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nifH Sequences and Nitrogen Fixation in Type I and Type II Methanotrophs

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Some methane-oxidizing bacteria (methanotrophs) are known to be capable of expressing nitrogenase and utilizing N_2 as a nitrogen source. However, no sequences are available for *nif* genes in these strains, and the known nitrogen-fixing methanotrophs are confined mainly to a few genera. The purpose of this work was to assess the nitrogen-fixing capabilities of a variety of methanotroph strains. *nifH* gene fragments from four type I methanotrophs and seven type II methanotrophs were PCR amplified and sequenced. Nitrogenase activity was confirmed in selected type I and type II strains by acetylene reduction. Activities ranged from 0.4 to 3.3 nmol/min/mg of protein. Sequence analysis shows that the *nifH* sequences from the type I and type II strains cluster with *nifH* sequences from other gamma proteobacteria and alpha proteobacteria, respectively. The translated *nifH* sequences from three *Methylomonas* strains show high identity (95 to 99%) to several published translated environmental *nifH* sequences PCR amplified from rice roots and a freshwater lake. The translated *nifH* sequences from the type II strains show high identity (94 to 99%) to published translated *nifH* sequences from a variety of environments, including rice roots, a freshwater lake, an oligotrophic ocean, and forest soil. These results provide evidence for nitrogen fixation in a broad range of methanotrophs and suggest that nitrogen-fixing methanotrophs may be widespread and important in the nitrogen cycling of many environments.

Methanotrophs, or methane oxidizers, are a group of bacteria capable of growth on methane as their sole source of carbon and energy. These bacteria can be divided into two major phylogenetic groups, the type I methanotrophs (gamma proteobacteria) and the type II methanotrophs (alpha proteobacteria) (15). These two groups are thought to differ in several ways, foremost among which is their carbon assimilation pathway. The type I methanotrophs use the ribulose monophosphate pathway, while the type II methanotrophs utilize the serine cycle (1).

Groups of methanotrophs have also been classified based on the types of methane monooxygenase (MMO) that they produce. Until recently, most type I methanotrophs were thought capable of producing only the membrane bound or particulate MMO (pMMO), whereas type II methanotrophs and the type I *Methylococcus* strains were known to also produce a different, cytoplasmic enzyme, or soluble MMO (sMMO) (15). However, recent work has shown that several type I strains, including members of the genera *Methylomonas* and *Methylomicrobium*, can also produce sMMO (2, 13, 18, 26, 27). The type of MMO expressed is of environmental significance because sMMO shows rates of oxidation of halogenated solvents such as trichloroethylene (TCE) that are 100- to 1,000-fold higher than those of pMMO (10, 22).

Nitrogen fixation capabilities in methanotrophs have also been thought to distinguish these two groups (20). Type II methanotrophs and members of the type I genus *Methylococcus* have been shown to be capable of nitrogen fixation, while other type I methanotrophs are not (9, 20, 21). However, some evidence from DNA hybridization studies and acetylene reduction assays has suggested that some members of the type I genus *Methylomonas* and the type I strain *Methylobacter marinus* A45 (formerly known as *Methylomonas methanica* A4) may also be capable of nitrogen fixation (4, 21, 24). However, acetylene reduction by *Methylomonas* and *Methylobacter* strains was not detected in the second study, and in the last study, the only acetylene reduction rate measured in whole cells for a type I strain (*Methylomonas rubra*) was very low (3.1 nmol/h/mg of cells, recalculated to be approximately 0.11 nmol/min/mg of protein). Thus, the significance of nitrogen fixation in type I methanotrophs other than *Methylococcus* has been unclear.

In order to efficiently degrade TCE in contaminated environments, methanotrophs require a sufficient nitrogen source in addition to their substrates of methane and oxygen. However, in some vadose zone and aquifer environments, fixed nitrogen may be limiting (5), and methanotrophs capable of nitrogen fixation would have an advantage. Evidence also exists that nitrogen-fixing methanotrophs have an increased capacity for TCE oxidation (5, 6, 7). Because both type I and type II methanotrophs are now known to possess sMMO, both groups may be important in the bioremediation of TCE. For these reasons, we were interested in assessing the nitrogen fixation capabilities of both type I and type II methanotroph strains. This work provides genetic and biochemical evidence for the presence of nitrogenase, the key enzyme involved in nitrogen fixation, in both type I and type II methanotrophs. In addition, sequence analysis of *nifH*, the gene that encodes the highly conserved Fe protein of nitrogenase, suggests that nitrogenase genes from type I and type II methanotrophs may be present in a variety of environments, indicating that nitrogen-fixing methanotrophs may be widespread.

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MATERIALS AND METHODS

Strains and media. The methane-oxidizing strains used in this study were described previously. They included strains isolated from Lake Washington, Seattle, Wash., (2), and the type strains *Methylosinus trichosporium* OB3b, *Methylobacter marinus* A45, *Methylomonas methanica* S1, *Methylomonas rubra*, and *Methylomicrobium albus* BG8 (4). For chromosomal DNA extraction, strains were grown on nitrate mineral salts medium (NMS) (31) with a vitamin solution (12) and 10 μM $CuSO_4 \cdot 5H_2O$ and incubated at 30°C under a 50% methane–50% air atmosphere (vol/vol). To promote nitrogen fixation, strains were grown on nitrate-free mineral salts (NFMS) medium with vitamins and 10 μM $CuSO_4 \cdot 5H_2O$ and incubated at 30°C under an 80% methane–20% air atmosphere (vol/vol). Cycloheximide and nystatin were dissolved in dimethyl sulfoxide (DMSO) and added to plates to final concentrations of 20 and 10 μg/ml, respectively, to minimize mold contamination. For assays, strains were inoculated from NFMS plates and grown in 160-ml serum vials with liquid NFMS medium containing vitamins and 10 μM $CuSO_4 \cdot 5H_2O$ and incubated at 30°C shaking at 200 rpm under a 90% methane–10% air atmosphere (vol/vol).

Acetylene reduction assays. Nitrogen fixation was estimated using the method of acetylene reduction described previously (8, 29) with a few modifications. Samples (1 ml) of liquid culture were removed from the growth vials and added to 21-ml serum vials. Methanol was added to a final concentration of 2% (vol/vol), and cell samples were incubated for 10 min with shaking at 30°C under a 90% argon–10% air atmosphere (vol/vol). Then 0.2 ml of acetylene was injected, and 0.5-ml samples of the headspace were removed at 0 min and approximately every 7 min up to 35 min postinjection. The rate of ethylene production was linear under these conditions. The amount of ethylene present in each sample was determined using a Carle analytical gas chromatograph (model 211) at 50°C equipped with a flame ionization detector, a 10-ft column (packed with a mixture of Porapak N and Porapak Q), and a Waters data module (model 740). To determine the protein concentration in the cultures, a sample of each culture was lysed by adding NaOH and sodium dodecyl sulfate to final concentrations of 1 N and 1%, respectively, and heating to 70°C for 15 min. The samples were then diluted fivefold with distilled water, and aliquots were used in the BCA protein assay (Pierce, Rockford, Ill.) as per the manufacturer's protocol. Bovine serum albumin samples were treated similarly and used as standards for the protein assay.

PCR amplification of *nifH*. Chromosomal DNA was isolated from liquid cultures or from plates using methods described previously (25, 28). *nifH* gene fragments were amplified from chromosomal DNA samples using primers described previously (37). These degenerate primers, based on all known *nifH* genes at the time of design, were chosen from highly conserved amino acid sequences that required less than 200-fold degeneracy of the DNA coding sequences; the primers were synthesized with every possible combination of the base sequences, resulting in a mixture of 128 and 96 oligonucleotides for the upstream and downstream primers, respectively (37). For most strains, the reactions were carried out in an MJ Research PTC-200 thermocycler, with an initial denaturation step of 30 s at 94°C, followed by 30 cycles of 92°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 5 min. For most strains, the PCRs contained final concentrations of 1×PCR buffer (Gibco-BRL, Rockville, Md), 1.5 mM $MgCl_2$ (Gibco-BRL), 333 nM nifH-f, 333 nM nifH-r, 0.167 mM each deoxynucleoside triposphate (Boehringer Mannheim), and 2.5 U of *Taq* polymerase (Gibco-BRL) in a total volume of 30 μl. For LW5 and *M. trichosporium* OB3b, the reannealing temperature was lowered to 55°C, and DMSO was added to a final concentration of 5%. For PCR-positive strains, the *nifH* fragments were then cloned into pCR2.1 using the Topo-TA cloning kit (Invitrogen, San Diego, Calif.).

DNA sequencing and analysis. DNA sequencing of the *nifH* PCR products was carried out on both strands using the ABI Prism BigDye terminator sequencing kit (PE Applied Biosystems, Foster City, Calif.). The sequencing reactions and analyses were performed by the University of Washington Department of Biochemistry Sequencing Facility using an Applied Biosystems automated sequencer. Analyses and translation of DNA sequences were performed using the Genetics Computer Group programs (Madison, Wis.). NifH sequences were aligned with translated *nifH* sequences obtained from the GenBank database using SeqPup (Indiana University) and GeneDoc (www.psc.edu/biomed/genedoc). Dendrograms were constructed using the programs PROTDIST, PROTPARS, NEIGHBOR, SEQBOOT, and CONSENSE from PHYLIP version 3.5c (11), and tree files were analyzed using Tree View (23). Related environmental *nifH* sequences were obtained using the tblastn program in BLAST version 2.1 (www.ncbi.nlm.nih.gov/BLAST).

Nucleotide sequence accession numbers. The GenBank accession numbers for the *nifH* gene sequences described in this study are AF378714 to AF378724.

TABLE 1. Evidence for nitrogen fixation in methanotrophs

Strain	PCR product obtained	Growth on N-free medium	Acetylene reduction activity (nmol/min/mg of protein)
Methylocystis sp. LW2	+	+	3.30
Methylocystis sp. LW5	+	+	0.43
Methylosinus sp. LW3	+	+	0.59
Methylosinus sp. LW4	+	+	NT[a]
Methylosinus sp. LW8	+	+	0.72
Methylosinus sp. PW1	+	+	1.84
Methylosinus trichosporium OB3b	+	+	NT[a]
Methylobacter sp. A45	+	NT[c]	NT[a]
Methylomonas sp. LW13	+	+	2.76
Methylomonas sp. LW15	+	+	NT[a]
Methylomonas methanica S1	+	+	NT[a]
Methylobacter sp. LW14	−	−	NT[b]
Methylomonas sp. LW21	−	−	NT[b]
Methylomonas rubra	−	−	NT[b]
Methylomicrobium albus BG8	−	−	NT[b]

[a] These strains grew poorly in NFMS and were not tested for acetylene reduction.
[b] No attempts to grow these strains in liquid NFMS and perform acetylene reduction assays were made because these strains did not grow on NFMS plates.
[c] This strain was difficult to maintain and was subsequently lost.

RESULTS

PCR amplification of *nifH*. *nifH* encodes the Fe protein of nitrogenase and has been used as a marker for nitrogenase (36). Phylogeny based on NifH sequences has been shown to parallel that based on 16S ribosomal DNA (rDNA) sequences (33, 34). In order to assess the nitrogen-fixing capabilities of both type I and type II methanotrophs, *nifH* was studied in several type strains as well as pure cultures recently isolated from Lake Washington. Existing degenerate *nifH* primers (37) were used to attempt amplification of an approximately 360-bp product from five type strains and 10 strains isolated from Lake Washington (2). *Methylosinus trichosporium* OB3b and *Methylomicrobium albus* BG8 were used as positive and negative controls, respectively, based on their known nitrogen fixation capabilities (21). PCR products were obtained for all type II strains tested, two *Methylocystis* strains and five *Methylosinus* strains, including *M. trichosporium* OB3b, the positive control (Table 1). In addition, PCR products were obtained for several type I strains, including three *Methylomonas* strains and one *Methylobacter* strain. PCR products could not be obtained for several type I strains, including two *Methylomonas* strains, a *Methylobacter* strain, and *M. albus* BG8, the negative control. These PCR products were sequenced and translated, and the amino acid alignments are highly similar, with 78% identity overall (Fig. 1). Translated PCR products were aligned with NifH sequences from other proteobacteria, revealing high conservation (67% identity overall). However, within the variable residues, some signature sequences were conserved only in specific groups of methanotrophs. For example, the combination of S, Q, and D at residues 19, 22, and 27 in the translated PCR products appears to be diagnostic of *Methylomonas* NifH sequences, while the combination of E and G at residues 27 and 82 appears to be a marker for type II methanotroph NifH sequences (*Methylosinus* and *Methylocystis*). The alignments were used to generate a phylogenetic tree (Fig. 2). The phylogeny of the translated PCR products corresponded to that pre-

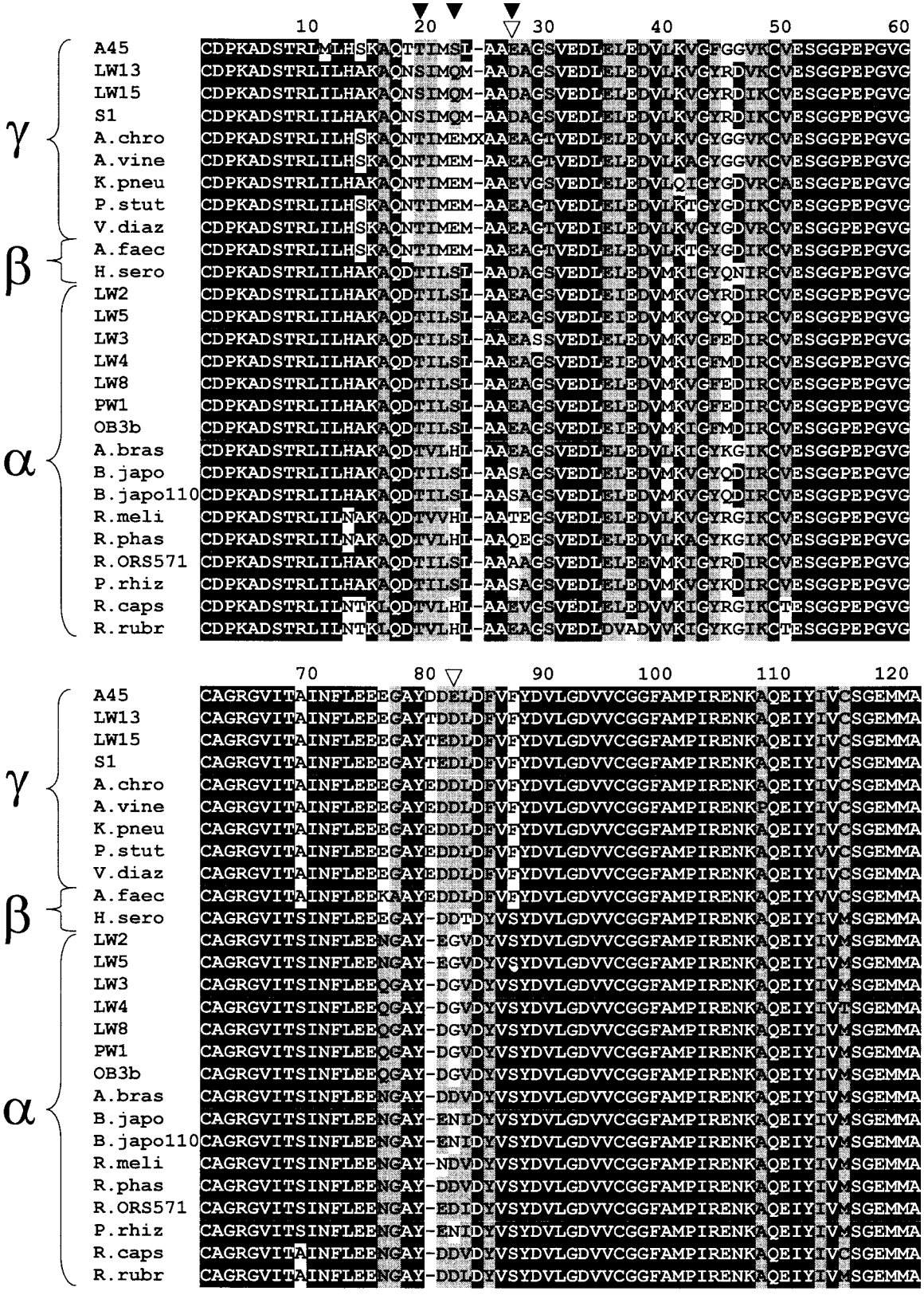

FIG. 1. Alignment of deduced amino acid sequences of the approximately 360-bp partial *nifH* genes from *Methylobacter marinus* A45, *Methylomonas* sp. strain LW13, *Methylomonas* sp. strain LW15, *M. methanica* S1, *A. chroococcum* (accession no. M73020), *A. vinelandii* (M11579), *K. pneumoniae* (J01740), *P. stutzeri* (AJ297529), *V. diazotrophicus* (AF111110), *A. faecalis* (X96609), *H. seropedicae* (Z54207), *Methylocystis* sp. strain LW2, *Methylocystis* sp. strain LW5, *Methylosinus* sp. strain LW3, *Methylosinus* sp. strain LW4, *Methylosinus* sp. strain LW8, *Methylosinus* sp. strain PW1, *M. trichosporium* OB3b, *A. brasilense* (M64344), *B. japonicum* (E00713), *B. japonicum* USDA 110 (K01620), *R. meliloti* (V01215), *R. phaseoli* (M10587), *Rhizoboium* sp. strain ORS571 (M16710), *P. rhizobium* (K00487), *R. capsulatus* (M15270), and *R. rubrum* (M33774). Identical residues are in black boxes, and similar residues are in gray boxes. Methanotroph strain names are in bold face. Solid triangles indicate signature amino acids for *Methylomonas* strains, while open triangles indicate signature amino acids for type II strains.

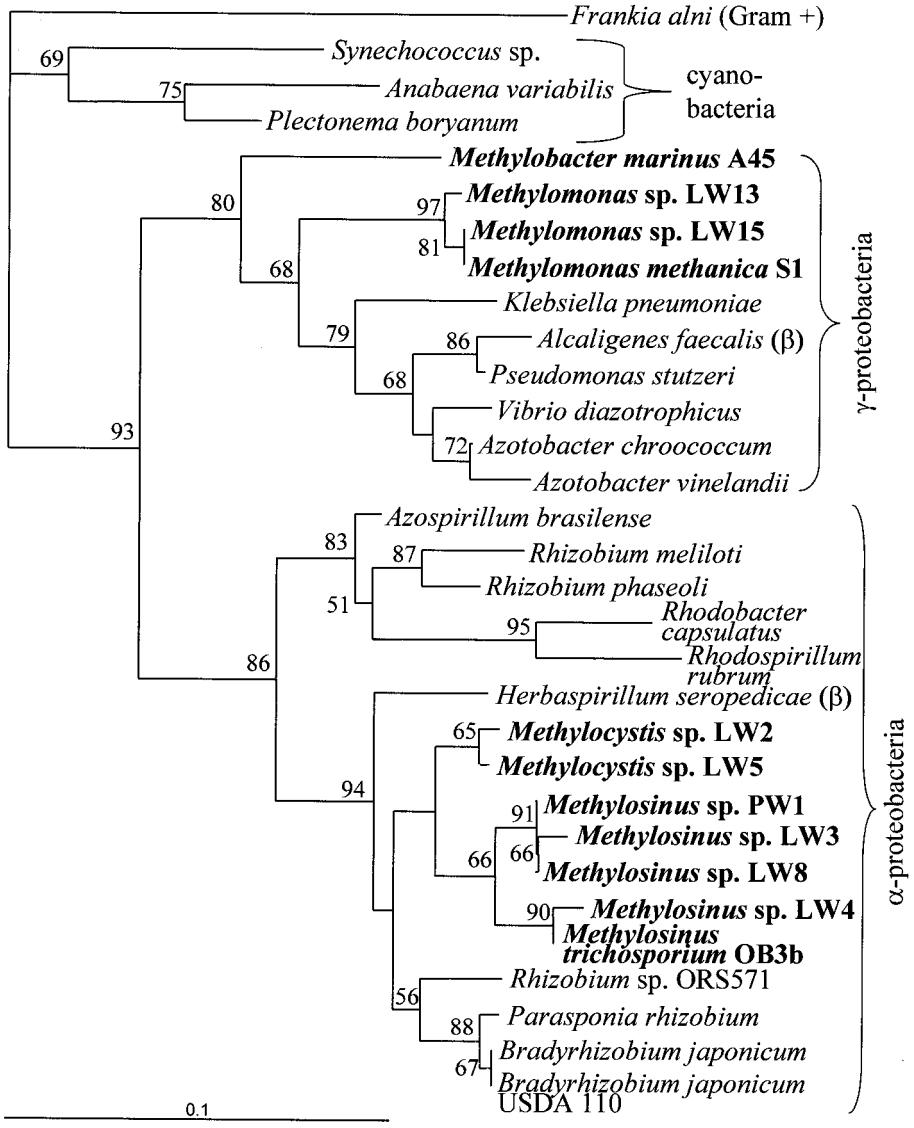

FIG. 2. Phylogenetic analysis of the derived amino acid sequences of *nifH* genes. Bootstrap values of >50% are shown near the clades. The bar represents 10% sequence divergence, as determined by the lengths of the horizontal lines connecting any two species. The tree includes sequences shown in Fig. 1 as well as *F. alni* L41344, *Synechococcus* sp. strain U22146, *A. variabilis* U89346, and *P. boryanum* D00666.

dicted by 16S rDNA sequences, with the NifH sequences from type I strains clustering together within a group of sequences from other gamma proteobacteria and the NifH sequences from type II strains also clustering together within a group of sequences from other alpha proteobacteria.

Nitrogenase activity. To confirm the nitrogen-fixing capabilities of methanotroph strains that were PCR positive for *nifH*, these strains were grown on NFMS plates under low oxygen tension (see Materials and Methods). The PCR-negative strains were used as negative controls and showed no growth on these plates. All PCR-positive strains grew slowly under these conditions (Table 1), in some cases taking up to 10 days to form small isolated colonies. Acetylene reduction has been shown to be a suitable assay for nitrogen fixation in methanotrophs (8, 20, 29). Because acetylene is a potent MMO inhibitor, 2% methanol was provided as an alternate oxidizable substrate (8). Representative strains that grew best in nitrogen-free liquid medium were assayed for their ability to reduce acetylene (Table 1). Several type II strains as well as a type I *Methylomonas* strain showed activity, ranging between 0.43 and 3.30 nmol/min/mg of protein. These acetylene reduction activities fall into the range of previously reported activities for type II methanotrophs (20, 29).

Sequence comparison to environmental clones. Environmental clone banks of *nifH* sequences have been generated from a number of different environments (19, 30, 32, 35, 38). In order to assess whether any of these environmental clones might have originated from nitrogen-fixing methanotrophs, translated BLAST nucleotide searches were performed with the methanotroph NifH sequences against the nonredundant nucleotide GenBank database. Translated environmental *nifH* sequences were found that were more closely related to *Methylomonas* NifH sequences than any others in the database, showing 95 to 99% identity at the amino acid level (Table 2).

TABLE 2. *nifH* PCR product identities for *Methylomonas* and related bacteria[a]

Clone or culture	*M. methanica* S1	*Methylomons* sp. LW13	*Methylomonas* sp. LW15	H-RIC7 **D26290**	H-RIC8 **D26291**	H-RIC13 **D26296**	H-RIC16 **D26299**	H-RIC18 **D26301**	H-RIC22 **D26305**	H-RIC23 **D26306**	LG1111 H **AF212874**	LG1112 H **AF212875**	*K. pneumoniae* **J01740**	*A. chroococcum* **M73020**	*A. vinelandii* **M11579**	*V. diazotrophicus* **AF111110**	*P. stutzeri* **AJ297529**	*A. faecalis* **X96606**	*M. purpuratum* **AF059648**
M. methanica S1		98.2 (109)	100 (109)	**97.6 (85)**	**95.3 (85)**	**96.5 (85)**	**95.3 (85)**	**97.6 (85)**	**96.5 (85)**	**96.5 (85)**	**97.2 (109)**	**97.2 (109)**	89.0 (109)	90.0 (110)	89.0 (109)	89.9 (109)	90.8 (109)	89.0 (109)	94.5 (109)
Methylomons sp. LW13	93.6 (328)		98.2 (109)	**95.3 (85)**	**96.5 (85)**	**98.8 (85)**	**95.3 (85)**	**97.6 (85)**	**98.8 (85)**	**98.8 (85)**	**95.4 (109)**	**95.4 (109)**	90.8 (109)	91.8 (110)	90.8 (109)	91.7 (109)	90.8 (109)	89.0 (109)	92.7 (109)
Methylomonas sp. LW15	95.4 (328)	92.7 (328)		**97.6 (85)**	**95.3 (85)**	**96.5 (85)**	**95.3 (85)**	**97.6 (85)**	**96.5 (85)**	**96.5 (85)**	**97.2 (109)**	**97.2 (109)**	89.0 (109)	90.0 (110)	89.0 (109)	89.9 (109)	90.8 (109)	89.0 (109)	94.5 (109)
H-RIC7 **D26290**	88.6 (255)	85.9 (255)	88.2 (255)		94.0 (116)	94.0 (116)	94.8 (116)	95.7 (116)	94.8 (116)	93.1 (116)	95.3 (85)	95.3 (85)	87.1 (116)	87.2 (117)	86.8 (91)	86.2 (116)	89.7 (116)	86.8 (91)	91.8 (85)
H-RIC8 **D26291**	86.3 (255)	84.3 (255)	85.9 (255)	87.4 (349)		97.4 (116)	97.4 (116)	97.4 (116)	98.3 (116)	96.6 (116)	91.8 (85)	91.8 (85)	88.8 (116)	89.7 (117)	89.0 (91)	88.8 (116)	90.5 (116)	87.9 (91)	90.6 (85)
H-RIC13 **D26296**	86.7 (255)	85.9 (255)	85.9 (255)	86.2 (348)	90.2 (348)		96.6 (116)	98.3 (116)	99.1 (116)	98.3 (116)	92.9 (85)	92.9 (85)	90.5 (116)	91.5 (117)	91.2 (91)	90.5 (116)	91.4 (116)	89.0 (91)	91.8 (85)
H-RIC16 **D26299**	86.3 (255)	84.7 (255)	85.9 (255)	87.4 (348)	98.9 (349)	90.2 (348)		96.6 (116)	97.4 (116)	95.7 (116)	92.9 (85)	92.9 (85)	88.8 (116)	89.7 (117)	89.0 (91)	88.8 (116)	90.5 (116)	89.0 (91)	91.8 (85)
H-RIC18 **D26301**	91.8 (255)	91.0 (255)	91.0 (255)	88.2 (348)	92.0 (348)	91.1 (348)	92.0 (348)		99.1 (116)	97.4 (116)	94.1 (85)	94.1 (85)	89.7 (116)	90.6 (117)	90.1 (91)	89.7 (116)	93.1 (116)	90.1 (91)	92.9 (85)
H-RIC22 **D26305**	89.8 (255)	89.8 (255)	89.8 (255)	84.5 (348)	86.8 (348)	89.1 (348)	87.4 (348)	89.1 (348)		98.3 (116)	92.9 (85)	92.9 (85)	90.5 (16)	91.5 (117)	91.2 (91)	90.5 (116)	92.2 (116)	89.0 (91)	91.8 (85)
H-RIC23 **D26306**	89.8 (255)	88.6 (255)	88.2 (255)	85.9 (348)	87.9 (348)	90.5 (348)	87.9 (348)	89.4 (348)	87.6 (348)		92.9 (85)	92.9 (85)	91.4 (116)	92.3 (117)	91.2 (91)	92.2 (116)	92.2 (116)	89.0 (91)	91.8 (85)
LG1111H **AF212874**	85.3 (326)	83.7 (326)	84.0 (326)	84.3 (255)	81.6 (255)	83.1 (255)	82.4 (255)	85.5 (255)	83.1 (255)	82.4 (255)		97.2 (109)	88.1 (109)	89.1 (110)	88.1 (109)	89.0 (109)	89.0 (109)	88.1 (109)	93.6 (109)
LG1112H **AF212875**	86.2 (326)	84.7 (326)	85.6 (326)	85.5 (255)	84.3 (255)	85.1 (255)	85.1 (255)	83.5 (255)	83.1 (255)	85.1 (255)	89.3 (326)		88.1 (109)	89.1 (110)	88.1 (109)	89.0 (109)	89.9 (109)	88.1 (109)	93.6 (109)
K. pneumoniae **J01740**	74.8 (326)	74.2 (326)	74.5 (326)	76.7 (347)	75.8 (347)	75.5 (347)	76.1 (347)	74.6 (347)	74.6 (347)	76.1 (347)	76.1 (326)	76.1 (327)		88.7 (291)	88.6 (289)	91.3 (229)	93.0 (158)	87.7 (292)	89.0 (109)
A. chroococcum **M73020**	80.5 (329)	80.2 (329)	81.5 (329)	81.1 (350)	78.1 (352)	79.4 (350)	77.7 (350)	78.6 (350)	82.3 (350)	78.0 (350)	79.9 (329)	81.2 (330)	79.8 (875)		95.5 (288)	93.5 (230)	96.2 (159)	91.8 (291)	91.8 (110)
A. vinelandii **M11579**	78.5 (326)	79.4 (326)	79.1 (326)	80.4 (347)	77.4 (349)	79.5 (347)	76.9 (347)	77.8 (347)	81.6 (347)	78.4 (347)	78.5 (326)	80.7 (327)	80.2 (868)	88.0 (1286)		93.0 (229)	96.2 (158)	91.3 (289)	90.8 (109)
V. diazotrophicus **AF111110**	81.0 (326)	82.5 (326)	81.0 (326)	79.0 (348)	77.6 (348)	79.9 (348)	78.2 (348)	79.6 (348)	77.6 (348)	79.3 (348)	79.5 (327)	77.9 (326)	78.5 (688)	78.0 (691)	77.8 (688)		94.9 (158)	92.6 (229)	91.7 (109)
P. stutzeri **AJ297529**	76.1 (326)	76.4 (326)	77.0 (326)	78.7 (347)	75.8 (347)	77.8 (347)	76.1 (347)	77.8 (347)	78.4 (347)	76.1 (347)	77.6 (326)	78.3 (327)	83.8 (475)	80.3 (676)	83.2 (612)	77.5 (475)		98.7 (158)	92.7 (109)
A. faecalis **X96609**	75.2 (326)	75.5 (326)	76.1 (326)	77.8 (347)	74.9 (347)	77.2 (347)	75.5 (347)	76.7 (347)	77.5 (347)	76.1 (347)	76.7 (326)	78.0 (327)	81.6 (870)	81.9 (1071)	83.5 (1005)	75.3 (688)	98.3 (1081)		90.8 (109)
M. purpuratum **AF059648**	75.5 (326)	75.2 (326)	75.5 (326)	75.6 (254)	72.0 (254)	75.2 (254)	72.4 (254)	75.2 (254)	74.4 (254)	74.4 (254)	78.2 (326)	78.6 (327)	84.4 (327)	84.5 (330)	85.0 (327)	74.8 (326)	88.4 (327)	88.1 (327)	

[a] Identities for the *nifH* PCR product obtained for pure cultures of *Methylomonas* strains, environmental clones, and related bacteria. Values above the diagonal are percent amino acid identity for the translated *nifH* products. Values below the diagonal are percent nucleotide identity. The number in parentheses is the number of the residues compared. Environmental amino acid identities with NifH from *Methylomonas* pure cultures greater than identities with NifH from other bacteria are shown in bold.

TABLE 3. *nifH* PCR product identities for type II methanotrophs and related bacteria[a]

Clone or culture	*Methylocystis* sp. LW2	*Methylocystis* sp. LW5	*Methylosinus* sp. LW3	*Methylosinus* sp. LW4	*Methylosinus* sp. LW8	*Methylosinus* sp. PW1	*M. trichosporium* OB3b	PO3120 **AF059644**	PO3133 **AF059645**	LG1120 **AF212882**	LG1122 **AF212884**	LG2369 **AF212891**	H-RIC20 **D26303**	AO12 **AF016615**	B13 **AF099789**	*B. japonicum* USDA 110 **K01620**	*P. rhizobium* **K00487**	*Rhizobium* sp. ORS571 **M16710**	*H. seropedicae* **Z54207**
Methylocystis sp. LW2		99.1 (108)	94.4 (108)	94.4 (108)	95.4 (108)	95.4 (108)	**95.4 (108)**	**99.1 (108)**	**95.4 (108)**	**98.1 (108)**	**98.1 (108)**	**97.2 (108)**	**95.2 (84)**	**95.4 (108)**	**93.5 (108)**	95.4 (108)	95.4 (108)	94.4 (108)	91.7 (108)
Methylocystis sp. LW5	94.5 (325)		94.4 (108)	94.4 (108)	95.4 (108)	95.4 (108)	95.4 (108)	**98.1 (108)**	**94.4 (108)**	**97.2 (108)**	**97.2 (108)**	**96.3 (108)**	**95.2 (84)**	**95.4 (108)**	92.6 (108)	96.3 (108)	95.4 (108)	93.5 (108)	92.6 (108)
Methylosinus sp. LW3	89.5 (325)	88.6 (325)		95.4 (108)	99.1 (108)	99.1 (108)	96.3 (108)	95.4 (108)	91.7 (108)	94.4 (108)	94.4 (108)	93.5 (108)	**94.0 (84)**	**93.5 (108)**	89.8 (108)	92.6 (108)	92.6 (108)	90.7 (108)	91.7 (108)
Methylosinus sp. LW4	91.4 (325)	91.1 (325)	91.7 (325)		96.3 (108)	96.3 (108)	99.1 (108)	93.5 (108)	89.8 (108)	92.6 (108)	92.6 (108)	91.7 (108)	**95.2 (84)**	**94.4 (108)**	89.8 (108)	90.7 (108)	90.7 (108)	90.7 (108)	91.7 (108)
Methylosinus sp. LW8	90.2 (325)	89.2 (325)	98.5 (325)	92.3 (325)		100 (108)	97.2 (108)	96.3 (108)	92.6 (108)	95.4 (108)	95.4 (108)	94.4 (108)	**95.2 (84)**	**94.4 (108)**	90.7 (108)	93.5 (108)	93.5 (108)	91.7 (108)	92.6 (108)
Methylosinus sp. PW1	90.4 (356)	89.8 (325)	98.2 (325)	93.2 (325)	98.5 (325)		97.2 (108)	96.3 (108)	92.6 (108)	95.4 (108)	95.4 (108)	94.4 (108)	**95.2 (84)**	**94.4 (108)**	90.7 (108)	93.5 (108)	93.5 (108)	91.7 (108)	92.6 (108)
M. trichosporium OB3b	92.6 (325)	92.6 (325)	91.1 (325)	95.7 (325)	91.7 (325)	91.7 (325)		94.4 (108)	90.7 (108)	93.5 (108)	93.5 (108)	92.6 (108)	**95.2 (84)**	**95.4 (108)**	90.7 (108)	91.7 (108)	91.7 (108)	91.7 (108)	92.6 (108)
PO3120 **AF059644**	88.0 (324)	85.2 (324)	84.9 (324)	84.6 (324)	85.5 (324)	84.6 (324)	85.2 (324)		96.3 (108)	99.1 (108)	99.1 (108)	98.1 (108)	96.4 (84)	94.4 (108)	94.4 (108)	96.3 (108)	96.3 (108)	95.4 (108)	92.6 (108)
PO3133 **AF059645**	85.8 (324)	83.3 (324)	82.7 (324)	83.4 (325)	83.3 (324)	82.4 (324)	83.0 (324)	97.8 (324)		95.4 (108)	95.4 (108)	94.4 (108)	91.7 (84)	90.7 (108)	90.7 (108)	92.6 (108)	92.6 (108)	93.5 (108)	88.9 (108)
LG1120 **AF212882**	87.7 (324)	84.9 (324)	84.6 (324)	84.3 (324)	85.2 (324)	84.3 (324)	84.9 (324)	99.7 (324)	97.5 (324)		98.1 (108)	97.2 (108)	96.4 (84)	93.5 (108)	93.5 (108)	95.4 (108)	95.4 (108)	94.4 (108)	91.7 (108)
LG1122 **AF212884**	87.7 (324)	84.9 (324)	84.6 (324)	84.3 (324)	85.2 (324)	84.3 (324)	84.9 (324)	99.7 (324)	97.5 (324)	99.4 (324)		97.2 (108)	95.2 (84)	93.5 (108)	93.5 (108)	95.4 (108)	95.4 (108)	94.4 (108)	91.7 (108)
LG2369 **AF212891**	87.0 (324)	84.3 (324)	84.0 (324)	83.6 (324)	84.6 (324)	83.6 (324)	84.3 (324)	99.1 (324)	96.9 (324)	96.8 (324)	98.8 (324)		95.2 (84)	92.6 (108)	92.6 (108)	94.4 (108)	94.4 (108)	93.5 (108)	90.7 (108)
H-RIC20 **D26303**	84.5 (252)	85.7 (252)	86.1 (252)	86.5 (252)	86.9 (252)	86.5 (252)	86.9 (252)	86.9 (251)	84.5 (251)	86.9 (251)	86.5 (251)	86.5 (251)		94.0 (84)	92.9 (84)	92.2 (115)	93.0 (115)	90.4 (115)	91.3 (115)
AO12 **AF016615**	85.8 (323)	86.7 (323)	85.8 (323)	86.7 (323)	86.7 (323)	86.7 (323)	87.9 (323)	82.0 (323)	81.1 (323)	81.7 (323)	81.7 (323)	81.1 (323)	85.7 (251)		90.7 (108)	91.7 (108)	91.7 (108)	91.7 (108)	89.8 (108)
B13 **AF099789**	84.9 (325)	83.7 (325)	82.2 (325)	82.5 (326)	83.4 (325)	83.4 (326)	82.8 (326)	81.8 (324)	80.9 (324)	81.5 (324)	81.5 (324)	80.9 (324)	78.0 (273)	84.9 (324)		91.7 (109)	91.7 (109)	92.7 (109)	90.8 (109)
B. japonicum USDA 110 **K01620**	79.6 (324)	80.6 (324)	79.9 (324)	78.1 (324)	79.6 (324)	79.3 (324)	79.0 (324)	80.2 (323)	79.3 (323)	79.9 (323)	79.9 (323)	79.3 (323)	77.9 (344)	79.6 (324)	79.1 (368)		96.9 (294)	92.5 (293)	87.0 (293)
P. rhizobium **K00487**	81.8 (324)	82.7 (324)	80.6 (324)	80.9 (324)	80.9 (324)	80.9 (324)	82.1 (324)	81.7 (323)	80.8 (323)	81.4 (323)	81.4 (323)	80.8 (323)	79.9 (344)	82.7 (324)	77.2 (368)	85.9 (1274)		93.5 (293)	86.3 (293)
Rhizobium sp. ORS571 **M16710**	87.4 (325)	88.9 (325)	84.0 (324)	87.1 (325)	84.9 (325)	84.6 (325)	88.0 (325)	85.5 (324)	85.2 (324)	85.2 (324)	85.2 (324)	84.6 (324)	82.3 (344)	83.6 (323)	82.6 (368)	78.5 (933)	82.1 (914)		86.3 (293)
H. seropedicae **Z54207**	85.8 (325)	86.8 (325)	84.6 (325)	87.4 (325)	85.5 (325)	85.2 (325)	86.8 (325)	86.7 (324)	84.6 (324)	86.4 (324)	86.4 (324)	85.8 (324)	82.0 (344)	81.1 (323)	81.8 (368)	78.7 (890)	77.8 (901)	80.0 (935)	

[a] Identities for the *nifH* PCR product obtained for pure culture type II methanotrophs, environmental clones, and related bacteria. Environmental amino acid identities with NifH from type II methanotroph pure cultures greater than identities with NifH from other bacteria are shown in bold. Also see Table 2, footnote *a*.

These environmental sequences were initially PCR amplified from rice roots and a freshwater lake (30, 35). Translated environmental *nifH* sequences were also found that were more closely related to type II methanotroph NifH sequences than any others in the database, showing 94 to 99% identity at the amino acid level (Table 3). These environmental sequences were PCR amplified from an oligotrophic ocean, a freshwater lake (by reverse transcription [RT]-PCR), rice roots, and a Douglas fir forest soil (30, 32, 35, 38). The signature amino acid combinations indicative of either *Methylomonas* or type II NifH sequences were found in the *Methylomonas*-like or type II-like environmental sequences, respectively, in all of these cases.

DISCUSSION

The ability to utilize N_2 as a sole nitrogen source is an important trait for the use of methanotrophs for in situ bioremediation as well as for understanding the role of methanotrophs in nitrogen cycling in different environments. However, previous results had suggested that only type II and the type I moderately thermophilic *Methylococcus* strains were capable of N_2 fixation. Therefore, type I strains have been assumed to be unable to fix N_2 in mesophilic environments. The presence of both *nifH* gene fragments and acetylene reduction activity in a variety of type I and type II strains provides genetic and biochemical evidence that nitrogen fixation capabilities are broadly distributed among methanotrophs (Table 1). So far the only major group of mesophilic methanotrophs for which N_2-fixing strains have not been identified are the *Methylomicrobium* strains, and it is possible that this is due to the small number of strains tested.

Comparison of the translated *nifH* sequences obtained in this study with translated *nifH* sequences in environmental clone banks suggests that nitrogen-fixing methanotrophs may be more widespread than was previously thought. *Methylomonas*-like *nifH* fragments were amplified from rice roots and a freshwater lake (30, 35). Although we cannot be certain these *nifH* fragments are from methanotrophs, they are more similar to methanotrophic *nifH* sequences than any others in the database, and they do contain the signature amino acids that are so far specific to methanotrophs. In addition, these are environments in which type I methanotrophs are generally present (15, 16, 17).

Type II-like *nifH* fragments were amplified from a greater variety of environments. The translated type II *nifH* fragments showed high identity to translated *nifH* fragments from rice roots, a freshwater lake, a Douglas fir soil site, and an oligotrophic ocean (30, 32, 35, 38). Type II strains are generally thought to be present in rice roots, freshwater environments, and soils (15). However, their role in nitrogen fixation in these environments has not been studied in detail. The fact that the type II-like *nifH* fragment was amplified via RT-PCR from the freshwater lake environment suggests that type II methanotrophs may play a significant role in nitrogen cycling in this environment (35). Further work will be necessary to address this question.

Comparison of type II translated *nifH* sequences with environmental *nifH* sequences also showed the presence of type II-like sequences in both Atlantic and Pacific ocean samples (38). This is surprising because marine environments are generally thought to be dominated by type I strains (15). However, one study has suggested that type II methanotrophs are present in such habitats (14), so it is possible that these *nifH* sequences originated in methanotrophs.

Traditionally, it has been assumed that in natural populations, type II strains will be the dominant sMMO-containing population, and most in situ bioremediation protocols involving methanotrophs focus on type II strains (3). However, both type I (LW13, LW15, and the thermophilic *Methylococcus capsulatus* Bath) and type II strains (LW3, LW4, LW8, PW1, and *Methylosinus trichosporium* OB3b) have been found that can both express sMMO and fix nitrogen (2, 4, 13, 18, 21, 24, 26, 27). The correlation between increased capacity for TCE oxidation and nitrogen fixation in methanotrophs suggests that both type I and type II strains may be suitable for the bioremediation of TCE (5, 6, 7).

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