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Characterization of Diazotrophs Containing Mo-Independent Nitrogenases, Isolated from Diverse Natural Environments▽

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Molybdenum-independent nitrogenases were first described in the nitrogen-fixing bacterium *Azotobacter vinelandii* and have since been described in other diazotrophic bacteria. Previously, we reported the isolation of seven diazotrophs with Mo-independent nitrogenases from aquatic environments. In the present study, we extend these results to include diazotrophs isolated from wood chip mulch, soil, "paraffin dirt," and sediments from mangrove swamps. Mo-deficient, N-free media under both aerobic and anaerobic conditions were used for the isolations. A total of 26 isolates were genetically and physiologically characterized. Their phylogenetic placement was determined using 16S rRNA gene sequence analysis. Most of the isolates are members of the gamma subdivision of the class *Proteobacteria* and appear to be specifically related to fluorescent pseudomonads and azotobacteria. Two other isolates, AN1 and LPF4, are closely related to *Enterobacter* spp. and *Paenibacillus* spp., respectively. PCR and/or Southern hybridization were used to detect the presence of nitrogenase genes in the isolates. PCR amplification of *vnfG* and *anfG* was used to detect the genetic potential for the expression of the vanadium-containing nitrogenase and the iron-only nitrogenase in the isolates. This study demonstrates that diazotrophs with Mo-independent nitrogenases can be readily isolated from diverse natural environments.

Azotobacter vinelandii, an aerobic, free-living, nitrogen-fixing soil bacterium, was the first diazotroph shown to have three distinct nitrogenases (6): the molybdenum (Mo)-containing nitrogenase (nitrogenase 1), the vanadium (V)-containing nitrogenase (nitrogenase 2), and the iron-only nitrogenase (nitrogenase 3).

Nitrogenase 1 is expressed when Mo is present. This enzyme complex consists of two components: dinitrogenase reductase 1 (Fe protein) and dinitrogenase 1 (Mo-Fe protein) (21, 22, 25). The structural genes that encode nitrogenase 1 subunits are organized as the *nifHDK* operon; the dinitrogenase reductase 1 is a homodimer whose subunit is encoded by *nifH*, while the α- and the β-subunits of dinitrogenase 1 are encoded by *nifD* and *nifK*, respectively (8).

Nitrogenase 2 is a V-containing enzyme complex that is synthesized under diazotrophic conditions lacking Mo but containing V (13, 23, 24, 28, 42–44). This enzyme complex consists of two components: dinitrogenase reductase 2 and dinitrogenase 2. The genes encoding nitrogenase 2 proteins are split between two operons: *vnfHFd* and *vnfDGK*. *vnfH* encodes dinitrogenase reductase 2 subunits, and *vnfFd* encodes a ferredoxin-like protein that is required for nitrogenase 2-dependent diazotrophic growth (42). The *vnfDGK* operon encodes the subunits for dinitrogenase 2. *vnfD* encodes the α-subunit, *vnfK* encodes the β-subunit, and *vnfG* encodes the small δ-subunit (28, 43, 44).

Nitrogenase 3 is made under Mo- and V-deficient conditions and does not appear to contain either Mo or V (10, 40). The components of this enzyme complex are dinitrogenase 3 and dinitrogenase reductase 3. The structural genes encoding nitrogenase 3 in *A. vinelandii* are located in the operon *anfHDGKOR* (27, 38). *anfH* encodes the subunits of dinitrogenase reductase 3, while *anfD* and *anfK* encode the α- and β-subunits of dinitrogenase 3, respectively, and *anfG* encodes the δ-subunit of dinitrogenase 3 (40). *anfO* and *anfR* are located immediately downstream of *anfK* and are cotranscribed with the *anfDGK* genes into one polycistronic mRNA (41). Both AnfO and AnfR are required for nitrogen fixation in the absence of both molybdenum and vanadium (38).

Over the years it has become clear that Mo-independent nitrogenases are present in a diverse group of diazotrophic microorganisms which include *Clostridium pasteurianum* (55), *Rhodobacter capsulatus* (46, 47), *Anabaena variabilis* (30, 50), *Rhodospirillum rubrum* (11, 32), *Heliobacterium gestii* (31), *Azospirillum brasilense* (9), *Azotobacter salinestris* (34), *Azotobacter paspali* (34), *Azomonas macrocytogenes* (34), *Rhodopseudomonas palustris* (39), and *Methanosarcina acetivorans* (19). Loveless et al. (35) demonstrated that diazotrophs with Mo-independent nitrogenases were easily isolated from aquatic environments using Mo-deficient, nitrogen-free media under aerobic conditions. In that study, seven isolates were shown to have Mo-independent nitrogenase systems. Analysis of the 16S rRNA gene sequences showed that these isolates fall into the gamma subdivision of the class *Proteobacteria* and seem to be specifically related to the fluorescent pseudomonads and azotobacteria. The ability to isolate diazotrophs with Mo-independent nitrogenases from these environments, including those that are known to have molybdenum concentrations sufficient for nitrogen fixation, suggests that factors other than molybdenum concentrations in the macroenvironment may be important in determining the presence of these organisms. For

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example, the concentration of molybdenum in a wastewater treatment plant was 90 nM, and in a salt marsh it was approximately 110 nM (unpublished observations).

In the present study, we demonstrate the widespread presence of Mo-independent nitrogenases in diazotrophs isolated from diverse environments by using Mo-deficient, nitrogen-free media under both aerobic and anaerobic conditions.

MATERIALS AND METHODS

Isolation of diazotrophic bacteria from natural environments. Modified Burk medium (BM) containing 2% (wt/vol) glucose without any additions (−Mo, −N BM) (5) or supplemented with either 1 μM Na_2MoO_4, (+Mo, −N BM) or 1 μM V_2O_5 (−Mo, +V, −N BM) was used for growth and enrichment procedures with most of the isolates (35). All chemicals and reagents used were analytical grade and were ≥99.9% molybdenum free. To remove trace Mo contamination, glucose and phosphate buffer stock solutions for preparation of BM were extracted by the 8-hydroxyquinolone technique as described previously (5, 14). To remove trace metals, all glassware was base and acid washed as described by Benemann et al. (3). Enrichment was done under both aerobic and anaerobic conditions. Aerobic enrichment was accomplished by placing a spatula full of wood chip mulch, soil, or sediment in 10 ml of N-free, Mo-deficient BM (−Mo, −N BM) in a sterile 50-ml plastic centrifuge tube and incubating at 30°C with shaking until a noticeable increase in turbidity was observed. Approximately, six to seven transfers followed the initial enrichment by placing 100 μl of inoculum in 9.9 ml of −Mo, −N BM. Incubation conditions were as described above. A 100-μl aliquot from the sixth or seventh transfer was spread on −Mo, −N BM agar. Pure cultures were isolated from single colonies as previously described (35).

For anaerobic enrichment, the same samples (wood chip mulch, soil, or sediment) were placed in sterile 18- by 150-mm Bellco anaerobic culture tubes containing 9 ml of −Mo, −N BM. The tubes were sealed with a rubber stopper clamped with an aluminum ring. The sealed tubes were flushed with N_2 and incubated at 30°C until growth was observed. Following the initial enrichment, the culture was transferred five to six times. Transfers were conducted as described above except that 10 ml of −Mo, −N BM was used and 100 μl of inoculum was transferred using a 1-ml syringe. After six transfers, 100 μl of inoculum was spread on −Mo, −N BM agar followed by incubation in a Coy anaerobic chamber (Coy, Ann Arbor, MI) with an anaerobic gas mixture of 10% H_2, 5% CO_2, and 85% N_2 until growth was observed. Solid medium to be used for plates was poured inside the anaerobic chamber and allowed to equilibrate for 24 to 48 h prior to inoculation.

Growth experiments. Growth of each strain was tested in −Mo, −N BM or −Mo, −N BM supplemented with 1 μM Na_2MoO_4, 1 μM V_2O_5, or 10 mM ammonium acetate. Each culture was grown overnight in the same medium in order to monitor growth. Inoculum culture (0.5 to 1.0 ml) was transferred to 30 ml of −Mo, −N BM in a sidearm flask (300 ml) and placed on a shaker at 30°C. Growth was monitored with a Klett-Summerson colorimeter equipped with a no. 66 filter (red). Generation times were calculated by log-linear regression.

Acetylene reduction assays. To estimate nitrogenase activity, acetylene reduction assays were conducted on freshly grown cultures that had been starved for molybdenum by repeated transfers (at least three times) in molybdenum-deficient liquid media. Bellco sidearm flasks previously treated to remove metals were used to grow 30-ml cultures in modified BM. The media contained either no additive, 1 μM Na_2MoO_4, or 1 μM V_2O_5. To repress nitrogenase expression, ammonium acetate was added to the media at 1, 10, or 28 mM. A 10-ml volume of each culture grown to a density of 75 to 90 Klett units was then transferred to a 52- by 95-mm serum bottle and fitted with a rubber stopper. Two percent of the headspace gas was removed before injecting that same volume of acetylene into the bottle. A 3-ml volume was removed from the sample headspace and analyzed for ethylene and ethane using a Shimadzu GC-17 gas chromatograph after 0, 30, and 60 min of shaking at 200 rpm at 30°C. The gas chromatograph was fitted with a 1-ml sample loop on the injector port, a column of 50/80 Porapak N (182.88 cm by 3.175 mm), and a flame ionization detector. The temperatures of the injector, detector, and oven were 40°C, 200°C, and 35°C, respectively. Ethylene standards consisted of a mixture of ethylene and ethane, with various amounts of ethylene in increasing incremental amounts across seven tubes keeping ethane constant across all seven. For ethane standards, the amount of ethane was increased incrementally across seven tubes while the amount of ethylene remained constant. Incubation conditions were the same for both standards and samples.

Two isolates, AN1 and LPF4, failed to grow in Burk nitrogen-free liquid media. Therefore, in order to assay for acetylene reduction, the isolates were grown on slants prepared in anaerobic culture tubes. Strain LPF4 (*Paenibacillus*) was grown on modified Line's acetylene reduction medium (49) consisting of glucose (10 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), $C_6H_5FeO_7 \cdot H_20$ (0.01 g), $CaCl_2 \cdot 2H_2O$ (0.07 g), K_2HPO_4 (2 g), KH_2PO_4 (0.5 g), thiamine (0.001 g), biotin (0.001 g), sodium thioglycolate (0.5 g), Jurgensen micronutrient solution (1 ml) (29), purified agar (15 g), and deionized water to a final volume of 1 liter. For AN1 (*Enterobacter*), the solid media consisted of −Mo, −N BM with 1.5% purified agar or the same medium supplemented with either 1 μM Na_2MoO_4, 1 μM V_2O_5, or 10 mM ammonium acetate. Slants were inoculated from an aerobic overnight culture grown in the same medium. The slants were incubated 24 to 48 h at 30°C in an anaerobic chamber or a Gas Pak anaerobic jar with an anaerobic system envelope generating H_2 plus CO_2. Once growth was observed, the culture tube was fitted with a rubber stopper and 0.5 ml of acetylene was added. After anaerobic incubation at 30°C for 24 h, 0.5-ml headspace samples were analyzed in duplicate as described above.

Determination of protein concentration. Total cell protein concentration for the acetylene reduction assays was determined using a Pierce bicinchoninic acid protein assay kit (Rockford, IL), according to the protocol provided by the supplier.

DNA extraction. DNA for all bacterial strains was prepared using the CTAB method (1).

PCR amplification and phylogenetic analysis of 16S rRNA genes. The universal primers 515F and 1492R were used for 16S rRNA gene amplification (2). Amplification was accomplished using the protocol and reagents of the Qiagen *Taq* PCR core kit. A Bio-Rad iCycler thermal cycler or an Ericomp PowerBlock was used for the amplifications. The cycler settings were those described below for the *anfG* and *vnfG* amplifications. PCR products were sequenced at the biotechnology facilities of Iowa State University (Ames, IA).

A subalignment of 16S rRNA sequences from azotobacteria and diazotrophic *Pseudomonas* species was extracted from the Ribosomal Database Project (PubMed ID 17090583). The 'Sequence Match' function was used to identify the most similar sequence from a named organism for each of the isolates' 16S rRNA sequences; in the cases of AN1 and LPF4, the isolates were not azotobacteria, and so these relatives and a related well-known representative were included in the extracted alignment. The 16S rRNA sequences of the diazotrophic isolates were added to the alignment manually, and the alignment was refined manually. Trees were generated by the neighbor-joining method using Phylip v3.6 (18); these trees were not substantially different than those generated by maximum likelihood or unrooted parsimony.

PCR amplification of Mo-independent nitrogenase genes and phylogenetic analysis of AnfG and VnfG. Primers for PCR amplification of *vnfDGK* and *anfDGK* DNA sequences were previously described (34). In addition, the 18-mer reverse primer K3r, 5′ GCAGTCGTACATCGGGTT 3′ (*vnfK* priming site positions 4312 to 4323 in reference to the *Azotobacter vinelandii vnfDGK* numbering [27, 28]), was used for the amplification of *vnfG* with forward primer D6f.

Amplification of *vnfG* and *anfG* was accomplished using the protocol and reagents of an Epicenter Fail Safe kit (Epicenter Biotechnologies, Madison, WI). The programmed temperature sequence for *vnfG* and *anfG* amplification was 94°C for hot start followed by 92°C for 1.5 min, 50°C for 1.5 min, and 72°C for 0.5 min. The temperature sequence was run for 30 cycles. The final product extension was conducted at 72°C for 7 min followed by a 4°C temperature hold. PCR products were isolated from a 0.4% (wt/vol) agarose gel using a GeneClean II kit (Bio 101, Inc., Vista, CA). PCR products were cloned using a Promega Easy vector kit and the associated protocols. Cloned PCR products were sequenced at the biotechnology facilities of Iowa State University (Ames, IA).

AnfG and VnfG sequences from the diazotrophic isolates and named bacterial species downloaded from the NCBI/GenBank database were added to the AnfG/VnfG protein family (PFAM 03139.13) alignment (PubMed ID 16381856), and nonconserved sequences at the N-termini were removed. Trees were generated by the neighbor-joining method using Phylip v3.6 (18); these trees were not substantially different than those generated by maximum likelihood or unrooted parsimony.

PCR amplification of *nifH* gene. Primers for PCR amplification of the *nifH* gene were previously described (54).

Southern hybridization. DNA fragments containing *A. vinelandii nifD* (0.8-kb *nifD* insert of pTMR18 [7]), *vnfD* (1.4-kb *vnfD* insert of pVDSJ1 [26]), and *anfD* (1.08-kb *anfD* insert of pPJD3A2 [41]) were used as hybridization probes. Probes were labeled with digoxigenin (Boehringer Mannheim), and the Southern blot procedure was performed as described by the supplier. Hybridization temperatures ranged from 50 to 60°C.

Nucleotide sequence accession numbers. The GenBank accession numbers for the nucleotide sequences of the nitrogen fixation genes are as follows: BR2*anfG*, EF694542; BR3*anfG*, EF694543; BR5*anfG*, EF694544; BR6*anfG*, EF694545;

BR7*anfG*, EF694546; BR8*anfG*, EF694547; BR10*anfG*, EF694548; DP6*anfG*, EF694549; DP7*anfG*, EF694550; LPF4*anfG*, EF694551; MU4*anfG*, EF694552; MU5*anfG*, EF694553; MU11*anfG*, EF694554; MU12*anfG*, EF694555; MU13*anfG*, EF694556; NC2*anfG*, EF694557; PRM1*anfG*, EF694558; MU7*anfG*, EF694559; ARB2*vnfG*, EF694560; ARB3*vnfG*, EF694561; BR2*vnfG*, EF694562; BR3*vnfG*, EF694563; BR5*vnfG*, EF694564; BR6*vnfG*, EF694565; BR7*vnfG*, EF694566; BR8*vnfG*, EF694567; BR10*vnfG*, EF694568; DP6*vnfG*, EF694569; DP7*vnfG*, EF694570; LPF4*vnfG*, EF694571; MB4*vnfG*, EF694572; MU1*vnfG*, EF694573; MU2*vnfG*, EF694574; MU4*vnfG*, EF694575; MU6*vnfG*, EF694576; MU7*vnfG*, EF694577; MU11*vnfG*, EF694578; MU12*vnfG*, EF694579; MU13*vnfG*, EF694580; NC2*vnfG*, EF694581; PB3*vnfG*, EF694582; and AN1*anfHDGK*, EF694583. The GenBank accession numbers for the 16S rRNA gene sequences are as follows: ARB2, AY588643.1; ARB3, AY590431.1; NC2, AY590432.1; MU2, AY590433.1; MU4, AY590434.1; MU5, AY590435.1; MU7, AY590436.1; BR7, AY590437.1; BR3, AY590438.1; BR10, AY590439.1; BR5, AY590440.1; MU11, AY590441.1; MB4, AY590442.1; MU6, AY590443.1; MU1, AY590444.1; DP7, AY590445.1; MU13, AY590446.1; MU12, AY590447.1; PRM1, AY590448.1; BR8, AY590449.1; PB3, AY590450.1; BR2, AY590451.1; DP6, AY590452.1; BR6, AY590453.1; LPF4, AY590454.1; and AN1, AY590455.1.

RESULTS AND DISCUSSION

Isolation of environmental isolates. A total of 26 diazotrophic strains were isolated from soil samples from Brazil, Puerto Rico, and the United States using Mo-deficient, N-free medium (Table 1). Group 1 consists of 19 isolates where genes for nitrogenases 1, 2, and 3 were detected. Group 2 consists of 6 isolates where genes for nitrogenases 1 and 2 were detected. Group 3 consists of only 1 isolate where genes for nitrogenases 1 and 3 were detected. Modified BM was used for growth and enrichment procedures for most of the isolates (5). Strains AN1 and LPF4 were isolated using anaerobic enrichment conditions.

The ability of diazotrophs with Mo-independent nitrogenases to grow in a wide variety of environments, including those known to have sufficient Mo concentrations for Mo-dependent nitrogen fixation, might be determined by other environmental factors (e.g., temperature) in addition to the concentration of Mo in the macroenvironment (4, 36). As such, we were able to isolate strains with Mo-independent nitrogenases using enrichment media containing 1 μM Na_2MoO_4 (strains MU1 and MU2). Another possible environmental factor that may be involved in the requirement of Mo-independent nitrogenases is temperature. Several studies suggest that the V-containing nitrogenase may function more efficiently at low temperatures than does the Mo-containing nitrogenase (37, 51).

Previously, we suggested that diazotrophic growth of *A. vinelandii* on a solid medium surface might generate Mo-depleted microzones due to the organism's powerful Mo uptake system. This could lead to a competitive advantage by generating ecological niches that exclude diazotrophs lacking Mo-independent nitrogenases. Consistent with this hypothesis are the results of competition experiments where wild-type *A. vinelandii* had an advantage over a mutant lacking Mo-independent nitrogenase 3 on Mo-sufficient solid media but not in liquid media (36). If this hypothesis holds for other diazotrophs with Mo-independent nitrogenases, then it is reasonable to expect to find these diazotrophs in macroenvironments that have sufficient Mo concentrations for Mo-dependent nitrogen fixation. The fact that diazotrophs with Mo-independent nitrogenases were isolated from many different soil environments suggests that this is probably the case, although we did not measure molybdenum concentrations in the samples used in this study.

TABLE 1. Isolates containing Mo-independent nitrogenases and their environmental sources

Group and isolate[a]	Environmental source	Enrichment[b]
Group I		
BR2	Soil, Foz do Iguazu, Brazil	−Mo, −N BM
BR3	Soil, Foz do Iguazu, Brazil	−Mo, −N BM
BR5	Soil, Foz do Iguazu, Brazil	−Mo, +V, −N BM
BR6	Soil, Foz do Iguazu, Brazil	−Mo, −N BM
BR7	Soil, Foz do Iguazu, Brazil	−Mo, −N BM
BR8	Soil, Foz do Iguazu, Brazil	−Mo, −N BM
BR10	Soil, Foz do Iguazu, Brazil	−Mo, −N BM
DP6	Wastewater treatment plant, Durham, NC	−Mo, +V, −N BM
DP7	Wastewater treatment plant, Durham, NC	−Mo, +V, −N BM
LPF4	"Paraffin dirt," Avery Island, LA	−Mo, −N BM (anaerobic)
MU4	Wood chip mulch, Durham, NC	−Mo, +V, −N BM
MU5	Wood chip mulch, Durham, NC	−Mo, +V, −N BM
MU6	Wood chip mulch, Durham, NC	−Mo, +V, −N BM
MU7	Wood chip mulch, Durham, NC	−Mo, +V, −N BM
MU11	Wood chip mulch, Durham, NC	−Mo, −N BM
MU12	Wood chip mulch, Durham, NC	−Mo, −N BM
MU13	Wood chip mulch, Durham, NC	−Mo, −N BM
NC2	Creek sediment, Raleigh, NC	−Mo, −N BM
PRM1	Mangrove sediment, Boqueron, Puerto Rico	−Mo, −N BM
Group II		
ARB2	Wood chip mulch, Durham, NC	−Mo, −N BM
ARB3	Wood chip mulch, Durham, NC	−Mo, −N BM
MB4	Mangrove sediment, Boqueron, Puerto Rico	−Mo, −N BM
MU1	Wood chip mulch, Durham, NC	+Mo, −N BM
MU2	Wood chip mulch, Durham, NC	+Mo, −N BM
PB3	Soil, Raleigh, NC	−Mo, −N BM
Group III		
AN1	Wood chip mulch, Durham, NC	−Mo, −N BM (anaerobic)

[a] Group I, genes for nitrogenases 1, 2, and 3 were detected; group II, genes for nitrogenases 1 and 2 were detected; and group III, genes for nitrogenases 1 and 3 were detected.
[b] "−Mo, −N BM," N-free, Mo-deficient Burk medium; "−Mo, +V, −N BM," N-free, Mo-deficient, +V Burk medium; and "+Mo, −N BM," N-free, +Mo Burk medium.

Phylogenetic analysis. Phylogenetic analysis of 16S rRNA sequences places all but two of the diazotrophic isolates among the gammaproteobacteria, and more specifically as members of the *Pseudomonas/Azotobacteria* "fluorescent pseudomonad" clade (Fig. 1). The genus *Pseudomonas* is ubiquitous in soil, and its members are capable of growing on diverse carbon sources and under diverse environmental conditions (12). *Pseudomonas* is phenotypically similar to the genera *Azotobacter* and *Azomonas*, and the phylogenetic relationship between these organisms has not been well defined (52, 53) (PubMed ID 15133068). Some diazotrophic strains of *Pseudomonas stutzeri*, a nonfluorescent pseudomonad, have been described, and for this reason the azotobacteria have been placed in this group (53). This study as well as previous work (35) show that *Pseudomonas/Azotobacteria* is the predominant group isolated using −Mo, −N BM, a medium commonly used

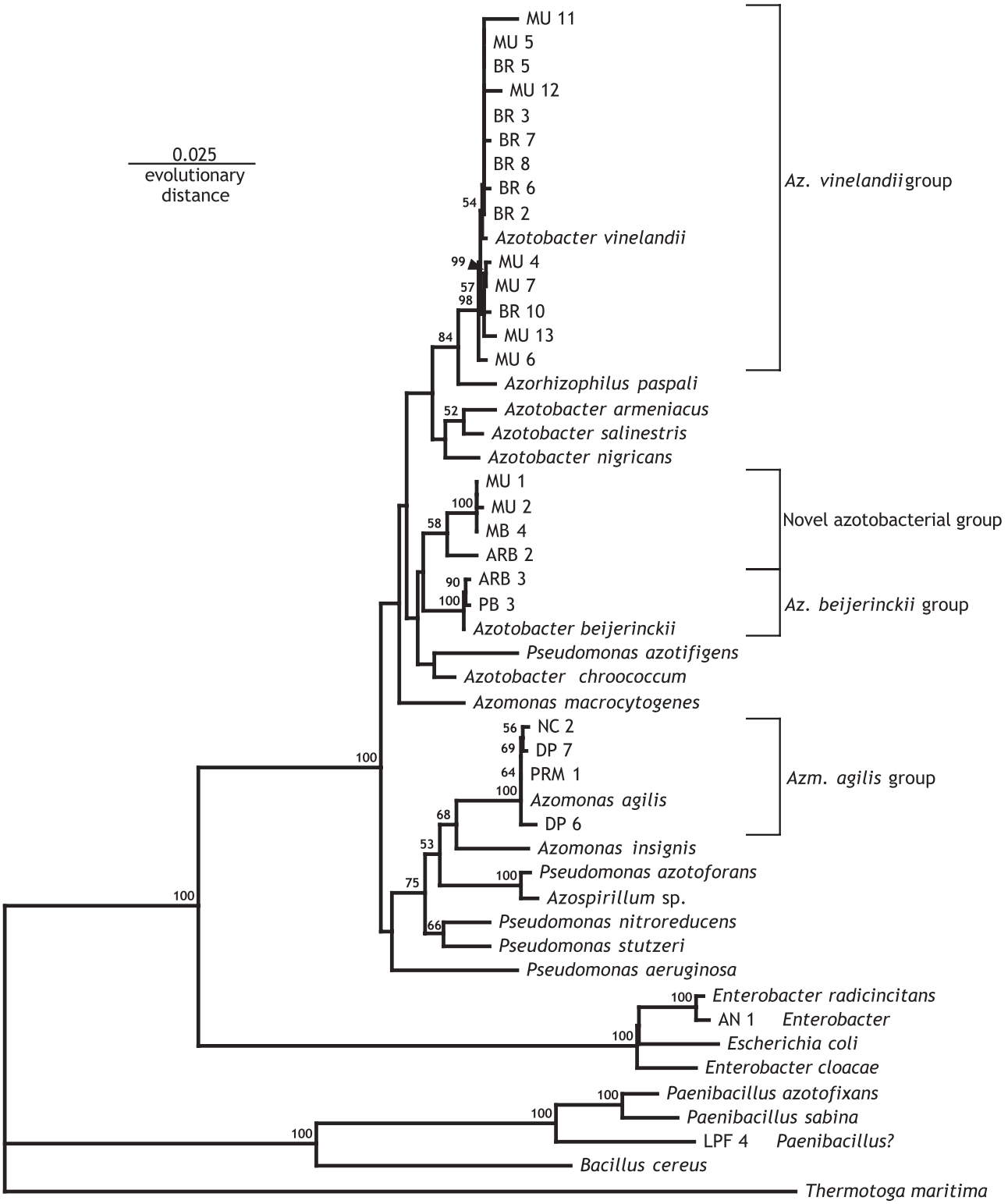

FIG. 1. 16S rRNA phylogenetic analysis of the environmental isolates and azotobacteria/diazotrophic *Pseudomonas* species. A subalignment of 16S rRNA sequences from azotobacteria and diazotrophic *Pseudomonas* species was extracted from the Ribosomal Database Project (PubMed ID 17090583). The 'Sequence Match' function was used to identify the most similar sequence from a named organism for each of the isolated 16S rRNA sequences; in the cases of AN1 and LPF4, these were not azotobacteria, and so these relatives and a related well-known representative were included in the extracted alignment. The 16S rRNA sequences of the diazotrophic isolates were added to the alignment manually. Trees were generated by the neighbor-joining method using Phylip v3.6.

for growth and maintenance of *Azotobacter* species. Approximately half of the isolates (MU11, MU5, BR5, MU12, BR3, BR7, BR8, BR6, BR2, MU4, MU7, BR10, MU13, and MU6) obtained in this study are very closely related to the well-studied *Azotobacter vinelandii*; at least in terms of rRNA similarity, these might be considered strains of this species. Likewise, two isolates (ARB3 and PB3) are more similar to strains of *Azotobacter beijerinckii*, and four isolates (NC2, DP6, DP7, and PRM1) are more similar to strains of *Azomonas agilis*. An additional four isolates (MU1, MU2, MU4, and ARB2) form a clade that, although clearly falling among the azotobacteria, was not specifically affiliated with any characterized species.

Isolate AN1 also is a member of the gammaproteobacteria, but it is most closely related to species of the genus *Enterobacter*, and specifically to *E. radicincitans*, a known diazotrophic and plant growth-promoting species (PubMed ID 15900968). Nitrogen-fixing enterics have also been found as common inhabitants of soils (33), plant material (16), decaying wood (48), and pulp and paper mill effluents (20). However, alternative nitrogenases have not previously been observed in this group.

The only nonproteobacterial strain was LPF4 isolated from "paraffin dirt" (Avery Island, LA). LPF4 is a member of the Firmicutes (low G+C gram-positive bacteria) and is most closely related to species of the genus *Paenibacillus*, although this affiliation is more distant than that of the other isolates and is not specific to any particular species of the genus. Diazotrophic *Paenibacillus* species (e.g., *P. azotofixans*) are common soil inhabitants, but alternative nitrogenases have not previously been identified in this group (15, 45, 49, 53).

Nitrogenase genes. The presence of the different nitrogenase genes, *nifH*, *nifD*, *vnfD*, *vnfG*, *anfD*, and *anfG*, was detected using Southern blot hybridization and/or PCR amplification (Table 2). In each of the isolates, except AN1, LPF4, and MB4, we were able to detect genes that encode the Mo-nitrogenase using Southern blot hybridization. Detection of the Mo-nitrogenase *nifH* gene on AN1, LPF4, and MB4 was done using PCR. In most of the isolates, the molecular characterization of the Mo-independent nitrogenases to detect the δ-subunit (*vnfG*/*anfG*) of the V-nitrogenase and iron-only nitrogenase was done using PCR. Southern blot hybridization was used to characterize several isolates for the α-subunit (*vnfD*/*anfD*) of the V-nitrogenase and iron-only nitrogenase (respectively).

The *vnfG* PCR products obtained with the D6f/K1r primer set were 1,700 bp in size, and those obtained with D6f/K3r were 1,213 bp in size. The *anfG* PCR product generated by D7f/K2r was approximately 760 bp. A comparison of the predicted amino acid sequences for VnfG and AnfG indicates a high degree of identity among the gene products for most of the isolates examined.

The molecular characterization of the nitrogenase genes either by Southern blotting and/or PCR (Table 2) clearly supports our 16S phylogenetic analysis (Fig. 1). In all of the isolates within the *Azotobacter vinelandii* group and the *Azomonas agilis* group, except for isolate MU6, genes for the three nitrogenases were detected. The presence of all three nitrogenases in *Azotobacter vinelandii* and *Azomonas agilis* had been previously reported and well characterized (4, 17). In isolate MU6 of the *A. vinelandii* group, although a nitrogenase 3 gene was not detected, it is possible that an Fe-only nitrogenase is present, based on diazotrophic growth and the acetylene reduction assay as discussed below. In the *Azotobacter beijerinckii* group, which includes strains ARB3 and PB3, only the genes for nitrogenases 1 and 2 were detected. These results are consistent with those reported by Fallik et al. for *Azotobacter beijerinckii* (17). In strains MU1, MU2, MB4, and ARB2, which are included in the novel azotobacteria group of the 16S phylogenetic tree (Fig. 1), only nitrogenase 1 and 2 genes were detected (Table 2).

TABLE 2. Detection of nitrogenase genes in the environmental isolates using PCR and Southern hybridization

Isolate	DNA fragment size(s) (kb)[a]		
	nifD/*nifH*	*vnfD*/*vnfG*	*anfD*/*anfG*
AN1	+	ND	3.1
ARB2	15	4.4, 5.0	ND
ARB3	12	2.3, 4.3	ND
BR2	1.4	+	+
BR3	1.5	+	+
BR5	1.4	+	+
BR6	1.5	+	+
BR7	1.4	+	+
BR8	1.5	+	+
BR10	+	+	+
DP6	9	+	+
DP7	8	+	+
LPF4	+	+	+
MB4	+	1.7, 4.5	ND
MU1	>10	5.0, 4.5	ND
MU2	>10	5.0, 4.5	ND
MU4	4.4, 1.4	4.9, 3.0, 1.3	6.2, 3.4
MU5	1.4	3.0, 1.3	3.4
MU6	1.5	+	ND
MU7	1.4	3.0, 1.3	3.4
MU11	1.4	+	+
MU12	1.4	+	+
MU13	1.7	+	+
NC2	7.1	+	+
PB3	>10	2.2	ND
PRM1	7.8	2	>10

[a] DNA fragment size(s) in EcoRI digests of genomic DNAs hybridizing to nitrogenase gene probes listed in references 7, 26, and 41. +, products cloned and sequenced using primers listed in references 27, 28, 34, and 54; ND, not detected.

For PCR amplification of *anf* genes in AN1, degenerate primers D2f/D5r (34) were used. The only product detected was *anfD*. Similar results were obtained with Southern blot hybridizations using a probe containing *A. vinelandii anfD* (1.08-kb *anfD* insert of pPJD3A2 [41]). Blast sequence comparison of the *anfD* sequence shows that it is 85% identical to that of *Azotobacter vinelandii*.

Phylogenetic analysis of *vnfG* and *anfG*. All of the PCR amplification products obtained using either *anfDGK* or *vnfDGK* PCR primers contained sequences encoding proteins related to AnfG or VnfG, respectively (Fig. 2), and these correspond to the nutritional requirements of the organisms for Mo-independent growth that are presented below. The relationships between the *anfG* and *vnfG* sequences of characterized species do not correspond to the inferred relationships between these organisms in the 16S rRNA phylogenetic tree, as seen previously (34). This suggests that *anf* and *vnf* operons have been frequently transferred horizontally. How-

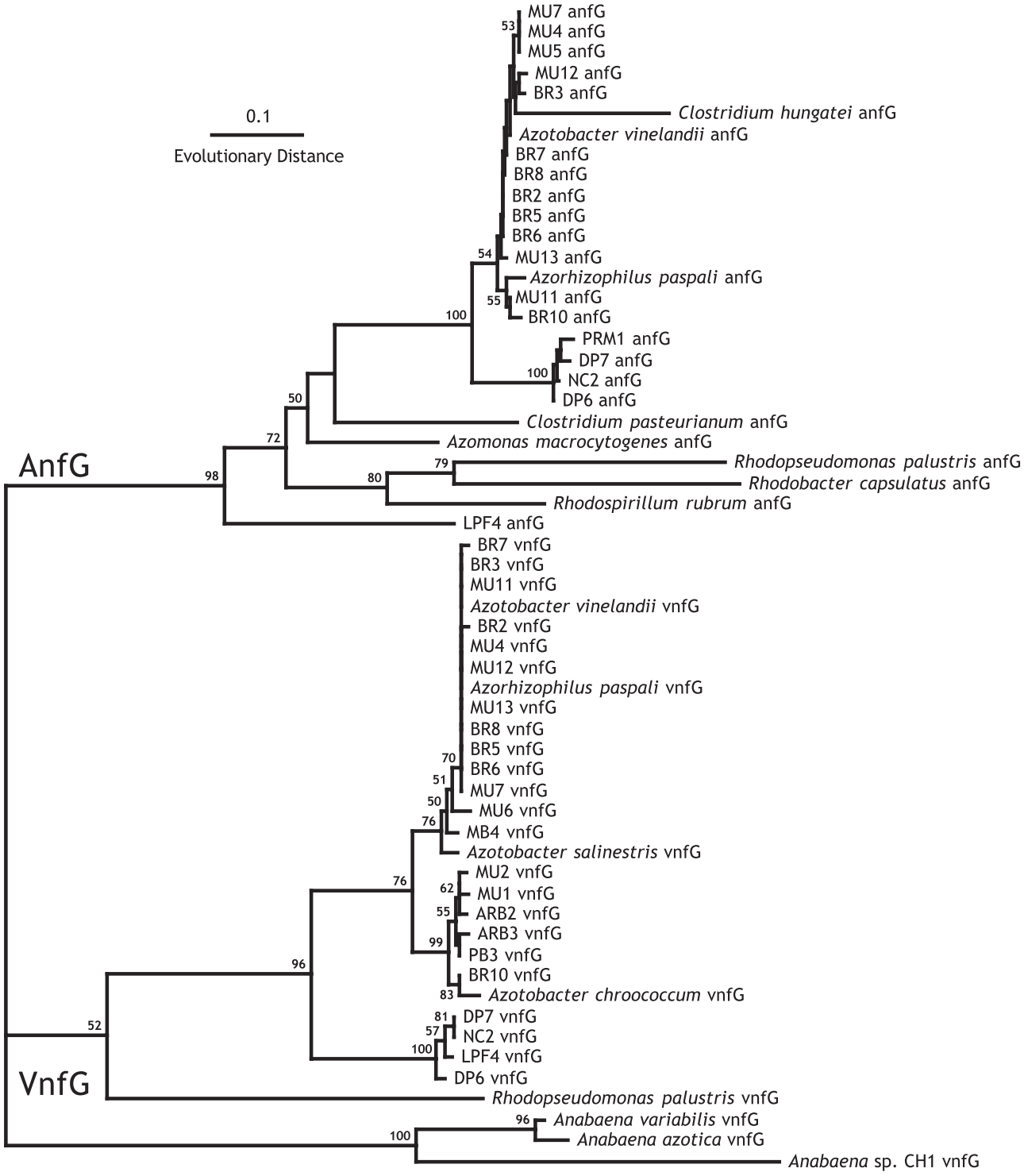

FIG. 2. Phylogeny of the AnfG and VnfG sequences. AnfG and VnfG sequences from the environmental isolates and named bacterial species downloaded from the NCBI/GenBank database were added to the AnfG/VnfG protein family (PFAM 03139.13) alignment (PubMed ID 16381856), and nonconserved sequences at the N-termini were removed. Trees were generated by the neighbor-joining method using Phylip v3.6.

ever, the relationships between the *anfG* and *vnfG* sequences of the newly isolated diazotrophs are generally consistent with the phylogenetic placement of the organisms on the basis of 16S rRNA sequence. *anfG* and *vnfG* sequences from isolates of the *A. vinelandii* group are both also closely related to *anfG* and/or *vnfG* of *A. vinelandii*, with the single exception of the *vnfG* sequence from isolate BR10, which is more closely related to the distinct *vnfG* of *Azotobacter chroococcum*. All of

TABLE 3. Diazotrophic growth and whole-cell nitrogenase activity in liquid media

Isolate	Addition to −Mo, −N BM	Generation time (h)	Nitrogenase activity ± SD (*n*)[a]	
			nmoles C_2H_4 · mg protein^{-1} · 30 min^{-1}	nmoles C_2H_6 · mg protein^{-1} · 30 min^{-1}
A. vinelandii CA	NH_4^+ (28 mM)	2.83	−	−
	Na_2MoO_4 (1 μM)	3.49	2,542 ± 27 (2)	−
	V_2O_5 (1 μM)	5.20	255 ± 28 (2)	6 ± 1 (2)
	None	5.92	66 ± 1 (2)	5 ± 1 (2)
ARB3	NH_4^+ (10 mM)	2.4	−	−
	Na_2MoO_4 (1 μM)	1.8	672 ± 52 (2)	− (2)
	V_2O_5 (1 μM)	3	100	6
	None	24	24	4
BR5	NH_4^+ (10 mM)	3.0	8	−
	Na_2MoO_4 (1 μM)	3.1	1,328	−
	V_2O_5 (1 μM)	4.5	31	2
	None	5.3	15	3
BR6	NH_4^+ (10 mM)	2.3	7 ± 1.0 (2)	− (2)
	Na_2MoO_4 (1 μM)	2.7	1,277	− (2)
	V_2O_5 (1 μM)	8.6	40 ± 4 (2)	3 ± 0.2 (2)
	None	8.4	27 ± 15 (2)	4 ± 1 (2)
BR7	NH_4^+ (10 mM)	4	−	−
	Na_2MoO_4 (1 μM)	4.3	1,293 ± 302 (2)	− (2)
	V_2O_5 (1μM)	3.7	65 ± 29 (3)	4 ± 0.4 (3)
	None	4.9	30 ± 11 (3)	4 ± 1 (3)
BR8	NH_4^+ (10 mM)	2.5	36 ± 4 (2)	ND[b]
	Na_2MoO_4 (1 μM)	2.4	1,369 ± 55 (2)	ND
	V_2O_5 (1 μM)	3.5	794	16
	None	3.5	89 ± 10 (2)	5 ± 2 (2)
BR10	NH_4^+ (10 mM)	1.1	−	ND
	Na_2MoO_4 (1 μM)	2.3	1,616 ± 97 (2)	ND
	V_2O_5 (1 μM)	2.5	781	25
	None	ND	320	14
DP6	NH_4^+ (10 mM)	4.7	−	ND
	Na_2MoO_4 (1 μM)	3.6	1,324 ± 13 (2)	ND
	V_2O_5 (1 μM)	3.7	136 ± 7 (2)	ND
	None	4.0	67 ± 7 (2)	ND
DP7	NH_4^+ (10 mM)	4.0	−	ND
	Na_2MoO_4 (1 μM)	3.4	836 ± 134 (2)	ND
	V_2O_5 (1 μM)	3.8	61 ± 36 (2)	ND
	None	3.7	42 ± 7 (2)	ND
MB4	NH_4^+ (10 mM)	4.8	−	−
	Na_2MoO_4 (1 μM)	4.6	900 ± 319 (3)	−
	V_2O_5 (1 μM)	4	52 ± 7 (2)	5 ± 1 (2)
	None	20.4	18 ± 2	−
MU4	NH_4^+ (10 mM)	3.7	42 ± 34 (2)	ND
	Na_2MoO_4 (1 μM)	4.8	481 ± 19 (2)	ND
	V_2O_5 (1 μM)	6.1	352 ± 89 (2)	10 ± 2 (2)
	None	7.0	234	8
MU5	NH_4^+ (10 mM)	4.1	50 ± 10 (2)	ND
	Na_2MoO_4 (1 μM)	4.7	310 ± 9 (2)	ND
	V_2O_5 (1 μM)	7.4	45 ± 2 (2)	ND
	None	9.4	138 ± 6 (2)	ND
MU6	NH_4^+ (10 mM)	1.2	− (3)	− (3)
	Na_2MoO_4 (1 μM)	1.5	1,513 ± 636 (3)	− (3)
	V_2O_5 (1 μM)	2.7	84 ± 33 (2)	5 ± 2 (2)
	None	2.4	24	4
MU11	NH_4^+ (10 mM)	2.5	−	−
	Na_2MoO_4 (1 μM)	2.6	1,355	−
	V_2O_5 (1 μM)	3.1	92 ± 19 (2)	4 ± 1 (2)
	None	5.4	18 ± 9 (2)	3 ± 1 (2)
MU13	NH_4^+ (10 mM)	2.6	−	−
	Na_2MoO_4 (1 μM)	2.9	865	−
	V_2O_5 (1 μM)	4.3	32	3
	None	4.8	16	3
NC2	NH_4^+ (10 mM)	1.9	−	ND
	Na_2MoO_4 (1 μM)	2.3	2,841 ± 17 (2)	ND
	V_2O_5 (1 μM)	2.7	431 ± 3 (2)	ND
	None	3.1	123 ± 1 (2)	ND
PB3	NH_4^+ (10 mM)	2.4	−	ND
	Na_2MoO_4 (1 μM)	2.3	1,142 ± 34 (2)	ND
	V_2O_5 (1 μM)	3.5	1,575	30
	None	11.1	693	43
PRM1	NH_4^+ (10 mM)	2.5	85 ± 10 (2)	ND
	Na_2MoO_4 (1 μM)	2.5	1,877 ± 338 (2)	ND
	V_2O_5 (1 μM)	3.2	656 ± 39 (2)	ND
	None	2.9	196 ± 18 (2)	ND

[a] −, measured activity was no greater than activity of the blank (−Mo, −N BM); *n*, number of acetylene reduction assays on different days.
[b] ND, not determined.

the *anfG* and *vnfG* sequences from the *A. beijerinckii* and "novel azotobacteria" groups form clusters in both the *anfG* and *vnfG* subtrees; in the case of the *vnfG* sequences, these clusters are very similar to that of *A. chroococcum*; this may reflect a relationship between these organisms that is not resolved in the 16S rRNA phylogeny. The single exception is the *vnfG* of strain MB4, which is related to those of the large *A. vinelandii* group. The *anfG* and *vnfG* sequences from the *A. agilis* group form clusters distinct from any characterized *anfG* or *vnfG* sequence, consistent with the 16S phylogenetic tree. The isolate LPF4 (*Paenibacillus*) *anfG* sequence forms a novel, deep branch in the *anfG* portion of the tree, as might be expected, but its *vnfG* sequence falls among those of the *A. agilis* group. Taken together, these relationships support the vertical evolution of the alternative nitrogenase operons generally, interspersed with an occasional horizontal transfer.

Diazotrophic growth and acetylene reduction. Table 3 summarizes the diazotrophic growth and whole-cell nitrogenase activity of representative isolates selected according to their placement in the 16S phylogenetic tree (Fig. 1). Nitrogenase activity as measured by acetylene reduction followed the usual pattern, where it was highest during growth in the presence of Mo and lowest under Mo-deficient conditions. As mentioned previously, most of the isolates in this study are closely related to *Azotobacter vinelandii*. All of the strains tested within this clade—BR5, BR6, BR7, BR8, BR10, MU4, MU5, MU6, MU11, and MU13—grew diazotrophically in the presence of Mo or V, or in the absence of these metals. When grown on +Mo, −N BM, all isolates reduced acetylene to ethylene and no ethane was produced. When grown on −Mo, +V, −N BM and −Mo, −N BM, acetylene was reduced to both ethylene and ethane. These results suggest that all of these isolates utilize nitrogenases 1, 2, or 3. All isolates from the *Azomonas agilis* clade—NC2, DP6, DP7, and PRM1—reduced acetylene to ethylene; however, ethane production was not determined when grown on −Mo, +V, −N BM and −Mo, −N BM.

Isolates ARB3, PB3, and MB4 grew diazotrophically in the presence of V and in the absence of Mo and V. However, generation times when grown on −Mo, −N BM were 11.1 h for PB3, 20.4 h for MB4, and 24 h for ARB3. Southern blot hybridization and PCR amplification suggested that only nitrogenase 1 and 2 genes are present in these isolates (Table 2). Thus, it is possible that these strains are using dinitrogenase 2 containing an Fe-only cofactor when cells are grown under Mo- and V-deficient conditions. An analogous situation has been observed with a mutant of *A. vinelandii* lacking the structural genes for dinitrogenases 1 and 2 where FeMoCo was incorporated in dinitrogenase 3 to form an active nitrogenase complex (40). Further studies with isolates ARB3, MB4, and PB3 will be needed to test the cofactor substitution hypothesis.

All isolates grew with generation times of 1.8 to 4 h in +Mo, −N BM. Isolates BR10, MU4, MU5, MU11, and NC2 follow a growth rate pattern (NH_4^+ > Mo > V > −Mo) similar to that for *A. vinelandii* CA and that described for strains isolated in a previous study (6). Interestingly, this pattern is altered for other isolates; for example, ARB3, BR7, BR8, DP6, DP7, MB4, and PRM1 grow as fast or faster under at least one nitrogen-fixing condition than they do in the presence of 10 mM ammonium acetate.

BR10 grows diazotrophically in the presence of Mo or V. However, growth was not detected for BR10 in the absence of Mo and V even though *anfG* was detected using PCR. This strain also did not exhibit nitrogenase activity under Mo-deficient conditions. Thus, it is unclear as to whether BR10 lacks a full complement of genes for nitrogenase 3 expression or whether other conditions were not met during our attempts to grow it under diazotrophic conditions in the absence of Mo and V.

In most of the isolates, nitrogenase activity was greatly reduced or absent in the presence of 10 mM NH_4^+.

Two isolates, AN1 (closely related to *Enterobacter*) and LPF4 (closely related to *Paenibacillus*) failed to grow in liquid, nitrogen-free BM. Therefore, these isolates were grown on slants (BM or Line's acetylene reduction medium) for acetylene reduction studies. The finding that these isolates grew on N-free agar medium in the absence of Mo and V but not in liquid medium could be attributable to the presence of Mo and V (or other micronutrients) in the purified agar. Loveless et al. (35) previously described similar diazotrophic growth for isolates SM1, SM3, and WB3. Strains AN1 and LPF4 exhibited nitrogenase activity when cultured under diazotrophic conditions in the presence and absence of Mo, with the highest values obtained in the presence of Mo (data not shown). Nitrogenase activity was repressed by 10 mM NH_4^+. The acetylene reduction assays are consistent with the genetic characterization of these isolates and suggest the presence of at least one of the Mo-independent nitrogenases.

Conclusion. Twenty-six environmental isolates and the nitrogenases detected in them are summarized in Table 1. Of these, 19 isolates possessed the three nitrogenases, 6 isolates contained nitrogenases 1 and 2, and only 1 isolate, AN1, contained nitrogenases 1 and 3.

We were able to identify the presence of Mo-independent nitrogenases in strains closely related to *Enterobacter* spp. and *Paenibacillus* spp. as well as the fluorescent pseudomonads. The 16S phylogenetic analysis is consistent with a previous study and shows that the majority of isolates are closely related to the fluorescent pseudomonads and azotobacteria.

Lastly, the ability to isolate these types of diazotrophs directly from different geographical areas should expand our knowledge of bacteria that express Mo-independent nitrogenases.

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