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Phylogeny and Characterization of Three *nifH*-Homologous Genes from *Paenibacillus azotofixans*

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In this paper, we report the cloning and characterization of three *Paenibacillus azotofixans* DNA regions containing genes involved in nitrogen fixation. Sequencing analysis revealed the presence of *nifB1H1D1K1* gene organization in the 4,607-bp *Sac*I DNA fragment. This is the first report of linkage of a *nifB* open reading frame upstream of the structural *nif* genes. The second (*nifB2H2*) and third (*nifH3*) *nif* homologues are confined within the 6,350-bp *Hin*dIII and 2,840-bp *Eco*RI DNA fragments, respectively. Phylogenetic analysis demonstrated that NifH1 and NifH2 form a monophyletic group among cyanobacterial NifH proteins. NifH3, on the other hand, clusters among NifH proteins of the highly divergent methanogenic archaea.

Nitrogen fixation-related genes have been highly conserved throughout evolution even though they are widely distributed among eubacteria and archaea (4, 7, 11, 13, 15). In terms of their physical and biochemical properties, the mechanisms of the nitrogen fixation process are very similar among these organisms. The conventional dinitrogenase is composed of an $\alpha_2\beta_2$ tetramer; the α and β subunits are encoded by the *nifD* and *nifK* genes, respectively. Also included in the nitrogenase complex is nitrogenase reductase, which is encoded by the *nifH* gene. In most diazotrophs, the *nifHDK* genes are contiguous. Sequence and mutational analyses of nitrogen fixation-related genes of various diazotrophs indicate that the arrangement of *nif* and associated genes differs considerably among these organisms. Examples of organisms with a noncontiguous arrangement of structural *nif* genes are *Frankia* sp. strain FaC1, *Bradyrhizobium japonicum*, and *Rhizobium* sp. strain Irc78 (2, 14).

Paenibacillus azotofixans ATCC 35681 is a gram-positive, facultatively anaerobic diazotroph that falls into a broad cluster of nitrogen fixers in rRNA group 3; this cluster also includes *P. macerans* and *P. polymyxa* (3). Diazotrophic strains of *P. azotofixans* were shown to possess the ability to fix atmospheric dinitrogen with high efficiency (8, 25, 29). In contrast to the majority of diazotrophs, their ability to fix nitrogen is not affected by the presence of nitrate (29).

PCR amplification of the *nifH* gene fragment. The objective of identifying DNA fragments containing *nif* homologues was achieved by using the 380-bp *nifH* gene as a homologous probe. Alignment of NifH polypeptide sequences from representative diazotrophs was performed using ClustalX software (9). Based on these sequence alignments, *nifH*-degenerate oligonucleotides (5′-TAY GGN AAR GGN GGN ATN GGN AA-3′ and 5′-GCR AAN CCN CCR CAN ACN ACR TC-3′) were designed as primers.

Chromosomal DNA (40 ng/ml) was PCR amplified in a 50-μl reaction volume containing 1× PCR buffer (Promega), a 1 mM concentration of each primer, a 0.2 mM concentration of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, and 2.0 U of Weiss *Taq* DNA polymerase (Promega). The following PCR parameters were used: 94°C for 5 min; 30 thermal cycles of 94°C for 30s, 45°C for 30s, and 72°C for 30s; and a final extension step at 72°C for 10 min.

Screening of genomic library and Southern blot analysis. A genomic library of individual lambda clones from primary recombinants was screened according to standard procedures (28), using the PCR-amplified *nifH* probe. Following secondary and tertiary screenings, positively hybridized plaques were isolated and their DNA was extracted. The purified DNA was subjected to restriction enzyme digestions (*Eco*RI, *Hin*dIII, and *Sac*I). Southern analysis using the *nifH* probe revealed the presence of three distinctly different DNA digestion profiles (data not shown), suggesting the existence of three different *nif* gene-containing DNA regions, which were subsequently gel purified and ligated. Hybridization analysis using the digoxigenin-labeled *nifH* PCR probe was also performed with genomic DNA digested with the *Eco*RI and *Hin*dIII restriction enzymes (data not shown). The results obtained suggested the presence of more than one copy of the *nifH* gene in *P. azotofixans*, in agreement with previous studies by Oliveira et al. (21) and Rosado et al. (26).

Sequence analysis of *nifH1*, *nifH2*, *nifH3*, and other *nif* genes. The 4,607-bp *Sac*I fragment contained a 320-amino-acid partial *nifB1* coding region, the complete *nifH1* and *nifD1* open reading frames (ORFs), and the first 387 amino acids of *nifK1* (Fig. 1a). These alleles were designated *nifB1*, *nifH1*, *nifD1*, and *nifK1*, respectively. This is the first report of linkage of a *nifB* ORF upstream of the conventional structural *nif* genes. Analysis of the region immediately upstream of the *nifH1D1K1* ORFs revealed the presence of potential ribosome binding sites (RBSs; GAAGG, GAGG, and GAGG, respectively) (30) located between 8 and 11 bp from the ATG initiation codon of each ORF. The suggested *nifH1* RBS overlaps with the 3′ end of the *nifB1* coding region. Examination of the 143-bp *nifH1*-*nifD1* intergenic region revealed the presence of an 11-bp inverted-repeat structure that might have a regulatory function during *nifD1K1* transcription. Similar inverted repeats have been described for other diazotrophs (4, 11, 16). Comparison of its amino acid sequences with sequences in the database

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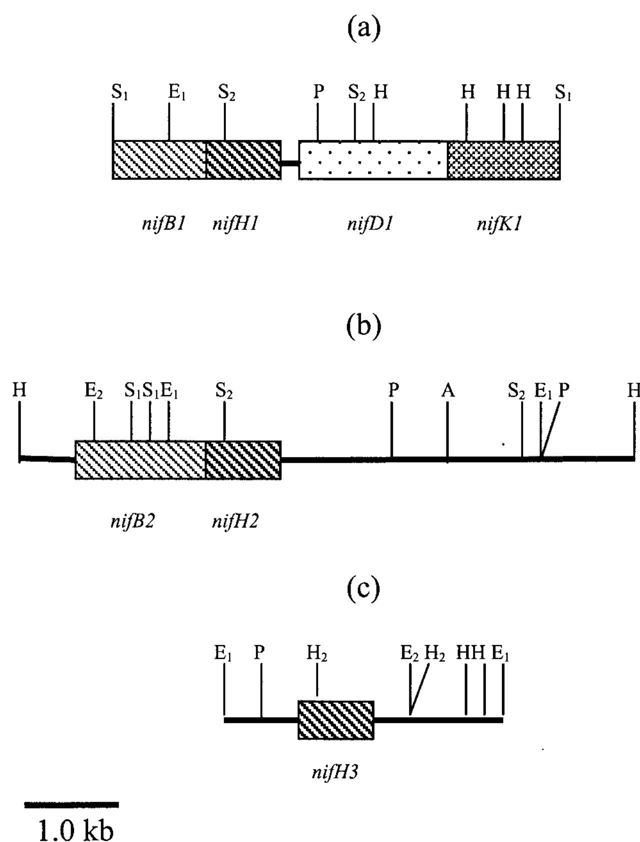

FIG. 1. Organization of *nif* genes in a 4.607-kb *Sac*I fragment from pCQC102 containing the entire *nifH1* and *nifD1 genes* and partial *nifB1 and nifK1* genes (a), a 6.350-kb *Hin*dIII fragment from pCQC100 containing *nifH2* and *nifB2* (b), and a 2.840-kb *Eco*RI fragment from pCQC101 containing *nifH3* (c). Restriction enzyme cutting sites are indicated as follows: A, *Hpa*I; H, *Hin*dIII; H_2, *Hinc*II; E_1, *Eco*RI; E_2, *Eco*RV; P, *Pst*I; S_1, *Sac*I; and S_2, *Sac*II.

revealed that this *nifD1* ORF has the highest degree of homology with members of the gram-positive, high-G+C-content genus *Frankia* (69% identity with *Frankia alni* strain ArI3 and 68% identity with *Frankia* sp. strain EUIK1). A 4-nucleotide overlap occurs between the 3′ end of *nifD1* and the 5′ end of *nifK1*, an indication of a possible translational coupling phenomenon (22). The sequence of *nifK1* was partial from the putative ATG, coding for 387 amino acids, with a putative RBS located 10 bp upstream (within the 3′ end of *nifD1*).

A second *nifB-nifH* cluster (designated *nifB2H2*) was found in a 6,350-bp *Hin*dIII fragment (Fig. 1b). As with NifH1, the protein coding region of NifH2 is 879 nucleotides in length and encodes a predicted 292-amino-acid polypeptide. Interestingly, as in *nifH1*, the putative RBS (30) for *nifH2* is located within the 5′ end of its corresponding *nifB2* gene. The *nifB2* ORF terminates with a single stop codon, TAA, which is followed by the initiation codon for *nifH2* 6 bp downstream. Unlike *nifB1H1*, this *nif* cluster does not have the *nifDK* genes within the 3.5-kb region downstream of the *nifH2* termination codon. Instead, two potential ORFs that appear to lack any known *nif*-related function are found. The closest homologies were with various transporter substrate-binding proteins.

The third *nifH* homologue (*nifH3)* was found within the 2,840-bp *Eco*RI fragment (Fig. 1c). No adjacent *nifB* or *nifDK* coding regions were found within close proximity. A putative ORF (truncated) that displayed homology to transporter ATP-binding proteins was found approximately 80 bp upstream of the *nifH3* start codon.

Amino acid alignment of NifH proteins. Figure 2 shows an alignment of the deduced amino acid sequences of the *P. azotofixans* NifH proteins. They are more divergent in their C termini. Stretches of 10 or more conserved amino acids were observed for residues 10 to 21, 97 to 109, and 129 to 141. When the amino acid residues of NifH1 and NifH2 were compared, seven were found to differ; this constitutes 97% identity. Comparing either NifH1 or NifH2 with NifH3 yielded a comparatively low 43% identity. A high (97%) identity was also observed when the partial reading frames of *nifB* genes were translated to their respective amino acids. At the nucleotide level, alignment of the two *nifBH* gene clusters also revealed a high degree of identity (94%), with no significant changes until 57 bases downstream of the presumptive termination codon of the *nifH* ORF (data not shown). These data led us to postulate that *nifBH* gene clusters of *P. azotofixans* had undergone a gene duplication process, resulting in the *nifB1H1* and *nifB2H2* gene organizations.

Phylogenetic analysis. The *nifH* phylogenetic tree had been well established (20, 26, 32, 35, 36) and is largely consistent with the 16S rRNA gene phylogeny (34). Our data (Fig. 3) are in agreement with the division of the NifH topology into four major clusters, as described by Chien and Zinder (5, 6). When the complete *nifH* coding sequences were used, the clustering of *P. azotofixans* NifH1 and NifH2 yielded several interesting observations. Earlier *nifH*-based phylogenetic analyses of *P. azotofixans* involved partial sequences of *nifH* fragments derived by PCR amplification (1, 26, 36). Discrepancies between our study and those of other investigators (1, 26, 36) in the placement of *P. azotofixans* NifH proteins were probably due to their use of short-length *nifH* fragments, which reduced the resolving power of the analyses. When phylogeny was based on partial *nifH* gene sequences, determined by Zehr et al. (36), *P. azotofixans* NifH did not cluster with NifH proteins of any group of bacteria. Further observations and the branching order of the NifH phylogeny seemed to suggest that *P. azotofixans* NifH lies within the cyanobacterial clade (1, 36).

Use of the complete DNA sequences of the three *nifH* genes in a reanalysis of NifH phylogeny demonstrated clustering of *P. azotofixans* NifH1 and NifH2 within the *Cyanobacteriaceae* grouping (Fig. 3). The NifH protein from a filamentous, nonheterocystous marine cyanobacterium, *Trichodesmium* sp. strain IMS101, showed the highest degrees of identity with *P. azotofixans* NifH1 (80%) and NifH2 (79%), respectively. Interestingly, neither NifH1 nor NifH2 clustered with the NifH proteins of other gram-positive diazotrophs, such as *Frankia* spp. (a high-G+C firmicute) and *Clostridium pasteurianum* (a low-G+C firmicute).

The third putative *nifH* gene product of *P. azotofixans* (NifH3) clustered with NifH proteins of members of the *Archaea* domain, *Methanothermococcus thermolithotrophicus* and *Methanothermobacter thermoautotrophicus*. Again, this putative NifH did not cluster with those of the other phyletically related gram-positive microorganisms, such as *Frankia* spp. or *C. pasteurianum*. This is the first report of a gram-positive diazotroph

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                  10        20        30        40        50        60
NifH1     MSKKPRQIAFYGKGGIGKSTTSQNTLAQLATTFGQKIMIVGCDPKADSTRLILNTKAQQT
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NifH2     MSKKPRQIAFYGKGGIGKSTTSQNTLAQLATTFGQKIMIVGCDPKADSTRLILNTKAQQT
          | ||  ||| |||||||||||  |  |      | | |  ||||| |||          |
NifH3     MAKKIKQIAIYGKGGIGKSTTTSNISAALS-VAGYKVMQFGCDPKSDSTNTLRGGEYIPT
          *:** :***:***********:.*  * *: . * *:* .*****:***. : . :   *

                  70        80        90       100       110       120
NifH1     VLHMAAELGSAEDLELEDVLATGFGDILCVESGGPEPGVGCAGRGIITSINFLEEQGAYD
          ||| |||||| |||||||| ||||||||||||||||||||||||||||||||||||||||
NifH2     VLHLAAELGSVEDLELEDVVATGFGGILCVESGGPEPGVGCAGRGIITSINFLEEQGAYD
          ||               ||   ||  | ||| ||| |||||||||||||   |  |
NifH3     VLDTLRDK---QIVRAHDVIFEGFNGIYCVEAGGPAPGVGCAGRGIITSVSLLKQQKVFE
          **.   :    : :. .**:  **..* ***:*** *************:.:*::* .::

                 130       140       150       160       170       180
NifH1     G--MDFISYDVLGDVVCGGFAMPIRENKAQEIYIVCSGEMMAMYAANNIARGILKYAQSG
          |  |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NifH2     G--MDFISYDVLGDVVCGGFAMPIRENKAQEIYIVCSGEMMAMYAANNIARGILKYAQSG
              |   ||||||||||||| | ||  |     | |   || |||||   || ||   |
NifH3     ELDLDYVIYDVLGDVVCGGFAVPVREGIAEHVFTVTSADFMALYAANNLFKGIHKYSTEG
             :*:: *************:*:**. *:.:: * *.::**:*****: :** **: .*

                 190       200       210       220       230       240
NifH1     SVRLGGLICNSRNTDREDELIMELARRLNTQMIHFVPRDNVVQHAELRRMTVTQYNPEHN
          ||||||||||||||||||||| |||||||||||||||||||||||||||||||||||||
NifH2     SVRLGGLICNSRKTDREDELITELARRLNTQMIHFVPRDNVVQHAELRRMTVTQYNPEHP
             ||| | ||       |       |  ||    |||   |  |||   |     |
NifH3     GALLGGVIANSINAPYAKEIVDDFVARTHTQVMEYVPRSVSVTQAELQGKTTIEADPNSK
          .. ***:*.** ::   .*:: ::. * :**::.:***.  * :***:  *. : :*:

                 250       260       270       280       290
NifH1     QANEYKQLADKILHNEMLNIPTPIEMDELEQLLIDFG-VVEDEETALKKLEAAGH-
          |||||||||||||||| | |||||||||||||||||| |||||||||||||||||
NifH2     QANEYKQLADKILHNEKLTIPTPIEMDELEQLLIDFG-VVEDEETAIKKLEAAGH-
          ||  || || ||        | | |  ||       |      |       |||
NifH3     QAQIYKSLAQKIVDHTESKVPVPLETSELREWASNWGKQLVELEAGVLSPAAAGNL
          **: **.**:**:.:   .:*.*:* .**.:   ::*  : : *:.: .  ***:
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FIG. 2. ClustalX (9) alignment of deduced amino acids sequences of the three NifH proteins of *P. azotofixans*. Amino acids are designated with one-letter abbreviations. Gaps (indicated by hyphens) were introduced for maximal matching. The conserved amino acids are indicated by stars below the alignment; periods and colons denote similar amino acids and acceptable substitutions, respectively, at the indicated amino acid positions. NifH2 amino acid residues which differ from those in the corresponding positions of NifH1 are in bold and underlined.

having a NifH protein that clusters with Nif proteins of confirmed methanogenic diazotrophs. Based on the NifH phylogenetic analysis (Fig. 3), *P. azotofixans* NifH3 also did not fall within the *anf* nitrogenase clade. Rosado et al. (26) reported a *nifH* phylogenetic tree in which the proteins of three *Paenibacillus* strains, *P. azotofixans* P3E20 and RBN4 and *P. durum* DSMZ1735, formed a cluster with the alternative (*anf*) nitrogenases. It is not known at this point whether the putative *anf* nitrogenase reported by Rosado et al. (26) exists in this *P. azotofixans* type strain as well. It has not yet been determined whether this *nifH3* gene product is a functional nitrogenase. It has been postulated that genes from this cluster are related to genes involved in bacteriochlorophyll synthesis and probably have a function unrelated to nitrogen fixation (5, 12).

The question of horizontal transfer of the *nifH* gene has been debated among evolutionists for the last 3 decades (10, 17, 18). The strongest evidence yet for horizontal *nifH* gene transfer came from the pioneering phylogeny studies of *nifH* genes (17, 18). Verification of a horizontal-transfer event is difficult, especially with the limited genetic data from *Paenibacillus* strains. Nevertheless, our data on NifH phylogeny revealed some unanticipated features that brought us to postulate that the gene transfer phenomenon exists. The most striking evidence for the occurrence of a gene transfer event was the unusual placement of NifH3 among the highly divergent members of the *Archaea*. Smith et al. (31) described a phylogenetic congruency test based on the assumption that a NifH tree corresponds to conventional NifH phylogenies (20, 26, 32, 35, 36); if there was any odd placement, a horizontal-gene-transfer event may have occurred. Furthermore, the low level of identity (43%) between *P. azotofixans* NifH3 and the other two NifH proteins likely indicates that there are two different groupings of orthologous gene products. The vast differences in the sequences among NifH3 proteins compared to NifH1 and NifH2 seemed to suggest that a duplication event was unlikely; otherwise, like the five *C. pasteurianum* NifH proteins (33), all three *P. azotofixans* NifH proteins would be grouped in the same cluster.

It is not presently known whether all three *nif* homologue clusters are located in the genome or on plasmids (if any even

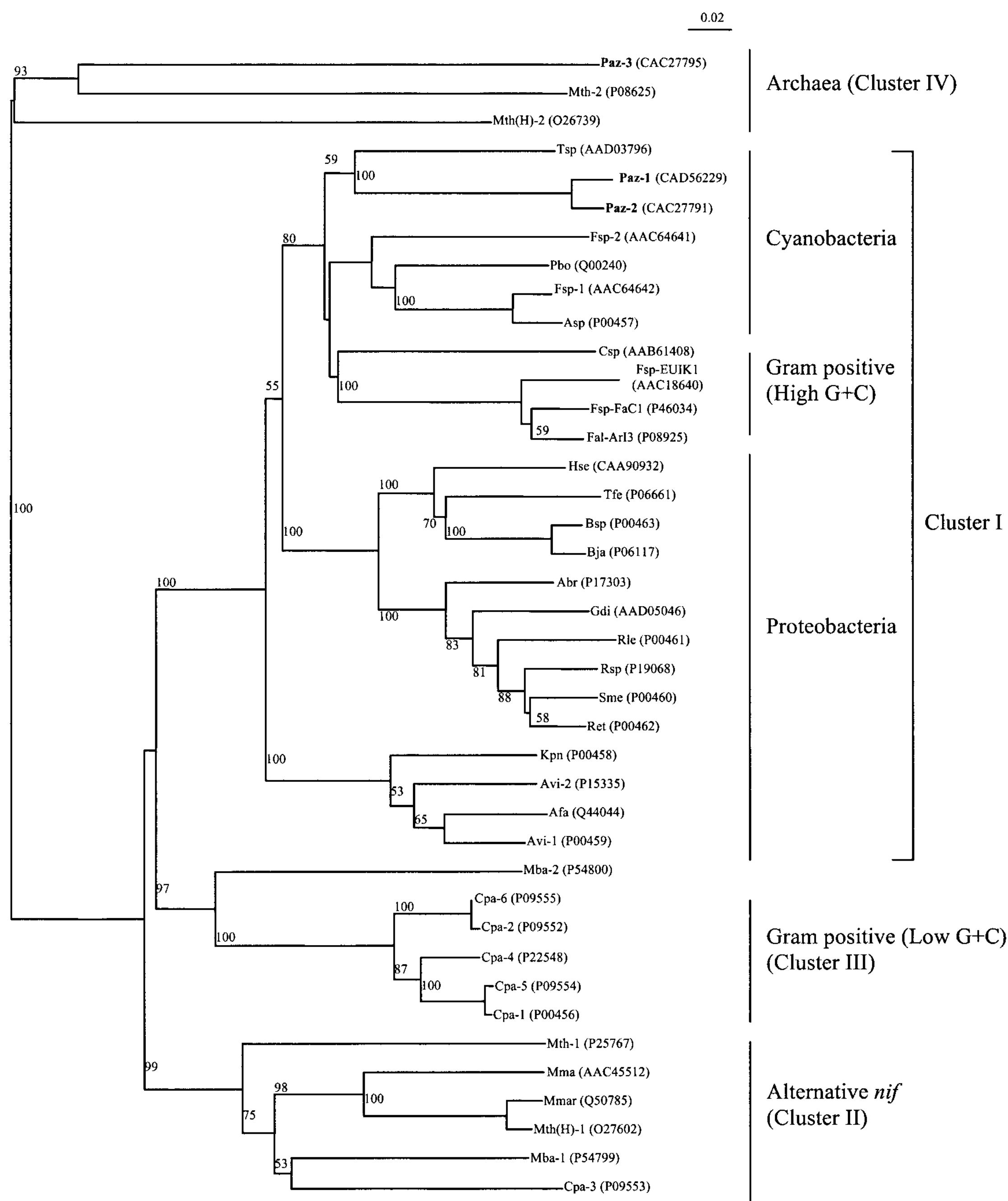

FIG. 3. Tree showing phylogeny of NifH polypeptide sequences, constructed by the neighbor-joining method (27). Graphic representation of the tree was made using *NJPlot* software (23). The database accession numbers are indicated after the abbreviations. Cluster I to IV assignments are described elsewhere (5, 6). The data was analyzed with 100 bootstrap values. The values presented above the nodes are the bootstrap values generated. Bootstrap values below 50% are not shown. The scale bar represents 0.02 substitution per site. Abbreviations: Abr, *Azospirillum brasilense*; Afa, *Alcaligenes faecalis*; Asp, *Nostoc* sp. strain PCC7120; Avi, *Azotobacter vinelandii*; Bsp, *Bradyrhizobium* sp. strain ANU289; Bja, *Bradyrhizobium japonicum*; Cpa, *Clostridium pasteurianum*; Csp, *Cyanothece* sp. strain ATCC 51142; Fal-ArI3, *Frankia alni* strain ArI3; Fsp-EUIK1, *Frankia* sp. strain EUIK1; Fsp-FaC1, *Frankia* sp. strain FaC1; Fsp, *Fischerella* sp. strain UTEX1931; Gdi, *Gluconacetobacter diazotrophicus*; Hse, *Herbaspirillum seropedicae*; Kpn, *Klebsiella pneumoniae*; Mba, *Methanosarcina barkeri*; Mma, *Methanococcus maripaludis*; Mth(H), *Methanothermobacter thermoautotrophicus* (ΔH); Mmar, *Methanothermobacter marburgensis* strain Marburg; Mth, *Methanothermococcus thermolithotrophicus*; Paz, *Paenibacillus azotofixans*; Pbo, *Plectonema boryanum*; Ret, *Rhizobium etli*; Rle, *Rhizobium leguminosarum*; Rsp, *Rhizobium* sp. strain NGR234; Sme, *Sinorhizobium meliloti*; Tfe, *Acidithiobacillus ferrooxidans*; Tsp, *Trichodesmium* sp. strain IMS101.

exist), as in some diazotrophic systems (19, 24). It will also be of interest to determine whether the phylogenies of complete *nifH* genes of other *Paenibacillus* strains conform to the conventional *nifH* phylogenetic topology.

Nucleotide sequence accession numbers. The sequencing data obtained in this study have been deposited in the EMBL database under the following accession numbers: AJ299453, AJ299454, and AJ515294.

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