybdenum. This medium was prepared anaerobically and dispensed in 50-ml amounts into 126-ml serum bottles under a pure N<sub>2</sub> atmosphere (16). After autoclaving, each bottle was supplemented with the following: methanol (8 M), 1 ml; sodium acetate (2.5 M), 1 ml; H<sub>2</sub>S (gas), 1 ml; NaHCO<sub>3</sub> (1%), 0.1 ml; ferric citrate (50 mM), 0.05 ml; titanium(III) citrate (83 mM) (30), 0.1 ml; and the relevant metal addition or ammonia addition. H<sub>2</sub>S gas was prepared by the acidification (5 ml of 1 M HCl) of 5 ml of anoxic 20% (wt/vol) Na<sub>2</sub>S in a 126-ml nitrogen-sparged sealed vial.

Mo-starved starter cultures were prepared in the Mo-free medium with the addition of limiting amounts of ammonium chloride (2 mM). After three transfers (2%, vol/vol), cultures grew more slowly and were suitable for metal addition experiments. All bottles were inoculated with 1 ml of *M. barkeri* grown in ammonia-supplemented Mo-free medium at 37°C. Growth was determined principally by methane production using a thermal conductivity gas chromatograph (16).

**Nitrogenase assays.** Whole-cell nitrogenase enzyme activity was determined by the acetylene reduction assay reaction in sealed 36-ml argon-filled acid-washed serum vials as previously described by Lobo and Zinder (16), except that a 1.5-m by 3.2-mm stainless steel column packed with 80/100 mesh alumina F-1

(Supleco, Bellefonte, Pa.) was used to resolve methane, ethane, ethene, and acetylene.

**Nucleotide sequence accession number.** The accession number for the sequences reported in this paper is AF254784.

#### RESULTS

**Analysis of the *nifHDK1* gene cluster in *M. barkeri* 227.** Figure 2 presents a map of the *M. barkeri nifI* region cloned and sequenced by Sibold et al. (25) and ourselves. Like all functional *nif* gene clusters in methanogens studied to date, there are two small ORFs, both resembling eubacterial *glnB* genes (25), located between *nifH1* and *nifD1*. The deduced *nifD1* gene was 1,398 bp long (including a TGA encoding a translation stop), has approximately 41 mol% G+C, and encodes a polypeptide of 465 amino acids with a predicted molecular mass of 52,807 Da. The predicted polypeptide lacked the 50-amino-acid insert present in the *M. barkeri nifD2* gene product and the *nifD1* gene product from *C. pasteurianum*.

Immediately following the TGA encoding the translation stop of the *nifD1* gene is an ATG encoding a translation start of another ORF which, as described in more detail below, shows clear homology to *vnfG* and *anfG* genes of alternative nitrogenases. Eight base pairs upstream of this ATG is an AGGAA encoding a potential Shine-Dalgarno translation initiation signal within the putative *nifD1* gene. This "nifG" ORF is 336 bp long, including a TAA encoding a translation stop, and encodes a predicted polypeptide with 111 amino acids and a molecular mass of 12,897 Da. Nineteen base pairs downstream from *nifG1* is the sequence encoding the potential translation start site of an ORF containing *nifK1*, of which there are 146 bp of data until the end of the cloned fragment.

We performed a phylogenetic analysis of the predicted gene product of the *nifD1* gene, and the results are presented in Fig. 3. It clearly clustered with the *vnfD* gene products from *Azotobacter vinelandii* and *Anabaena variabilis*, with a closer relationship with the latter, and was distant from the other methanogen *nifD* gene products. The identity at the amino acid level with the *A. variabilis vnfD* gene product was 80%, whereas it was 69, 58, and 35% with the *A. vinelandii vnfD*, *anfD*, and *nifD* gene products, respectively. Thus, there was a high degree of similarity with V nitrogenases. The eubacterial *anfD* gene products clustered with the *vnfD* gene products to the exclu-

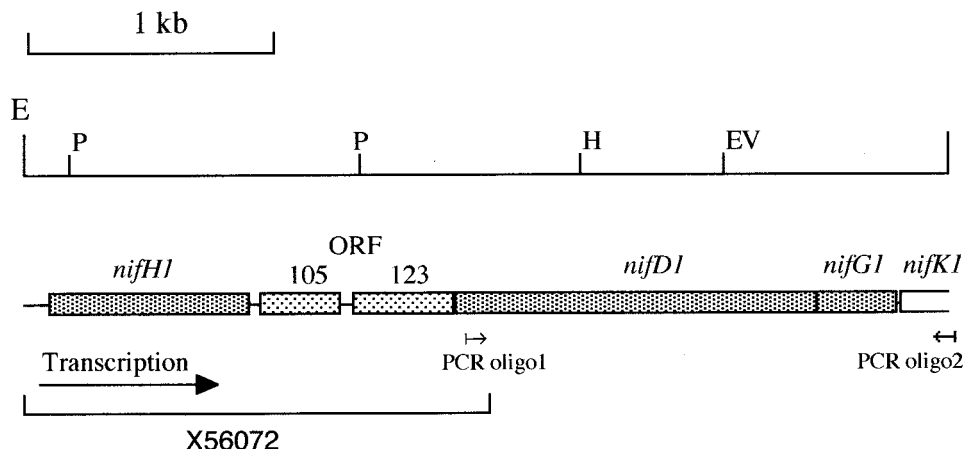


FIG. 2. Physical map of a 3.4-kb DNA fragment from *M. barkeri* 227 containing *nifHDGK1* genes. The original clone of Sibold et al. (25) contained *nifH1*, ORF105, ORF123, and a small part of *nifD1*. The PCR oligonucleotide primers (termed oligo1 and oligo2), used for PCR cloning of the *nifDGK1* genes, are indicated by arrows. The direction of transcription for the *nif* genes is shown by a large arrow. H, *Hind*III; E, *Eco*RI; P, *Pst*I; EV, *Eco*RV.

sion of the three methanogen *nifD* gene products in cluster II, which, it could be argued, form a separate cluster. As previously demonstrated (8), the *nifD2* gene product from *M. barkeri* was most closely related to that of *C. pasteurianum*.

Figure 4 shows the phylogenetic relationship between the *M. barkeri* “*nifG1*” gene product and the corresponding predicted sequences of various *vnfG* and *anfG* gene products. Similar to the case for the *nifD* gene products, the “*nifG*” gene product

from *M. barkeri* had the greatest similarity with the *vnfG* gene product from *A. variabilis*. However, the *nifG* gene product amino acid sequences were not nearly as conserved as were those for *nifD*. The *M. barkeri nifG1* predicted gene product had 44, 38, and 35% amino acid identity with the *A. variabilis vnfG* gene product and the *A. vinelandii vnfG* and *anfG* gene products, respectively. The *vnf* and *anf* genes clearly formed distinct phylogenetic clusters.

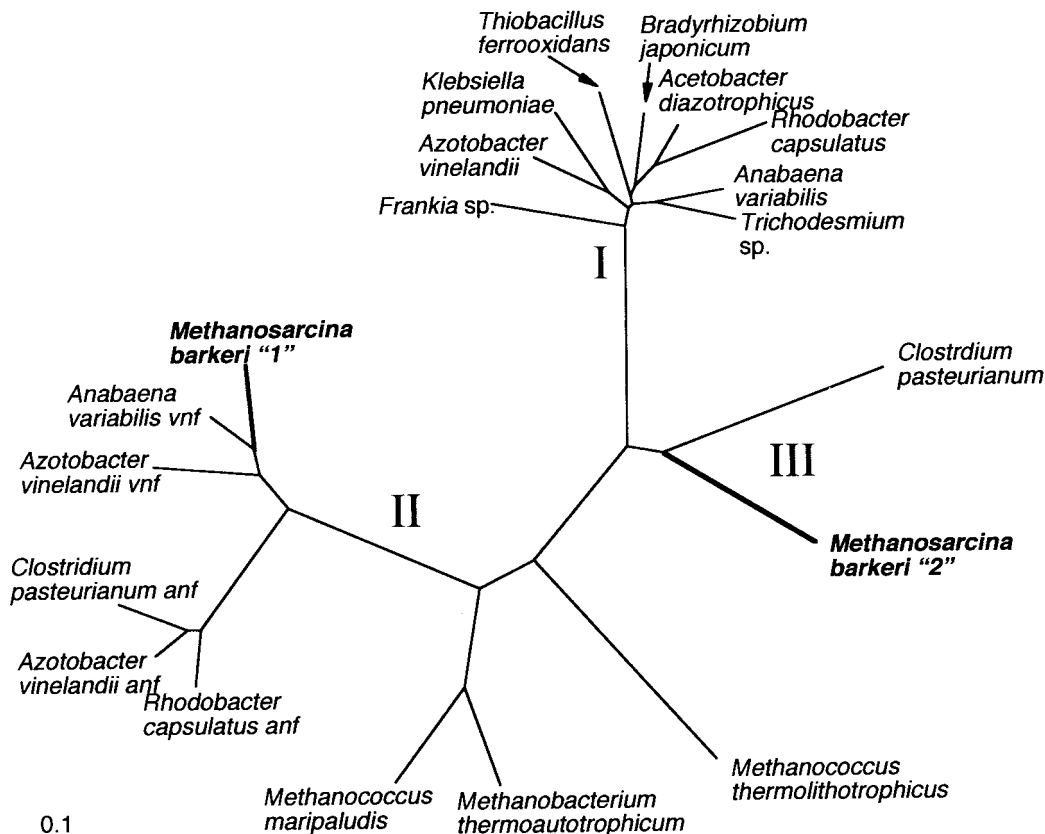


FIG. 3. Unrooted phylogenetic tree for *nifD* gene products prepared using the neighbor-joining method. The *nifD* gene products from *M. barkeri* 227 are in boldface.

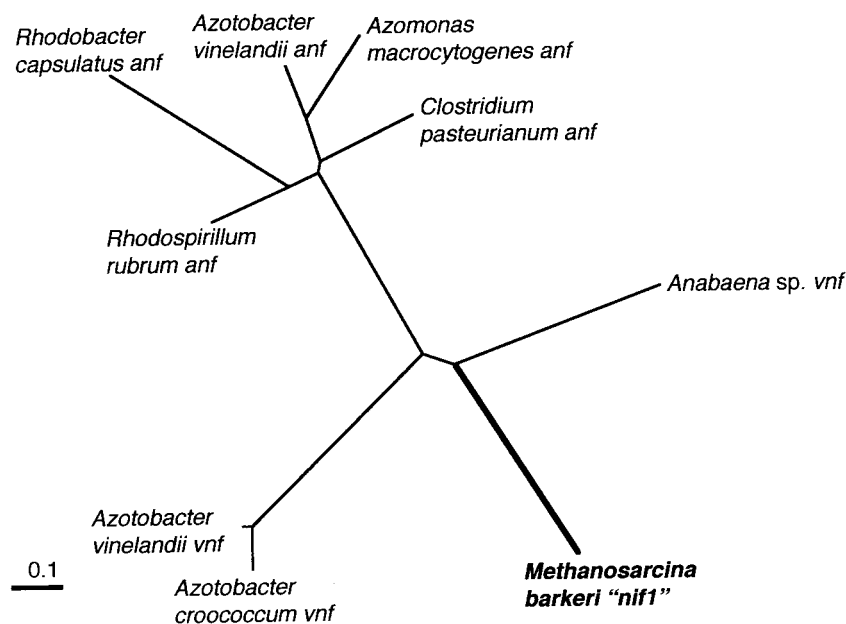


FIG. 4. Unrooted tree prepared using the neighbor-joining method showing phylogeny of *vnfG/anfG* gene products. The "*nifG1*" sequence from *M. barkeri* 227 is in boldface.

**Diazotrophy of *M. barkeri* 227 grown with V in the absence of Mo.** We have found it difficult to obtain reproducible Mo limitation of growth using our standard growth medium and conditions. In our original studies on trace element requirements for diazotrophy in *M. barkeri* 227, growth in the absence of added Mo was 60% of that with Mo. Examining the procedures developed by Scherer (23) for metal limitation of growth of *M. barkeri* 227, we found that one particularly important source of variability is sodium sulfide, which, despite our rinsing the crystals with distilled water, seems to be a major source of contamination. Replacing it with H<sub>2</sub>S gas generated by acidification of sodium sulfide appeared to limit the amount of variability. Indeed, we were eventually able to detect Mo limitation of growth with ammonia, which is expected since *M. barkeri* uses Mo as a prosthetic group in the enzyme formylmethanofuran dehydrogenase (13, 24), which carries out a reaction analogous to that of formate dehydrogenase. We also found that it took at least two transfers in medium lacking added Mo from our standard medium containing ca. 0.4 μM Mo before we detected growth limitation. Similar results were obtained in studies on *C. pasteurianum* by Hinton and Mortenson (11), who detected molybdenum storage proteins in that organism.

Using medium and conditions adapted from those of Scherer, we were able to demonstrate V stimulation of diazotrophic growth as measured by methane production (Fig. 5). We (16, 19) and Scherer (23) have found an excellent correlation between methanogenesis and growth in *Methanosarcina* spp. The culture was able to grow with ammonium in the absence of added Mo or V either because the amounts of these elements in the medium met the needs of the nondiazotrophic cells or, perhaps, because there was some contaminating Mo or V in the ammonium chloride used. Schmitz et al. (24) found that *M. barkeri* Fusaro could grow with methanol without added Mo, whereas growth with H<sub>2</sub>-CO<sub>2</sub>, which presumably required higher levels of the molybdoenzyme formylmethanofuran dehydrogenase, was Mo dependent. Under diazotrophic conditions, methanogenesis was severely limited in the absence of

added Mo or V, and there was slight stimulation by the vitamin solution that we added, similar to results of Scherer (23). Addition of Mo greatly stimulated methanogenesis under diazotrophic conditions, and V caused a smaller but still significant effect.

We also examined the effects of the Mo antagonist tungsten (W) (added as sodium tungstate) on methanogenesis when the organism was growing diazotrophically with Mo or V. W inhibits Mo nitrogenases but not alternative ones (3). Our previous results (16) demonstrated that W inhibited methanogenesis by *M. barkeri* 227 when neither Mo nor V was added to the medium under diazotrophic conditions but not when growth was with NH<sub>4</sub><sup>+</sup> as the nitrogen source. As shown in Fig. 6, the stimulation of methanogenesis by Mo under diazotrophic conditions was completely inhibited by adding a 100-fold excess of W. As before, adding V caused a smaller stimulation of methanogenesis under diazotrophic conditions, but addition of 100 μM W caused slight, if any, inhibition.

A hallmark of V-type alternative nitrogenases is that when they reduce acetylene, they produce ethane amounting to 1 to 3% of the amount of ethene produced, whereas ethane is barely detectable from Mo-type nitrogenases (3, 9). Methanogenic nitrogenases reduce acetylene at very low rates overall compared to eubacterial nitrogenases (2, 16, 17), so that results may not be completely comparable. In the case of *M. barkeri* 227 growing diazotrophically in the absence of Mo and in the presence of V, the ethane produced by cells was 1.24% ± 0.14% of the ethene produced, whereas ethane was undetectable in cells growing with Mo in the growth medium.

## DISCUSSION

The results presented here support the hypothesis that the *nifI* gene cluster from *M. barkeri* 227 encodes an alternative nitrogenase, most likely of the vanadium type. Evidence supporting the presence of an alternative nitrogenase includes the presence of a "*nifG*" gene (heretofore found only in alternative nitrogenases) in the *nifI* cluster, the sequence similarity be-

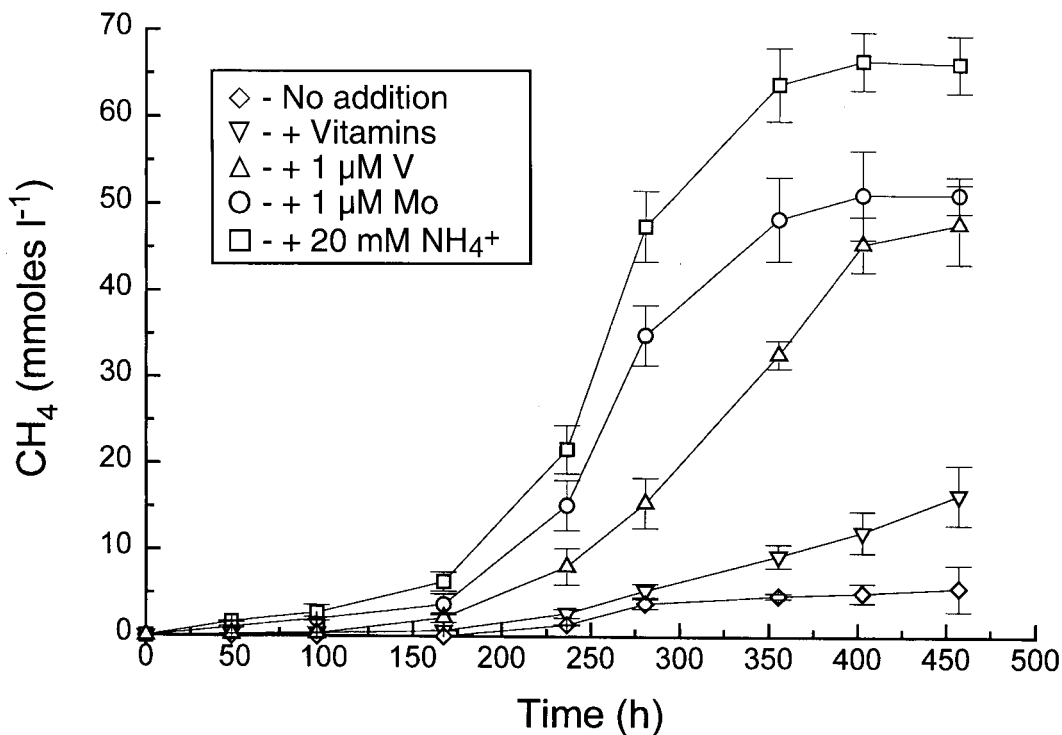


FIG. 5. Effects of additions of trace metals on methanogenesis by growing cultures of *M. barkeri* 227. All cultures except the culture designated No addition received a vitamin mixture (1). Except in the case of addition of NH<sub>4</sub><sup>+</sup>, the cultures were grown diazotrophically. Error bars indicate standard deviations.

tween the *nifG1* and *nifD1* gene products and their *vnf* counterparts in *A. variabilis*, the stimulation of diazotrophic growth by V added to the medium, the lack of inhibition by W of nitrogen-fixing cells grown with V, and the production of sig-

nificant amounts of ethane from acetylene. Direct demonstration that the purified enzyme is a vanadium nitrogenase would require much more cell material than we were able to obtain using Mo-depleted medium. Thus, our evidence for a V-con-

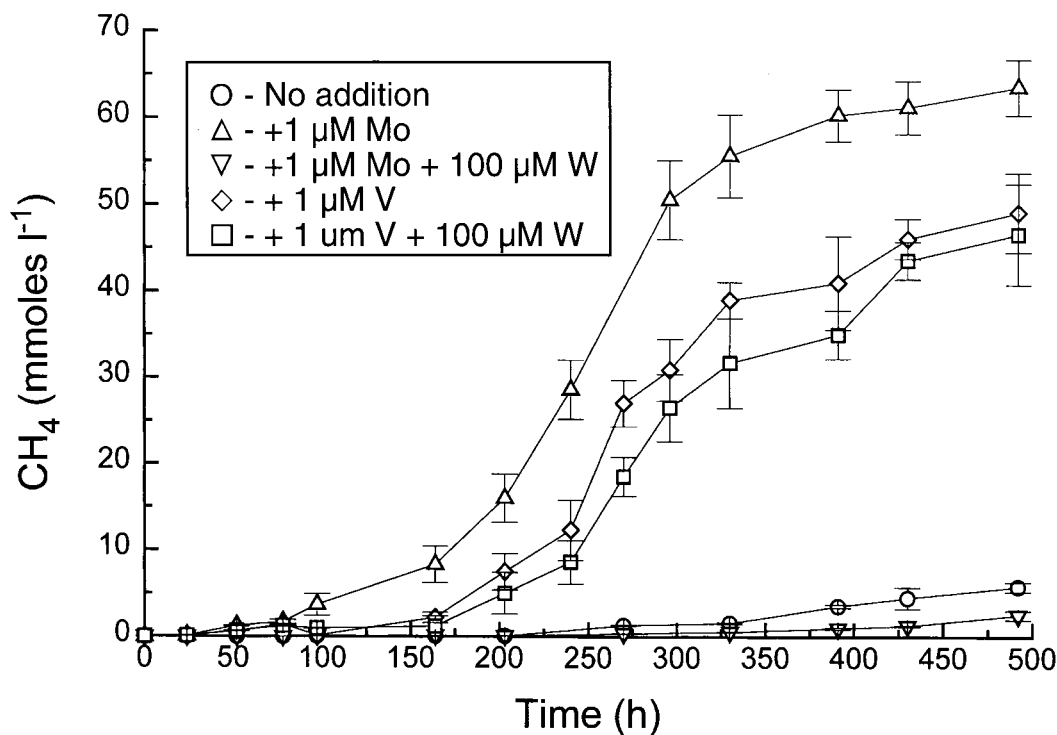


FIG. 6. Effects of W on methanogenesis by *M. barkeri* 227 grown diazotrophically in the presence of Mo or V. Error bars indicate standard deviations.

taining nitrogenase in *M. barkeri* is tentative, depending mainly on the stimulation of diazotrophic growth by V in Mo-depleted medium.

The *nifI* gene cluster in *M. barkeri* clearly resembles those of other methanogens (4, 25–27), containing two ORFs homologous to *glnB* located between *nifH* and *nifD*. These ORFs are not found in eubacterial *nif* gene clusters and have recently been shown to modulate ammonia switch-off of nitrogenase activity in *Methanococcus maripaludis* (14). However, unlike other methanogen *nif* gene clusters, but similar to eubacterial *vnf* and *anf* clusters, there is an ORF located between *nifD1* and *nifK1* in *M. barkeri*. The “*nifG1*” of *M. barkeri* clearly clusters with *vnfG* genes, with closest homology to *vnfG* of the cyanobacterium *A. variabilis*.

The 80% amino acid identity between deduced *nifD1* gene product from *M. barkeri* and the *vnfD* gene product from the cyanobacterium *A. variabilis* is one of the highest degrees of identity ever found between eubacterial and archaeal gene products (R. Doolittle, personal communication). To provide perspective, the identity between the *nifD* polypeptides in *A. vinelandii* and *Klebsiella pneumoniae*, two members of the gamma proteobacteria, is 72%. The moles percent G+C for the *M. barkeri nifD1* gene is 41%, essentially identical with the organism's value of 42% (1), whereas that for the *A. variabilis* ATCC 29413 *vnfDG* genes is 45.3%, similar to that of the organism's genomic DNA, which is 45% (22). It is difficult to imagine how these genes could be so highly conserved in the absence of genetic transfer, but it is not clear in which direction this putative transfer occurred. The fact that the *vnfD* gene product from the eubacterium *A. vinelandii* clusters outside the *M. barkeri* and *A. vinelandii* products suggests that the transfer occurred into an ancestor of *M. barkeri*; however, more *vnfD* sequences need to be obtained from diverse organisms to clarify this situation. For example, there are no *vnf* genes thus far detected in clostridia.

The situation regarding *vnfH* genes is interesting. Whereas the *vnfD* gene from *A. vinelandii* is clearly an alternative nitrogenase gene in cluster II (Fig. 3), the *A. vinelandii vnfH* gene is very closely related to *nifH* in cluster I (Fig. 1). This has been interpreted as a recruitment of a copy of *nifH* by the *vnfDGK* genes in *A. vinelandii* (3). In the case of the cyanobacterium *A. variabilis* (28), no *vnfH* was found immediately upstream or downstream of the *vnfDGK* genes, and all four *nifH*-homologous genes found in this organism fall within cluster I (T. Thiel, personal communication). Thus, an authentic *vnfH* has not been yet identified. The *M. barkeri nifH1* gene product clusters with *anfH* gene products to the exclusion of other methanogen *nifH* gene products in a neighbor-joining analysis (Fig. 1), suggesting that it may be an authentic *vnfH* gene. Analysis of the same data set shown in Fig. 1 using least-squares analysis (FITCH program [see Materials and Methods]) or parsimony analysis (PROTPARS program) gave similar results, but in a bootstrap analysis using neighbor joining, the *M. barkeri* product clustered with the *anfH* gene products to the exclusion of the methanogen sequences in only 59 of 100 replicates. Thus, it is possible that the *M. barkeri nifH1* gene was a methanogenic *nifH* recruited by incoming *vnf* genes, similar to the situation proposed for *A. vinelandii*.

The genes encoding *M. barkeri* nitrogenases are considerably different from those in other methanogens examined thus far, which have cluster II genes apparently encoding their Mo nitrogenases and lack alternative nitrogenases. In contrast, *M. barkeri* contains one set of *nif* genes resembling those of *C. pasteurianum* and another resembling eubacterial *vnf* genes, including the presence of a *vnfG*-homologous ORF. Despite these differences, both *nif* gene clusters contain two ORFs

homologous to *glnB*, making them similar to other methanogen *nif* gene clusters. It is difficult to hypothesize a scenario that can account for these similarities and differences, and perhaps more *nif* sequences from diverse archaea and eubacteria will clarify this situation.

Because the *nif2* genes in *M. barkeri* are most homologous to those encoding the Mo nitrogenase from *C. pasteurianum* and because we have demonstrated that they are expressed in Mo-containing medium (8), we propose calling them simply *nif* genes. Because of their similarity to *vnf* genes and the evidence for V stimulation of diazotrophic growth of *M. barkeri*, we propose calling the *nif1* genes in *M. barkeri* “*vnf*,” with the quotation marks indicating the tentative assignment.

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