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Cloning, DNA Sequencing, and Characterization of a *nifD*-Homologous Gene from the Archaeon *Methanosarcina barkeri* 227 Which Resembles *nifD1* from the Eubacterium *Clostridium pasteurianum*

YUEH-TYNG CHIEN AND STEPHEN H. ZINDER*

Section of Microbiology, Cornell University, Ithaca, New York 14853

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L. Sibold, M. Henriquet, O. Possot, and J.-P. Aubert (Res. Microbiol. 142:5–12, 1991) cloned and sequenced two *nifH*-homologous open reading frames (ORFs) from *Methanosarcina barkeri* 227. Phylogenetic analysis of the deduced amino acid sequences of the *nifH* ORFs from *M. barkeri* showed that *nifH1* clusters with *nifH* genes from alternative nitrogenases, while *nifH2* clusters with *nifH1* from the gram-positive eubacterium *Clostridium pasteurianum*. The N-terminal sequence of the purified nitrogenase component 2 (the *nifH* gene product) from *M. barkeri* was identical with that predicted for *nifH2*, and dot blot analysis of RNA transcripts indicated that *nifH2* (and *nifDK2*) was expressed in *M. barkeri* when grown diazotrophically in Mo-containing medium. To obtain *nifD2* from *M. barkeri*, a 4.7-kbp *Bam*HI fragment of *M. barkeri* DNA was cloned which contained at least five ORFs, including *nifH2*, ORF105, and ORF125 (previously described by Sibold et al.), as well as *nifD2* and part of *nifK2*. ORF*nifD2* is 1,596 bp long and encodes 532 amino acid residues, while the *nifK2* fragment is 135 bp long. The deduced amino acid sequences for *nifD2* and the *nifK2* fragment from *M. barkeri* cluster most closely with the corresponding *nifDK1* gene products from *C. pasteurianum*. The predicted *M. barkeri nifD2* product contains a 50-amino acid insert near the C terminus which has previously been found only in the clostridial *nifD1* product. Previous biochemical and sequencing evidence indicates that the *C. pasteurianum* nitrogenase is the most divergent of known eubacterial Mo-nitrogenases, most likely representing a distinct *nif* gene family, which now also contains *M. barkeri* as a member. The similarity between the methanogen and clostridial *nif* sequences is especially intriguing in light of the recent findings of sequence similarities between gene products from archaea and from low-G+C gram-positive eubacteria for glutamate dehydrogenase, glutamine synthetase I, and heat shock protein 70. It is not clear whether this similarity is due to horizontal gene transfer or to the resemblance of the *M. barkeri* and *C. pasteurianum* nitrogenase sequences to an ancestral nitrogenase.

Nitrogenase, the two-component enzyme complex responsible for reduction of N_2 to NH_3, shows high levels of conservation of structure, function, and amino acid sequence across wide phylogenetic ranges (11). In typical Mo-nitrogenases, component 1 (also called the MoFe-protein or dinitrogenase) is an $\alpha_2\beta_2$ tetramer encoded by *nifD* and *nifK*. It contains two unusual metal clusters, an 8Fe-8S cluster called the P cluster and the FeMo-cofactor, which has the proposed composition 7Fe-9S-Mo (22) and is considered to be the site of N_2 reduction. Component 2 (also called the Fe-protein or dinitrogenase reductase) is a homodimer with a single 4Fe-4S cluster linking the subunits (18) and is encoded by *nifH*. There is an especially high degree of sequence conservation in *nifH* gene products (11, 18). Two discoveries in the 1980s greatly increased the understanding of nitrogenase diversity: the discovery of alternative nitrogenases and the discovery of nitrogen fixation in methanogenic archaea (archaebacteria).

Bishop et al. (5) originally proposed the existence of alternative nitrogenases, generally expressed in the absence of Mo, which contain either V (vanadium) instead of Mo in their component 1 (V-nitrogenases) or contain only Fe (Fe-nitrogenases). V- and Fe-nitrogenase genes have been called *vnf* and *anf* respectively, or are given numbers such as *nif2* and *nif3*, respectively, in the case of *Azotobacter vinelandii*. Evidence for the presence of alternative nitrogenases or their genes has now been obtained for a wide variety of eubacteria (14, 15, 21, 24, 26, 51, 57). Alternative nitrogenases show biochemical properties and amino acid sequences which are distinct from those of Mo-nitrogenases (6).

In 1984 and 1985, nitrogen fixation was reported for the methanogenic archaea *Methanosarcina barkeri* (7, 34) and *Methanococcus thermolithotrophicus* (3), the first description of this process outside the eubacterial (bacterial) domain. Nitrogen fixation has since been detected in a wide range of methanogens (2, 29). Sibold and colleagues detected sequences in methanogen DNA which hybridized to eubacterial *nifH* probes (37, 45) and went on to clone and sequence several *nifH*-homologous open reading frames (ORFs) from methanogens (44, 46–48) and a *nifD* ORF from *M. thermolithotrophicus* (48).

The phylogeny of deduced *nifH* gene products, with an emphasis on alternative and methanogen nitrogenases, is depicted in Fig. 1. There are essentially four major clusters. Cluster I is defined by the standard eubacterial Mo-nitrogenases (such as *A. vinelandii nifH1*). Cluster II consists of alternative nitrogenases (*nifH3* from *A. vinelandii* and *nifH3* from *Clostridium pasteurianum*) and three methanogen nitrogenase ORFs. The appearance of *A. vinelandii nifH2* (*vnfH*) in the eubacterial Mo-nitrogenase cluster is anomalous, since the

* Corresponding author. Mailing address: Section of Microbiology, Wing Hall, Cornell University, Ithaca, NY 14853. Phone: (607) 255-2415. Fax: (607) 255-3094. Electronic mail address: shz1@cornell.edu.

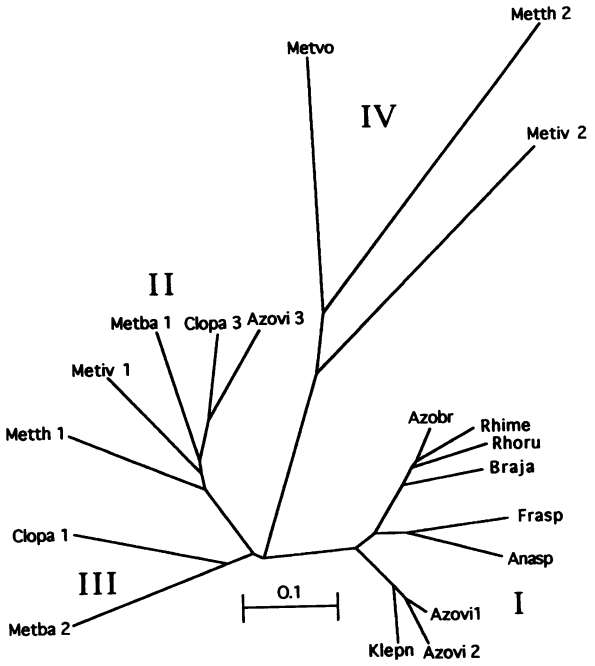

FIG. 1. Unrooted tree for *nifH* amino acid sequences analyzed by using PROTDIST and FITCH programs from the PHYLIP phylogenetic package (see Materials and Methods). The marker bar represents 0.1 expected substitution per amino acid position. For consistency, we have adopted the convention that all methanogen sequences which cluster with alternative nitrogenases (cluster II) are numbered 1. Metba 2, *M. barkeri* 2; Clopa 1, *C. pasteurianum* 1; Metth 1, *M. thermolithotrophicus* 1; Metiv 1, *M. ivanovii* 1; Metba 1, *M. barkeri* 1; Clopa 3, *C. pasteurianum* 3; Azovi 3, *A. vinelandii* 3 (*anfH*); Metvo, *M. voltae*; Metiv 2, *M. ivanovii* 2; Metth 1, *M. thermolithotrophicus* 1; Azobr, *Azospirillum brasiliense*; Rhime, *Rhizobium meliloti*; Rhoru, *R. rubrum*; Braja, *Bradyrhizobium japonicum*; Frasp, *Frankia* sp.; Anasp, *Anabaena* sp.; Azovi 1, *A. vinelandii* 1 (*nifH*); Azovi 2, *A. vinelandii* 2 (*vnfH*); Klepn, *Klebsiella pneumoniae*.

corresponding *nifDK* genes cluster with alternative nitrogenases (41). Cluster III consists of *nifH1* from *C. pasteurianum* (and five other closely related *nifH* ORFs in that organism which are apparently products of gene duplication [53]) and *nifH2* from *M. barkeri* (44). This is the first instance of clustering of a methanogen nitrogenase with a eubacterial Mo-nitrogenase. Cluster IV is highly divergent and consists of predicted gene products solely from methanogens, such as *Methanococcus voltae*. No evidence for nitrogenase function has been obtained for these genes.

So far, there is limited information on the structure and function of methanogen nitrogenases. In previous studies on *M. barkeri* 227, we showed that diazotrophic growth was stimulated by Mo (27) and that the partially purified nitrogenase complex from *M. barkeri* was binary, with low specific activity (28). We also showed that the component 2 from *M. barkeri* reacted in Western blots (immunoblots) with antibody to *Rhodospirillum rubrum* component 2 and that this protein was not detected in blots from ammonia-grown cells, suggesting repression. In this study, we show that *nifH2* is expressed in *M. barkeri* 227 when growing diazotrophically in standard Mo-containing medium. We also describe the cloning and sequencing of the *nifD2* gene and part of *nifK2* from *M. barkeri* and show that as with *nifH2*, their predicted amino acid sequences show greatest similarity with those from *C. pasteurianum*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *M. barkeri* 227 (ATCC 43241, DSM 1538, and OCM 35) was obtained from our own culture collection. *Escherichia coli* DH5α was used for transformation and DNA sequencing experiments and was obtained from S. Winans, Cornell University. Plasmid pUC19 was used for subcloning and DNA sequencing.

Medium and growth conditions. The growth medium for *M. barkeri* was that of Lobo and Zinder (27) as recently modified so that the culture headspace gas was N_2 and the medium was buffered by 12 mM phosphate (pH 7.0) rather than CO_2-HCO_3^- (10). Cultures for enzyme purification were grown in 10-liter amounts in a 14-liter fermentor vessel at 35°C. Once significant growth and methanogenesis occurred, the cultures were slowly sparged with N_2 gas (~10 ml/min), 5 ml of 20% (wt/vol) Na_2S was added every other day, and 1 ml of 1 M $FeSO_4 \cdot 7H_2O$ was added twice during the course of growth. Methanol was added as required to concentrations as great as 100 mM. Cells were harvested within 10 to 14 days when they reached an optical density at 600 nm of ca. 1.0 to 1.5 (1-cm cuvette), measured with a Beckman DU-50 spectrophotometer (Beckman Instruments, Fullerton, Calif.).

Nitrogenase purification and N-terminal sequence analysis. Both nitrogenase components were purified anaerobically as previously described (28), with some minor modifications (10). Briefly, the first step was changed from protamine precipitation (28), which provided variable results, to a 0 to 0.5 M NaCl step gradient using an Econo-Pac Q column (Bio-Rad, Richmond, Calif.), followed by desalting on an FPLC (fast protein liquid chromatography) Fast desalting column. Desalted extract from this step was loaded on a Poros Q/M perfusion anion-exchange column (PerSeptive Biosystems), and samples were eluted with an NaCl gradient, using an FPLC apparatus (Pharmacia LKB Biotechnologies, Piscataway, N.J.). Fractions containing nitrogenase components were further purified with a Superose 12 column. Once purified, the nitrogenase components were labile as previously described (28). The purified component 2 was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using a Hoefer Minigel apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) and transferred to a Bio-Rad polyvinylidene difluoride membrane, and the N-terminal sequence of the purified component 2 was determined by the Edman degradation performed at the Cornell Biotechnology Center Analysis Facility.

Western blot analysis. Western immunoblot analysis was performed as described by Maniatis et al. (31). A rabbit polyclonal serum raised against component 2 of *R. rubrum* was kindly provided by Paul Ludden, University of Wisconsin, and was diluted 1:5,000 for use. After incubation with the antiserum for 30 min, the Zeta-Probe membrane (Bio-Rad Laboratories) was washed and placed in a sealable bag containing a 1:5,000 dilution of alkaline phosphatase-coupled goat anti-rabbit antibody (Promega, Middletown, Wis.) and gently shaken for 30 min at room temperature. After the membrane was washed, the immunoreactive bands were visualized by soaking the membrane in 10 ml of alkaline phosphatase substrate (Promega) containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate. The color development was stopped by rinsing the membrane with water. A set of prestained SDS-PAGE standard proteins (Bio-Rad Laboratories) was used for molecular weight estimation.

Cloning and nucleotide sequencing. A fragment containing a portion of *M. barkeri nifH2* was amplified by PCR using oligonucleotides corresponding to positions 150 to 174 and 902

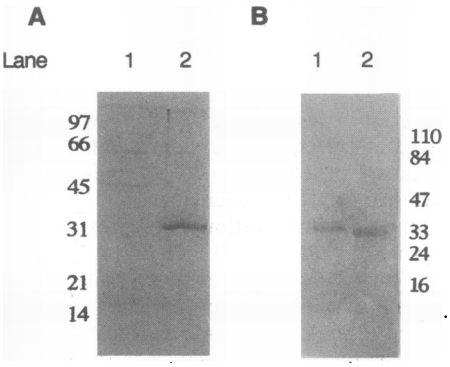

FIG. 2. Coomassie blue-stained SDS-polyacrylamide gel from the Superose 12 preparations containing component 2 (A) and the corresponding immunoblot of the same preparations by reaction with antibodies to *R. rubrum* component 2 (B). The sizes of the low-molecular-weight standards are 97.4, 66.2, 45, 31, 21.5, and 14.4 kDa (SDS-PAGE) and 110, 84, 47, 33, 24, and 16 kDa (immunoblot).

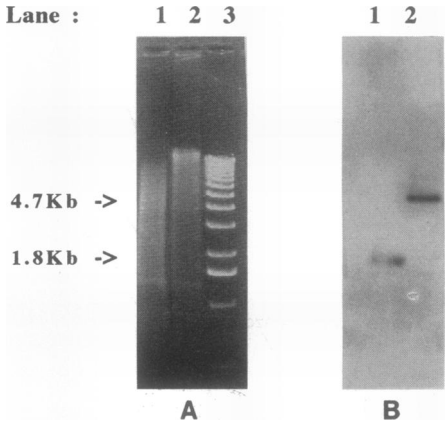

FIG. 4. Southern blot hybridization of DNA from *M. barkeri* 227, using a DIG-labeled *nifH2* probe. DNA was digested with *Hin*dIII (lane 1) and *Bam*HI (lane 2). (A) Ethidium bromide-stained agarose gel; (B) the corresponding hybridizations, showing bands of 1.8 and 4.7 kb, respectively, in the *Hin*dIII and *Bam*HI digestions.

to 925 of the published *nifH2* sequence from *M. barkeri* (44) (see Fig. 5). With these oligonucleotides, a 785-bp-long nucleotide fragment was obtained after PCR using a Hybaid thermal cycler (Labnet, Woodbridge, N.J.). The 5′ oligonucleotide had an extra nine bases at its 5′ end, giving a *Hin*dIII site and a three-base cap, and there was an *Acc*I site at position 774; thus, the fragment was cleaved with these enzymes and was cloned into pUC19. To ensure that it was the desired fragment, the 3′ and 5′ ends of this cloned fragment were sequenced by the dideoxy-chain termination method (42) (see below). The PCR fragment was labeled with digoxigenin (DIG) by using the Genius random priming labeling system (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and was used in Southern blot, RNA dot blot, and colony hybridization experiments. The hybridized DNA in these experiments was detected by the Genius chemiluminescence detection kit (Boehringer Mannheim) as described in the accompanying instructions.

M. barkeri DNA was digested with *Bam*HI, and the resulting fragments were ligated into *Bam*HI-phosphatase-treated pUC19. DNA was transformed into *E. coli* DH5α, and a *Bam*HI library of 1,200 clones was constructed. A positive clone, termed pYTC001, was detected by colony hybridization performed in the presence of 50% formamide at 42°C, using the DIG-labeled probe and chemiluminescence detection.

DNA sequencing was performed by the dideoxy-chain termination method (42), using a Sequenase DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio). DNA fragments were subcloned into pUC19, and double-stranded DNA sequencing was performed with universal oligonucleotide primers directed against the plasmid vector and oligonucleotides directed at previously sequenced parts of the cloned DNA.

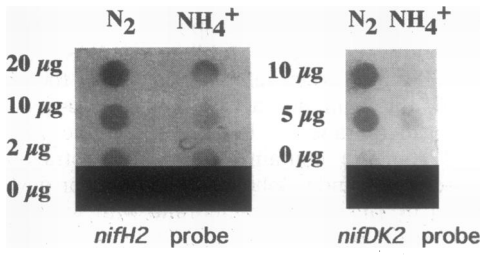

FIG. 3. Dot blot hybridization of total RNA isolated from NH_4^+- or N_2-grown *M. barkeri* 227 cells, using a DIG-labeled *nifH2* or *nifDK2* probe. The amount of total RNA applied on the membranes is indicated on the left.

RNA isolation. *M. barkeri* cells (20 ml), grown with either N_2 or NH_4^+, were harvested from exponential-phase cultures and mixed rapidly with 20 g of crushed ice and 15 mM sodium azide. After the ice melted, cells were sedimented at 20,000 rpm for 1 min. The pellet was frozen with liquid N_2, and the resulting brittle pellet was ground with a sterile mortar and pestle. The ground cells were resuspended in 6 ml of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) containing 250 mM sucrose and were treated with 600 ml of 10% SDS, 660 ml of 5 M NaCl, and 800 ml of hexadecyltrimethylammonium bromide–0.7 M NaCl (20). The lysate was extracted twice with hot phenol-chloroform (1:1) and once with chloroform-isoamyl alcohol (24:1) and precipitated with ethanol. The precipitated nucleic acids were redissolved in 20 mM Tris-HCl (pH 8.0)–10 mM $MgCl_2$–2 mM $CaCl_2$–100 mg of RNase-free DNase per ml.

After incubation for 60 min at 37°C, SDS, EDTA, and NaCl were added to final concentrations of 1%, 50 mM, and 0.2 M, respectively, and the remaining nucleic acids (RNA) were purified by phenol-chloroform extraction and ethanol precipitation. Pellets were dried in a SpeedVac SC 110 vacuum concentration system (Savant Instruments, Inc., Farmingdale, N.Y.) for 10 min, and the pellet was carefully resuspended in 100 μl of diethylpyrocarbonate (Sigma Chemical Co., St. Louis, Mo.)-treated H_2O. RNA samples were immediately frozen at −70°C.

Dot blot DNA-RNA hybridization. RNA samples (0.5 to 10 μl) isolated from N_2- or NH_4^+-grown cells were denatured by adding 0.5 ml of ice-cold 10 mM NaOH–1 mM EDTA. The denatured RNA was then applied onto a sheet of Zeta-Probe membrane (Bio-Rad), using the dot blot apparatus. The membrane was rinsed briefly in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate, [pH 7.0])–0.1% SDS and was dried at 80°C for 30 min in a vacuum oven. The mRNA was then detected by hybridization with DIG-labeled *nifH2* or *nifDK2* probe (5 ng/ml) overnight at 42°C. The *nifH2* probe was the cloned product of the PCR, while the *nifDK2* probe was a subclone of the 3′ end of the cloned fragment from the *Pst*I site to the *Bam*HI site (see Fig. 5). After hybridization, the membrane

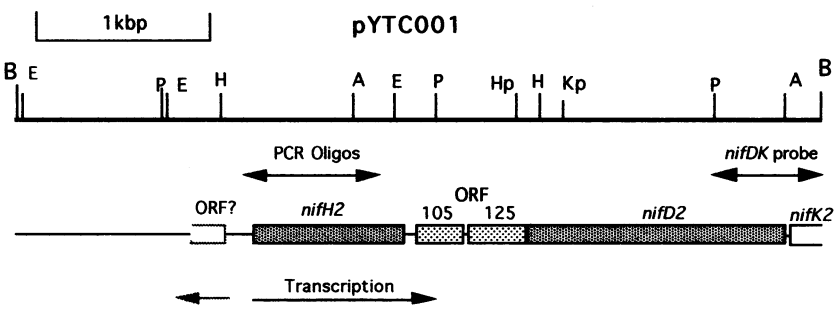

FIG. 5. Restriction map of a *Bam*HI fragment from *M. barkeri* containing *nifHDK2* genes cloned into pUC19(pYTC001). The original clone of Sibold et al. (44) is defined by the internal *Hin*dIII fragment. The DNA fragment (PCR Oligos) used as a probe in the hybridizations presented in Fig. 3 and 4 is indicated by arrows. B, *Bam*HI; H, *Hin*dIII; E, *Eco*RI; P, *Pst*I; A, *Acc*I; Hp, *Hpa*I; Kp, *Kpn*I.

was washed twice for 5 min in 2× SSC–0.1% SDS solution at room temperature and then twice for 15 min with 0.5× SSC–0.1% SDS at 65°C. The hybridized mRNA was then detected by the Genius chemiluminescence detection kit as described in the Genius (Boehringer Mannheim) protocol booklet.

Phylogenetic analysis. Amino acid sequences were used instead of DNA sequences to eliminate biases due to different G+C ratios (36) and were obtained from the combined GenBank/Swissprot/PIR database except for *M. barkeri nifH1* and *nifH2* and *Methanobacterium ivanovii nifH2*, which were obtained from Sibold et al. (44), and the *nifD* sequence for *Frankia alni* (36). The amino acid sequence of the *nifH2* product from *M. barkeri* (44) was corrected as described in Results.

Amino acid sequences were aligned by using PILEUP, part of the Genetics Computer Group software package (17), followed by a few minor changes made manually. Gaps present in more than five sequences were removed before phylogenetic analysis (49). The PHYLIP 3.5c phylogeny inference software package (16), in the form of compiled executable programs for Macintosh computers, was used for comparison of the protein sequences. The primary sequence analysis was done by using the PROTDIST program with a Dayhoff amino acid comparison matrix. This program gave a distances expressed in expected changes per amino acid position, including multiple forward and back mutations.

The resulting distance matrices were converted to phylogenetic trees by using the program FITCH, which uses the Fitch-Margoliash least-squares distance matrix method and does not assume a constant evolutionary clock. TREEDRAW was used to draw the unrooted phylogenetic trees presented. Unrooted trees produced by KITCH, NEIGHBOR, UPGMA, and PROPARS methods showed topologies essentially identical with those presented here. Dendrograms produced by DRAWGRAM resembled those previously published for a larger set of eubacterial and a smaller set of methanogen *nif* genes (36), but since there is no true outgroup sequence, unrooted trees were used in this analysis.

Nucleotide accession number. The nucleotide sequences for *nifD2* from *M. barkeri* and the first 135 bp of *nifK2* have been submitted to GenBank and have received accession number U11291.

RESULTS

Expression of the *nifH2* gene in *M. barkeri* grown diazotrophically in Mo-containing medium. Since the predicted products of the *nifH1* and *nifH2* genes in *M. barkeri* have different N-terminal amino acid sequences (MTRKIAFYGK and MRQIAIYGKG, respectively), we determined the N-terminal sequence of purified nitrogenase component 2 from *M. barkeri* grown in standard Mo-containing medium. Figure 2 shows that a single band was obtained in SDS-polyacrylamide gels from purified component 2 and that this band reacted with antibody to *R. rubrum* component 2 as shown previously (28). The first 10 amino acids of this purified polypeptide were identical with the sequence predicted for *M. barkeri nifH2*, indicating that *nifH2* was expressed in Mo-containing medium.

We also examined whether mRNA for *nifH2* could be detected in cells growing diazotrophically in Mo-supplemented medium. Figure 3 shows an RNA-DNA dot blot analysis demonstrating that RNA hybridizing to a *nifH2* probe or a probe containing fragments of both the *nifD* and *nifK* genes (see Fig. 5) could be detected in cells grown with N_2, while cells grown with NH_4^+ only showed background reactions with the probe which did not increase with increasing RNA concentration.

Cloning of *nifDK2* genes from *M. barkeri*. We generated and cloned a 786-bp fragment of the *nifH2* gene by using PCR (see Materials and Methods and Fig. 5) and used this fragment as a probe to clone downstream regions, since *nifD* genes are often downstream of *nifH* (11). To verify that the PCR product was the correct sequence, we sequenced its 5′ and 3′ ends. The sequence clearly was from the *nifH2* gene, but there were a few discrepancies between the sequence that we obtained and the published sequence (44), including a major one unlikely to be a PCR artifact since it involves the addition of three bases rather than a simple substitution. Starting with nucleotide 236 in the published sequence, the following sequence was given: 5′-GGGTGGGAATTGGAAAAATCC-3′, which encodes GWELEKS, amino acids 10 to 16 of the *nifH2* gene product. For the same region, we found a sequence lacking the three underlined nucleotide bases, encoding GGIGKS. The latter amino acid sequence is conserved in all other *nifH* sequences described to date and is believed to play a role in nucleotide binding (11, 18, 40).

The DIG-labeled *nifH2* PCR product was used as a probe to detect hybridizing fragments in Southern blots of restriction enzyme-digested *M. barkeri* DNA. As shown in Fig. 4, a 1.85-kb fragment from *Hin*dIII-digested DNA hybridized with the probe, as expected from previous results (44). A 4.7-kb fragment was detected in *Bam*HI digests. We cloned this 4.7-kb *Bam*HI fragment of *M. barkeri* DNA into pUC19, calling the new construct pYTC001. A map of the cloned fragment based on restriction analysis and subsequent sequencing analysis is shown in Fig. 5. The clone contains at least five ORFs which

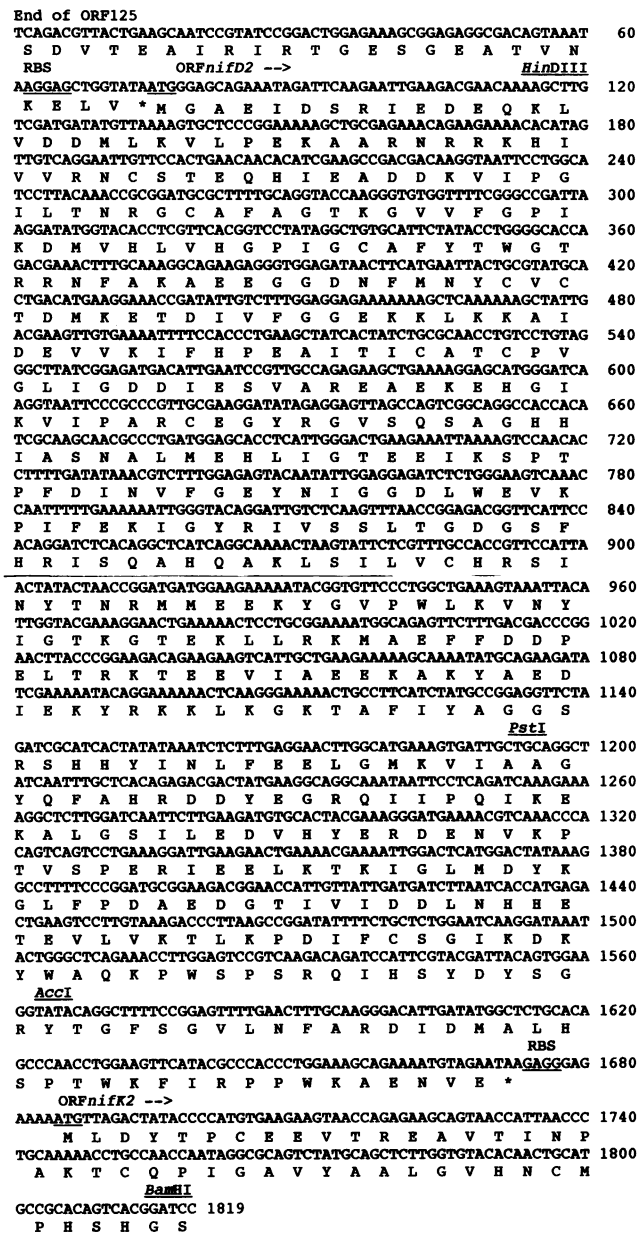

FIG. 6. Nucleotide sequence of the *nifD2* and 5′ end of *nifK2* region from *M. barkeri* 227. ORFs are indicated at the beginning of their translation start sites. Putative ribosome-binding sites (RBS) are underlined. The stop codons are indicated by asterisks. The restriction sites used for subcloning are also shown.

are transcribed in the same direction, in the order ORF*nifH2*-ORF105-ORF125 (previously described by Sibold et al. [44])-ORF*nifD2*-ORF*nifK2*. Preliminary analysis of sequences upstream of *nifH2* indicates a potential ORF beginning 184 bp upstream of *nifH2* and transcribed in the opposite direction from the *nifH2* gene.

Nucleotide sequence analysis of *nifD2* and the 5′ end of *nifK2*. The nucleotide and predicted amino acid sequences of *nifD2* and the 5′ end of *nifK2* are presented in Fig. 6. The predicted *nifD2* ORF is 1,596 bp long, begins using the final A of the UAA stop codon of ORF125, and has a potential ribosome-binding sequence (13) 9 bp upstream (5′-AGGAG-3′), which is within ORF125. The G+C content of *nifD2* was 43.7%, close to the value of 44.8% for the *nifH2* gene and 42% for the genomic DNA in *M. barkeri* (1). The ORF encodes a polypeptide 532 amino acids long with a predicted molecular mass of 60.3 kDa, close to the larger of the subunit molecular masses (57 and 62 kDa) detected by PAGE for the purified component 1 from *M. barkeri* (28). There is an 11-bp intergenic region after the *nifD2* ORF followed by the first 135 bp of the *nifK2* ORF. A GAGG sequence 8 bp upstream of the initial AUG codon of *nifK2* also provides a potential ribosome-binding site.

Figure 7 is an alignment of the amino acid sequences for the predicted *nifD2* gene product in *M. barkeri* and selected other *nifD* gene products. The predicted amino acid ligands for the FeMo-cofactor (11, 22, 23) are present in the predicted *M. barkeri nifD2* product, as are cysteine ligands which can coordinate the P cluster. Most noticeably, both the *M. barkeri nifD2* and *C. pasteurianum nifD1* deduced amino acid sequences have an insert of approximately 50 amino acids not present in other *nifD* products. The sequence identity in these inserts was near 33%, while that for the entire polypeptides is near 51%, and areas of identity and similarity could be detected throughout these proteins.

Figure 8 shows an alignment of the first 45 amino acids of the predicted product of the *M. barkeri nifK2* ORF with sequences of other *nifK* products. Both the *M. barkeri* and *C. pasteurianum* sequences lacked a long N-terminal region present in the *A. vinelandii* and *Frankia* sp. *nifK* products (as well as other eubacterial *nifK* products (11). There was a high degree of sequence identity in the N terminus of the *M. barkeri* and *C. pasteurianum* gene products, as well as the other *nifK* products.

Phylogenetic analysis of the *nifD2* product from *M. barkeri*. Figure 9 shows that the equivalents of clusters I, II, and III in Fig. 1 can also be defined for *nifD* gene products. No corresponding *nifD* products have been described for *nifH* cluster IV. As was the case for *nifH* products, the *nifD2* product from *M. barkeri* clustered with the *nifD1* product of *C. pasteurianum*. As has been noted before (6, 48), the *nifD1* product from *M. thermolithotrophicus* showed much less association with the alternative nitrogenases than did the *nifH1* product. All methods of phylogenetic analyses used (see Materials and Methods) gave similar tree topologies, and all showed association between *nifD2* from *M. barkeri* and *nifD1* from *C. pasteurianum*. Analysis of the 45-amino acid N-terminal fragment of the *M. barkeri nifK2* gene product also showed a close association with *nifK1* from *C. pasteurianum* (data not presented).

DISCUSSION

N-terminal sequencing analysis of purified nitrogenase component 2 and RNA-dot blot analysis both indicate that the *nifHDK2* genes were expressed in *M. barkeri* 227 cells grown in Mo-supplemented medium. While our results do not eliminate the possibility of expression of *nifHKD1* genes under these conditions, the N-terminal sequence data indicate that the *nifH2* gene product was responsible for most of the detectable (albeit low) nitrogenase activity detected in extracts. This is consistent with the similarity between *nifH2* in *M. barkeri* and *nifH1* in *C. pasteurianum*, which encodes the Fe-protein for its Mo-nitrogenase. An attractive hypothesis is that *nifH1* in *M. barkeri* encodes the Fe-protein of a V- or Fe-nitrogenase. V stimulation of diazotrophic growth of *M. barkeri* Fusaro and 227 has been reported (43).

The arrangement of the *nifHDK2* genes in *M. barkeri* (Fig. 5) shows similarity to the arrangement of *nifHDK1* genes in *M.*

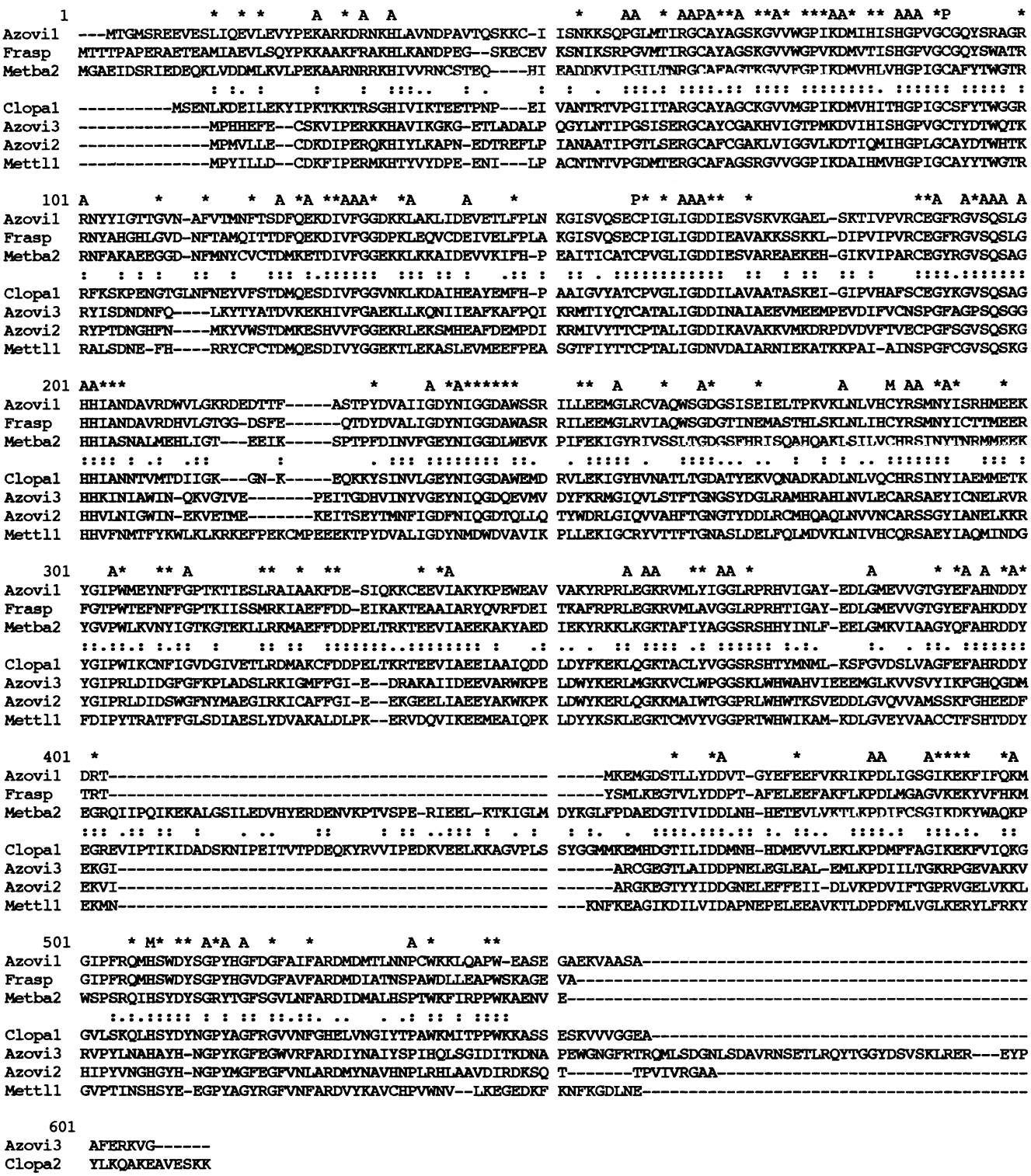

FIG. 7. Alignment of selected *nifD* amino acid sequences and sequence similarity between *M. barkeri nifD2* and *C. pasteurianum nifD1*. Organism names are as in Fig. 1. A, residues which are identical in all *nifD* products; *, residues which are considered to be conserved in *nifD* sequences from eubacterial Mo-nitrogenases, including *C. pasteurianum* (11); M, residues which are FeMo-cofactor ligands; P, residues which are P-cluster ligands; :, identical amino acid residues; ., conservative substitutions (I, L, V, M; D, E; K, R, H; S, T; G, A; F, Y; and N, Q).

```
      1
Azovi1  ------------------MSQQVDKIKASYPLFLDQDYKDMLAKKRDGFE
Frasp   MSGDDDPGNKQFRPAAGPRPQRAVQGRGLPEAVRGQARVRERQRRRRGRP

     51                    A       A **   **  PAA **      *
Azovi1  EKYPQDKIDEVFQWTTTKEYQELNFQREALTVNPAKACQPLGAVLCALGF
Frasp   GP-----------EWTPGWEYREKNFAREALTVNPAKACQPLGAVLAGLGF
Metba2  -----------MLDYTPCEE----VTREAVTINPAKTCQPIGAVYAALGV
                  ::: :: :     : : :. ::::::::::.::.::::::.
Clopa1  -----------MLDATPKEI----VERKALRINPAKTCQPVGAMYAALGI
Azovi3  ---------------MTCEVK--EKGRVG-TINPIFTCQPAGAQFVSIGI
Clopa2  ---------------MACEVK--EKDRTG-VINPIFTCQPAGAQYVSIGV
Azovi2  ------MSNCELTVLKPAEVKLSPRDREG-IINPMYDCQPAGAQYAGIGI
Mettl1  -----------MSEVNAGEICYVKKQRKG-TINPNKICQPIGAMWATVGV

     101    *   *****  * *    **  ** *
Azovi1  EKTMPYVHGSQGCVAYFRSYFNRHFREPVSCVSDSMTEDAAVFGGQQNMK
Frasp   EGTMPLVHGSQGCVAYFRSHFARHFKEPVPAASSSMTEDAAVFGGLNNLV
Metba2  HNCMPHSHGS
        :::.::::::
Clopa1  HNCLPHSHGSQGCCSYHRTVLSRHFKEPAMASTSSFTEGASVFGGGSNIK
Azovi3  KDCIGIVHGGQGCVMFVRLIFSQHYKESFELASSSLHEDGAVFGACGRVE
Clopa2  KDCIGLVHGGQGCVMFVRLIFSQHFKESFELASTSLHEDGAVFGALNRVE
Azovi2  KDCIPLVHGGQGCIMFVRLLFAQHFKENFDVASTSLHEESAVFGGAKRVE
Mettl1  KGTIPFVQGSQGCTTYVRYAFNRHFREPVSIATASFHEHAA
```

FIG. 8. Alignment of selected *nifK* sequences. Only the first 150 amino acids are shown. Organism names are as in Fig. 1; for details, see the legend to Fig. 7.

barkeri, *M. thermolithotrophicus*, and *M. ivanovii*, suggesting a methanogen-specific gene arrangement. All of these clusters have two small ORFs located between *nifH* and *nifD* encoding products 105 and 122 to 128 amino acids long (44, 48). The deduced products of both of these ORFs show sequence similarity to the *glnB* gene product in gram-negative bacteria (44). *glnB* encodes the P_{II} protein, which plays a role in the regulation of transcription of genes and activity of enzymes involved in nitrogen metabolism (39), suggesting a similar regulatory role for the products of these ORFs in methanogens. While the orders of these genes are similar, the spacings are not identical. For example, in the case of *M. thermolithotrophicus* (48), there was an 8-bp overlap between *nifD1* and *nifK1* and almost 200 bases between ORF 128 and *nifD*, while in *M. barkeri*, there was no overlap between *nifD2* and *nifK2* but there was a 1-bp overlap between ORF 125 and *nifD2*.

The presence of *nifH* mRNA only in N_2-grown cells is consistent with repression of *nif* genes by ammonia, as has been found in other free-living diazotrophs (33). In eubacteria, there is a specific regulatory sequence, often called the -24, -12, or σ^{54} consensus sequence, typically found upstream of nitrogen-related genes (33). No evidence for this element could be found upstream of *M. barkeri nif* genes, as is the case for other

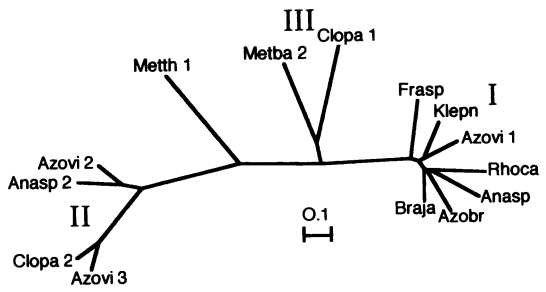

FIG. 9. Unrooted tree for *nifD* amino acid sequences drawn from analysis of the sequences as in Fig. 1. Organism names are as in Fig. 1 except Rhoca (*Rhodobacter capsulatus*) and Anasp 2 (Anabaena sp. 2 [*vnfD*]).

methanogen *nif* genes (44, 46, 47). In terms of potential archaeal promoters, in the 209 nucleotides preceding the *nifH2* gene (Fig. 3), the closest matches to a consensus methanogenic archaeal promoter of 5′-TTA(T/A)ATA-3′ (38) are 5′-TTA TAAA-3′ at 72 bp upstream and 5′-TTATTAA-3′ at 137 bp upstream of the start of the *nifH2* gene. There appears to be no promoter sequence upstream of the *nifD2* gene, similar to the *M. thermolithotrophicus nifD1* gene, which lacks an upstream promoter sequence even though it was apparently not cotranscribed with *nifH1* (48).

It has long been recognized that the *C. pasteurianum* Mo-nitrogenase is divergent from other eubacterial Mo-nitrogenases. *C. pasteurianum* nitrogenase has a higher K_i for inhibition of N_2 reduction by H_2 (30) and shows different specificities for nucleotides and nucleotide analogs (54). *C. pasteurianum* nitrogenase components fail to complement components from other eubacterial Mo-nitrogenases and can form inactive complexes with them (13). Previous analyses of sequences of *nif* structural genes in *C. pasteurianum* nitrogenase show them to be highly divergent from other eubacterial Mo-nitrogenases (11, 35, 56), including those from *Frankia* sp., a eubacterium in the high-G+C branch of the gram-positive line (55). It was recently proposed that the *C. pasteurianum* nitrogenase represents a separate gene family (36).

Our analysis has shown that the *nifH2* and *nifD2* gene products and the N-terminal 45 amino acids of the *nifK2* products from *M. barkeri* all show closest similarity to the corresponding *nifHDK1* gene products in *C. pasteurianum*. While it is not surprising that *nifD2* from *M. barkeri* should show the same phylogenetic affiliations as *nifH2*, this is not always the case, since *nifD2* in *A. vinelandii*, *nifD* in *Frankia* sp., and *nifD1* in *M. thermolithotrophicus* all show different branch positions from their corresponding *nifH* gene products (Fig. 1 and 9) (6, 36, 41, 48). The most striking similarity between the *M. barkeri nifD2* and *C. pasteurianum nifD1* sequences is the presence of an insert of approximately 50 amino acids near position 403, an insert heretofore found only in the *C. pasteurianum nifD1* gene product (9). Kim et al. (23) showed that the insert forms a loop spanning a cleft above the FeMo-cofactor in component 1 considered to be part of a region of contact between the two components. This loop causes the cofactor to be buried more deeply in the *C. pasteurianum* nitrogenase than in others, and the poor reactivity of the *C. pasteurianum* nitrogenase components toward other nitrogenases has been attributed to the presence of this loop (23). Of the amino acids in the insert which are solvent exposed and are proposed (23) to interact with the Fe-protein (Lys-412, Asp-414, Asp-416, and Asn-419 in Fig. 7), only Lys-412 (Lys-385 in the *C. pasteurianum* sequence) is conserved in *M. barkeri*, making it a likely candidate for interaction with component 2.

We have examined the phylogenetic proximity of the *M. barkeri nifHD* gene products to *nif* clusters I and II. Using the distances derived from the PROTDIST program, the *M. barkeri nifH2* gene product shows the closest similarity to the *nifH1* product from *C. pasteurianum* (0.36 amino acid change per position) and is roughly equidistant from the eubacterial Mo-nitrogenases (cluster I) (0.487 ± 0.034 [standard deviation] amino acid change per position) and the alternative nitrogenase cluster (cluster II) (0.455 ± 0.027 change per position). For the *nifD2* gene product from *M. barkeri*, the distance from *C. pasteurianum nifD1* is 0.75 change per position, while that from cluster I is 1.02 ± 0.07 change per position. The distances from cluster II were 1.46 ± 0.10 when the *nifD1* product from *M. thermolithotrophicus* was included in the cluster and 1.50 ± 0.05 if it was omitted. Similar results were obtained for *nifD1* genes from *C. pasteurianum*. Thus, the

nifD gene products from the *M. barkeri-C. pasteurianum* cluster show more affinity with eubacterial Mo-nitrogenases than with alternative ones, consistent with previous analysis of *C. pasteurianum* (11, 35, 36, 56).

One can advance various evolutionary scenarios to explain this similarity between the sequences of proteins between two organisms, *M. barkeri* and *C. pasteurianum*, which, according to phylogenetic analysis based on 16S rRNA sequences (55), have not had a common ancestor for over 3 billion years. One possible explanation is horizontal transfer of genes, which is given some credence by the fact that clostridia and methanogens are likely to be found in close physical proximity in anaerobic sediments. Arguing against a relatively recent transfer of genes between the two genera is the difference in the G+C values of the genes (ca. 35% for *C. pasteurianum* and ca. 44% for *M. barkeri*) and the presence of ORF105 and ORF125 located between the *nifH2* and *nifD2* genes in *M. barkeri* but absent in *C. pasteurianum* (9). It should be mentioned that the five nearly identical copies of *nifH1* in *C. pasteurianum* (53) are indicative of significant genetic rearrangement in this organism.

An alternative hypothesis, similar to one put forward by Young (56), is that the ancestor of the archaea and the eubacteria had two sets of *nif* genes, one encoding a Mo-nitrogenase resembling the present-day clostridial nitrogenase and the other resembling an alternative nitrogenase. Cluster I would represent a more recent radiation within the eubacteria, most likely from the ancestor of the clostridial nitrogenase. It is not clear where the extremely divergent cluster IV *nifH* genes in methanogens fit in the evolution of *nif* genes. That they may encode products which are not nitrogenases is suggested by their high degree of divergence relative to other *nifH* genes, the lack of *nifD*- or *glnB*-like ORFs found downstream from them, and the inability to detect nitrogen fixation by *M. voltae* (29, 47) or expression of *nifH2* in *M. thermolithotrophicus* (48). Recently, it was shown that the iron proteins of protochlorophyllide and chlorin reductases involved in bacteriochlorophyll synthesis show significant sequence similarity to *nifH* gene products (8).

There are other instances of similarity between gene sequences from archaea and members of the low-G+C gram-positive branch of the eubacteria, including the sequences of two enzymes which play a central role in nitrogen metabolism: glutamine synthetase I (25, 52) and glutamate dehydrogenase (4, 12). More than nitrogen-related genes may be involved in this phenomenon, since similarity between heat shock protein-70 sequences between archaea and gram-positive bacteria has also been found (19). This similarity is not universal, since other archaeal genes, such as those involved in tryptophan synthesis in methanogens (32), do not show any affinity with those in gram-positive organisms. As above, one can invoke gene transfer between the groups (or perhaps cell fusion) or a common heritage of duplicated copies of the genes in question. The impact of these findings, including those reported here, on our understanding of bacterial phylogeny is unclear at this point but should become clearer as more gene sequences from diverse organisms become available.

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