Microbial Ecology

Identification of Diazotrophs in the Culturable Bacterial Community Associated with Roots of *Lasiurus sindicus*, a Perennial Grass of Thar Desert, India

Soumitra Paul Chowdhury[1], Michael Schmid[2], Anton Hartmann[2] and Anil Kumar Tripathi[1]

(1) School of Biotechnology, Banaras Hindu University, Varanasi 221005, India
(2) Department of Microbe-Plant-Interactions, GSF-National Research Centre for Environment and Health, Neuherberg/Munich, Germany

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Abstract

Lasiurus sindicus is a highly nutritive, drought-tolerant, perennial grass that is endemic to the Thar Desert of Rajasthan, India. Analysis of 16S rRNA coding genes of the bacterial isolates enriched in nitrogen-free semisolid medium, from the surface-sterilized roots of *L. sindicus*, showed predominance of Gram-negative over Gram-positive bacteria. According to comparative sequence analysis of 16S rDNA sequence data, Gram-positive bacteria with low GC content (*Staphylococcus warneri* and *Bacillus* sp.) and high GC content (*Micrococcus luteus*, *Microbacterium* sp.) were identified. Gram-negative bacteria included *Azospirillum* sp., *Rhizobium* sp., *Agrobacterium tumefaciens*, and *Inquilinus limosus* (α-proteobacteria); *Ralstonia* sp., *Variovorax paradoxus*, and *Bordetella petrii* (β-proteobacteria); and *Pseudomonas pseudoalcaligenes*, *Stenotrophomonas* sp. (γ-proteobacteria). The occurrence of *nif*H sequences in *Azospirillum* sp., Rhizobium sp., and *P. pseudoalcaligenes* showed the possibility of supplying biologically fixed nitrogen by the root-associated diazotrophs to the host plant.

Introduction

The Thar Desert, situated in northwestern India, extends over 2.34 million km^2, and about 91% of it falls in the state of Rajasthan. The sandy soil of this desert is poor in organic matter, having rapid infiltration rates of water and rapid oxidation with very low productivity [9]. The climate induces frequent droughts, making farming difficult and unpredictable. Animal husbandry is one of the most important occupations in this area, and the livestock levels in the region are much beyond the carrying capacity of the land. As a consequence, severe overgrazing has caused changes in the natural succession of vegetation, destruction of the habitat, initiation of desertification, and eventual loss of biological diversity and productivity [21].

The climate, besides anthropogenic degradative activities, is a major threat to the sustainability of the fragile desert ecosystem of the Thar Desert where *Lasiurus sindicus* (locally known as "Sewan") is one of the most common and endemic perennial grasses growing in areas with annual rainfall <150 mm. *Lasiurus sindicus* has a high nutritive value and is preferentially consumed by cattle in the desert [21]. Besides contributing to the development of good rangeland in the Thar Desert, it also has a significant role in stabilizing the blowing sand dunes and expanding desert [9]. Although this agronomically important grass can tolerate prolonged droughts, it does not survive in relatively higher zones of rainfall and faces a serious threat of being an endangered species due to changes in the land-use patterns, increase in soil moisture regimes, and overgrazing. The rhizosphere microbiology of endemic grass like *L. sindicus* is important in view of the *in situ* conservation of biodiversity associated with such niches to sustain delicate ecological processes in the desert ecosystem.

There is very little knowledge on rhizosphere microbiology of the desert plants. Some previous reports have described the isolation and physiological characterization of specific microorganisms, viz., antibiotic producers or specific plant–microbe relationships [4]. Because desert plants survive extreme variations in temperature and

Correspondence to: Anil Kumar Tripathi; E-mail: tripathianil@rediffmail.com

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moisture and grow in typically poor soils with low organic content, with limited amounts of bioavailable inorganic nutrients, microorganisms living in association with desert plants should possess adaptive mechanisms to cope with frequent droughts and various other stresses such as starvation, high osmolarity, high temperature, and desiccation.

Biological nitrogen fixation is ecologically important in oligotrophic and stressed ecosystems such as the Thar Desert to supply nitrogen for biological productivity [2]. Some attempts have been made to understand the role of the associative nitrogen fixation in nitrogen-limited natural ecosystems [17]. The interest in diazotrophic endophytes has been stimulated by their potential use for plant-growth promotion, antagonistic effects against plant pathogens, or biological nitrogen fixation for sustainable agriculture [13].

Although several graminaceous plants have been investigated for the occurrence and diversity of endophytic nitrogen-fixing bacteria, most of them focused on agronomically important crops such as rice, wheat, sugarcane, maize, etc. [10]. In recent years, several studies have also addressed the importance and contribution of biological nitrogen fixation in ecologically unique habitats by focussing on the diversity of *nif*H sequences that encode one of the structural genes for the enzyme nitrogenase [32].

The aim of the present study was to investigate the composition of culturable bacteria associated with the roots of *L. sindicus*, which had survived three consecutive drought years, and to identify diazotrophs that might support nitrogen requirements under stressful conditions prevailing in the oligotrophic desert ecosystem. The 16S rRNA and *nif*H sequences, together with acetylene reduction assay of the isolates, were used to identify the culturable members associated with the roots of *L. sindicus* and to assess their nitrogen fixation potential.

Methods

Collection and Surface Sterilization of Plant Roots. The *L. sindicus* is an endemic grass, which grows naturally in sandy soils of the Thar Desert in Barmer and Jaisalmer districts of Western Rajasthan, India. The mean annual rainfall in this region is <150 mm and the mean aridity index is (–) 90.7 [9]. Roots from three *L. sindicus* plants that survived three consecutive droughts were collected from three different spots (at least 8 m apart from each other) from the sample collection site in the Jaisalmer district. To isolate and identify nitrogen-fixing members of the endophytic bacteria, which might contribute to the nitrogen requirements of *L. sindicus* in the oligotrophic desert environment, the roots were collected from such areas of the desert that did not show the occurrence of legumes. Root samples (15–20 cm) with adhering rhizosphere soil from each plant were transferred to separate sterile screw-capped plastic tubes and transported to the laboratory for further analysis. Roots were washed in sterile saline solution (0.85% NaCl) to remove the adhering soil. Surface sterilization was done with 1% Chloramine T for 15 min, followed by washing thrice with sterile triple distilled water. The efficiency of the root surface sterilization was demonstrated by the absence of any bacterial colonies on 0.1% tryptic soy agar plates, which were incubated at 30°C for 3 days after rolling surface-sterilized roots on the agar surface [16].

Enrichment and Isolation of Bacteria from Roots. The surface sterilized roots from three plant samples were thoroughly macerated and resuspended in 5 mL of phosphate buffered saline (pH 7.0, 0.1 M). The three suspensions were used after serial dilutions to inoculate semisolid NFb medium [1] in different sets. After 3 days of incubation at 30°C without shaking, a subsurface pellicle was observed, indicating the presence of potential diazotrophs. The medium used for the enrichment of nitrogen-fixing bacteria in the present study, containing malic acid as carbon source, has been frequently used to enrich and isolate different types of diazotrophs [13, 16, 25]. Thereafter, the enrichment culture was vortexed and 100 µL of this suspension was inoculated into 5 mL of semisolid NFb medium. Five more serial transfers in semisolid NFb medium were done to enrich nitrogen-fixing bacteria. After the fifth subculture, appropriate dilutions of the vortexed suspensions from the enrichments were spread on nutrient agar plates and incubated at 30°C. Twenty colonies representing all morphological types were randomly selected from each of the three sets (total of 60 colonies) and subjected to further studies.

Nucleic Acid Extraction and 16S rRNA Gene Amplification. Total DNA was extracted from the isolates by using the GFX™ DNA purification kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions. 16S-rRNA gene was amplified using universal primer pair 616f (5′ AGA GTT TGA TYM TGG CTC AG 5′) and 630r (5′ CAK AAA GGA GGT GAT CC 3′) corresponding to nucleotide positions 8–27 and 1528–1544 in 16S rDNA from *Escherichia coli* [20]. A 50-µL reaction mixture included 50 ng of bacterial DNA as template, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 100 µM each of diethylnitrophenyl thiophosphates, 200 ng of each primer, and 1.0 unit of Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania). The reactions were performed on a PTC100 Thermal Cycler (MJ Reseach, Watertown, MA, USA) and the reaction cycle included an initial denaturation of 5 min at 94°C followed by 35 cycles of 1 min

at 94°C, 1 min at 50°C, and 1 min at 72°C, with a final extension of 5 min at 72°C. The amplified product was analyzed in 0.8% agarose gel at 5 V cm^{-1} for 4 h and was visualized by UV excitation after staining with ethidium bromide (0.5 mg L^{-1}).

Polymerase Chain Reaction-RFLP Analysis of 16S-rRNA Genes (ARDRA). The 16SrDNA amplicons was purified using "QIAquick" polymerase chain reaction (PCR) purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A 10–15-μL aliquot of all the purified PCR products was used for restriction digestion with *Sau*3A I and *Alu*I (New England Biolabs, Ipswich, MA, USA) at 37°C for 3 h. Enzymes were inactivated by heating the reaction mixture to 65°C for 10 min. The reaction products were analyzed on a 2.5% agarose gel at 5 V cm^{-1} for 4 h and visualized in UV light after staining with ethidium bromide (0.5 mg L^{-1}).

Phylogenetic Analysis on the Basis of ARDRA Pattern. A dendrogram was constructed on the basis of restriction fragment pattern of the amplicons of 16S rDNA. A binary table or haplotype matrix for each strain was constructed linearly, composing presence (1)/absence (0) data derived from the analysis of the gel. The unweighted pair-group method with arithmetic mean algorithm was used for the calculation of phylogenetic distances with the NTSYS pc2.0 software (Applied Biostatistics, Setanket, NY, USA).

Acetylene Reduction Assay. Bacteria were pregrown at 37°C in minimal NFb-medium supplemented with 10 mM NH_4Cl, harvested at midlog phase, and washed twice with N-free minimal medium [1]. Equal amounts of cells of each strain were resuspended in 10 mL of semisolid N-free NFb-medium in 40-mL bottles, which were made airtight with suba-seal caps and incubated at 30°C for the formation of pellicle. After 48 h of incubation, air (10%, v/v) was replaced with acetylene gas, and the bottles were incubated for 24 h. These cultures were then used to assay acetylene-reducing activity by measuring the amount of ethylene produced from acetylene using a TRACOR 540 gas chromatograph equipped with a flame ionization detector and a Porapak R column.

Amplification of nifH Gene. A pair of universal degenerate primer [33], Zehr-nifHf (5′-TGY GAY CCN AAR GCN GA-3′) and Zehr-nifHr (5′-AND GCC ATC ATY TCN CC-3′), was used to amplify a 360-bp fragment of the *nif*H gene. These primers have been widely and successfully used to amplify the *nif*H gene from distantly related diazotrophs [27]. The samples were amplified in a PCR mixture containing 4 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 100 pmol of each primer, 2.5 U of *Taq* DNA polymerase with 1× buffer with $(NH_4)_2SO_4$ (MBI Fermentas) in 50 μL volumes for 40 cycles at 94°C for 1 min, 57°C for 2 min, and 72°C for 2 min on Primus 25 PCR-System Thermal Cycler (MWG-Biotech AG, Ebersberg, Germany).

Cloning and Sequencing Reactions. Both 16S rDNA and *nif*H gene amplicons were cloned using the TOPO-TA Cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Clone libraries were constructed in vector pCR2.1-TOPO, and positive clones were selected. Plasmids were prepared using Nucleospin Plasmid kit (Macherey Nagel GmbH, Düren, Germany) according to the manufacturer's instructions. Clones were screened for the correct size of the insert by *Eco*RI digestion. A 2-μL aliquot of the purified plasmid was used for restriction digestion with *Eco*RI (New England Biolabs) at 37°C for 3 h. Enzymes were inactivated by heating the reaction mixture to 65°C for 10 min. The reaction products were analyzed on a 1.5% agarose gel at 5 V cm^{-1} for 4 h and visualized by UV excitation after staining with ethidium bromide (0.5 mg L^{-1}). The plasmids were quantified using ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA). Sequencing was performed on both strands using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Rotkreuz, Switzerland) using M13 reverse and forward primers. Sequencing was done on ABI PRISM 3700 DNA Analyzer (Applied Biosystems).

Phylogenetic Analysis. The 16S rRNA gene sequences obtained from the clones were added to an existing database of small-subunit rRNA gene sequences by using fast alignment tool of the ARB software package [24] (http://www.arb-home.de). Alignments were manually verified by comparing the homologous positions of related sequences. Tree calculations were performed by applying maximum likelihood, maximum parsimony, and neighbor joining methods by use of respective tools implemented in the ARB software package. The *nif*H sequences obtained from the clones were added to a *nif*H database with aligned sequences created with so-far published *nif*H sequence data. The sequences were translated into amino acids and the deduced amino acid sequences were aligned with GDE 2.2 editor software. Nucleic acid sequences were then realigned according to the amino acid alignments. Phylogenetic analyses were performed as described above.

Nucleotide Sequence Accession Numbers. The 16S rRNA gene sequences obtained were submitted to NCBI GenBank (http://www.ncbi.nlm.nih.gov/) under the accession number DQ304792 to DQ304810, and the

*nif*H sequences were assigned the accession numbers DQ304811 to DQ304813.

Results

PCR Fingerprinting and Amplified Ribosomal DNA Restriction Analysis of Isolates. PCR amplification of 16S rRNA gene in the 60 bacterial isolates resulted in the amplification of a 1.5-Kb amplicon, which was subjected to restriction digestion with *Alu*I and *Sau*3A I. Both of the tetra-cutting restriction endonucleases yielded distinct banding patterns, which were used as molecular markers to construct a composite dendrogram to find phylogenetic similarities among the isolates. On the basis of the extent of similarity of ARDRA, the 60 isolates could be grouped into 19 clusters, designated as group 1 to group 19. The number of isolates belonging to each group is indicated in Table 1. One representative isolate from each group or genomovars was randomly selected for further studies and identification by 16S rRNA gene sequencing.

Acetylene-Reducing Ability of the Isolates. Acetylene-reduction assay was done to examine the nitrogen fixing ability of the 19 isolates representing different genomovars, which revealed that only two strains, C3 (group 3) and C3r1 (group 19), showed considerable acetylene reduction, with C3 and C3r1 showing 18 and 11.2 nmol C_2H_4 min^{-1} mg $protein^{-1}$, respectively.

Table 1. Summary of isolates obtained in this study, their ARDRA groups, taxonomic positions, and the closest affiliations of the representative isolates in the GenBank according to the 16S-rRNA gene sequences

Taxonomic position	*ARDRA group (no. of isolates represented)*	*Representative isolate*	*Nearest relative*	*% similarity*	*nifH amplification*
Gram-positive					
High G+C	Group 4 (4)	C4	*Micrococcus* sp. LMG19421 (AJ276811)	99.11	–
	Group 15 (2)	C15	*Microbacterium* sp. VKM AC-1389(AB042070)	97.93	–
Low G+C	Group 1 (5)	C1	*Bacillus* sp. (AJ509006)	98.07	–
	Group 2 (2)	C2	*Staphylococcus warneri* (L37603)	99.80	–
	Group 5 (3)	C5	*Bacillus megaterium* (AY167861)	98.92	–
Gram-negative					
α-Proteobacteria	Group 3 (7)	C3	*Azospirillum* sp. TS15 (AB114194)	98.17	+
	Group 6 (2)	C6	*Rhizobium* sp. ORS 1465 (AY500261)	97.85	+
	Group 7 (4)	C7	*Rhizobium* sp. ORS 1465 (AY500261)	97.93	–
	Group 8 (5)	C8	*Agrobacterium tumefaciens* (AJ389899)	99.65	–
	Group 9 (4)	C9	*Agrobacterium tumefaciens* (AJ389899)	100	–
	Group 14 (1)	C14	*Inquilinus limosus* (AY360342)	99.70	–
β-Proteobacteria	Group 10 (3)	C10	*Ralstonia* sp. (AF239160)	99.6	–
	Group 11 (2)	C11	*Variovorax paradoxus* (AF532868)	99.4	–
	Group 12 (4)	C12	*Variovorax paradoxus* (AF532868)	99.04	–
	Group 13 (1)	C13	*Bordetella petrii* (AJ870969)	98.72	–
γ-Proteobacteria	Group 16 (3)	C16	*Stenotrophomonas maltophilia* (AF100732)	99.86	–
	Group 17 (2)	C17	*Stenotrophomonas* sp. (AB183423)	99.07	–
	Group 19 (5)	C3r1	*Pseudomonas pseudoalkaligenes* (Z76675)	99.80	+
Bacteroides/ Flavobacteria	Group 18 (1)	C18	*Chryseobacterium defluvii* (AJ309324)	98.51	–

Identification of Isolates by 16S rRNA Gene Sequencing. One representative from each of the 19 groups was selected for cloning and sequencing of 16S rRNA gene so as to compare sequence similarity. A BLASTn search of these sequences with the most similar 16S rRNA sequences of the GenBank database (http://www.ncbi.nlm.nih.gov) revealed the closest sequence identities from the sequence database. All similarities are summarized in Table 1. Characterization of the isolates on the basis of their 16S rRNA coding genes revealed the presence of both Gram-positive and Gram-negative bacteria within the surface-sterilized roots although larger number was that of Gram-negative. Out of a total of 60 isolates, 44 belonged to Gram-negative, which included 23 α-proteobacteria and 10 each of β- and γ-proteobacteria; one isolate belonged to the Bacteroidetes group. The 16 Gram-positive isolates included eight each belonging to high and low DNA G+C groups. The representatives of Gram-positive isolates showed maximum similarity to *Staphylococcus warneri*, *Micrococcus luteus*, *Microbacterium* sp., and *Bacillus* sp. Gram-negative bacteria included those showing maximum similarity to *Variovorax paradoxus*, *Bordetella petrii*, *Pseudomonas pseudoalcaligenes*, *Ralstonia* sp., *Stenotrophomonas* sp., *Azospirillum* sp., *Rhizobium* sp., *Agrobacterium tumefaciens* and *Inquilinus limosus*.

nifH Phylogeny of the Representative Isolates. Three of the 19 representative strains, C3 (group 3), C6 (group 6), and C3r1 (group 19), generated a 360-bp *nif*H gene fragment. After determining the nucleotide sequence of the three *nif*H amplicons, a neighbor-joining analysis of partial NifH amino acid sequences was performed with other sequences from the *nif*H sequence database. The *nif*H nucleotide sequence from isolate C3r1 showed maximum similarity with the uncultured forest soil bacterium clone (AY912789), and the sequence from C6 showed maximum similarity with the sequence from C3, both of which showed maximum similarity with uncultured bacterium clone (AF389742) (Table 2). The phylogenetic tree revealed their close relatedness with members of cluster I of *nif*H genes [32], containing molybdenum nitrogenases from cyanobacteria and proteobacteria (Fig. 1).

Table 2. List of isolates showing *nif*H amplification and their closest affiliation in the database according to the *nif*H nucleic acid and NifH amino acid sequences

Isolate	*Nearest relative*	*% similarity (nucleic acid)*	*% identity (amino acid)*
C3	Uncultured bacterium (AF389742)	98.81	100.0
C6	Uncultured bacterium (AF389742)	99.4	100.0
C3R1	Uncultured forest soil bacterium (AY912789)	98.3	100.0

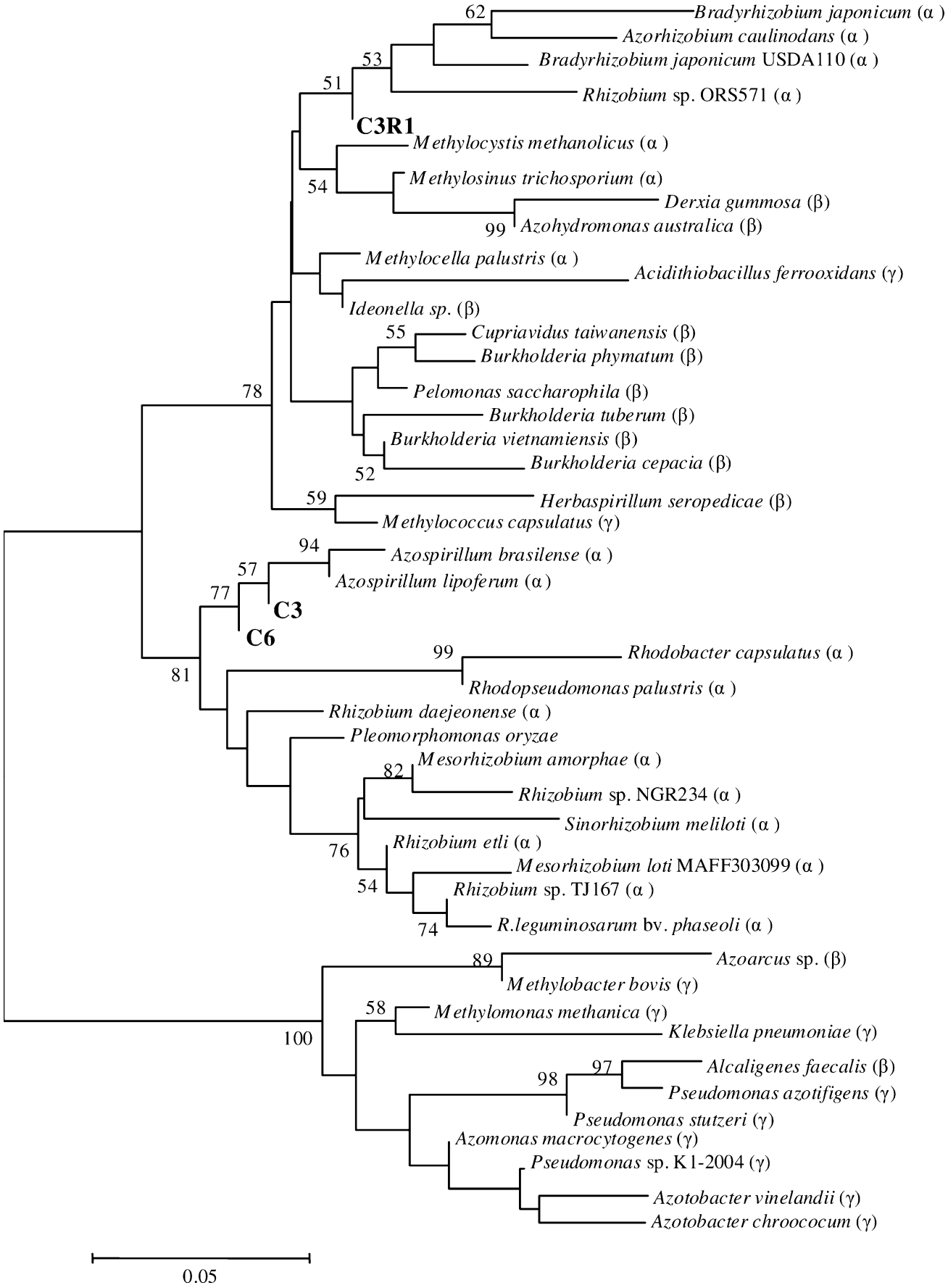
Discussion

Identification of the isolates on the basis of their 16S rRNA gene sequences and the results of ARA and *nif*H gene amplification show that the majority of the strains isolated from the enrichments in the N-free semisolid medium were nondiazotrophs. The occurrence and growth in the form of typical subsurface pellicle in N-free semisolid medium, not accompanied by acetylene reduction, has been reported earlier [28]. Such pellicles have revealed the presence of nondiazotrophic bacteria together with diazotrophs [28]. Approximately 90% bacteria isolated from surface-sterilized rice plants using N-deficient media have been shown to be nondiazotrophs [19]. Apparently, such nondiazotrophs could be nitrogen scavengers or oligotrophs, growing on the nitrogen fixed and released by diazotrophs during the enrichments.

Gram-positive isolates belonged to the phyla of Gram-positive organisms with high and low DNA G+C content. Members of the genus *Staphylococcus* are well-known human pathogens, but some species have been isolated from the rhizosphere of wheat, potato, and strawberry, suggesting their opportunistic mode of existence [6]. Bacteria belonging to *Bacillus* sp. have been identified as plant-growth-promoting rhizobacteria. Although, *nif*H genes have been detected in some species of *Bacillus cereus*, *Bacillus marisflavi*, and *Bacillus megaterium* [12], our isolates of *Bacillus* sp. showed neither acetylene-reducing ability nor amplifiation of *nif*H gene. The other Gram-positive species, *M. luteus*, was shown to have a potential for endophytic occurrence. An endophytic nature of *Microbacterium* species has also been reported in several plants, including agronomic crops and prairie plants [5, 34].

The members of α-, β-, and γ-proteobacteria represented the Gram-negative bacterial isolates equally.

Figure 1. Phylogenetic tree based on neighbor-joining analysis of partial amino acid sequences translated from *nif*H sequences of ▶ diazotrophic isolates of *L. sindicus* (indicated in *bold*). Significant bootstrap probability values are indicated at the *branching points*. α, β, and γ in *parentheses* indicate α, β, and γ subdivisions of proteobacteria.


62
Bradyrhizobium japonicum (α)
Azorhizobium caulinodans (α)
53
Bradyrhizobium japonicum USDA110 (α)
51
Rhizobium sp. ORS571 (α)
C3R1
Methylocystis methanolicus (α)
Methylosinus trichosporium (α)
54
Derxia gummosa (β)
99
Azohydromonas australica (β)
Methylocella palustris (α)
Acidithiobacillus ferrooxidans (γ)
Ideonella sp. (β)
55
Cupriavidus taiwanensis (β)
Burkholderia phymatum (β)
78
Pelomonas saccharophila (β)
Burkholderia tuberum (β)
Burkholderia vietnamiensis (β)
Burkholderia cepacia (β)
52
59
Herbaspirillum seropedicae (β)
Methylococcus capsulatus (γ)
94
Azospirillum brasilense (α)
57
Azospirillum lipoferum (α)
77
C3
C6
99
Rhodobacter capsulatus (α)
81
Rhodopseudomonas palustris (α)
Rhizobium daejeonense (α)
Pleomorphomonas oryzae
Mesorhizobium amorphae (α)
82
Rhizobium sp. NGR234 (α)
Sinorhizobium meliloti (α)
Rhizobium etli (α)
76
Mesorhizobium loti MAFF303099 (α)
54
Rhizobium sp. TJ167 (α)
74
R.leguminosarum bv. phaseoli (α)
89
Azoarcus sp. (β)
Methylobacter bovis (γ)
58
Methylomonas methanica (γ)
100
Klebsiella pneumoniae (γ)
97
Alcaligenes faecalis (β)
98
Pseudomonas azotifigens (γ)
Pseudomonas stutzeri (γ)
Azomonas macrocytogenes (γ)
Pseudomonas sp. K1-2004 (γ)
Azotobacter vinelandii (γ)
Azotobacter chroococum (γ)
0.05

The γ-proteobacteria was represented by *Stenotrophomonas* sp. and *P. pseudoalcaligenes*. The former is a well-known opportunistic human pathogen and is frequently isolated from the rhizospheres of diverse plants all over the world [6]. The isolate C3r1, showing highest 16S rDNA sequence similarity to *P. pseudoalcaligenes*, showed notable nitrogenase activity and presence of a *nif*H gene. Several species of *Pseudomonas*, including *Pseudomonas stutzeri* and recently described *Pseudomonas azotifigens*, have been shown to fix N_2 [18]. Although nitrogenase activity has previously been reported in *P. pseudoalcaligenes* [30], we have shown PCR amplification of *nif*H sequence from this species. The neighbor-joining analysis of partial NifH amino acid sequence (Fig. 1) showed that the sequence clustered together in subcluster 1K of the *nif*H phylogenetic cluster I [32]. This subcluster contains *nif*H sequences from various cultivated representatives of α-, β-, and some γ- (e.g., *Methylococcus capsulatus* and *Acidithiobacillus*) proteobacteria. It is interesting to note that the NifH from *P. psuedoalcaligenes* does not cluster together with NifH from *P. stutzeri* and *P. azotifigens*, which are highly similar to each other [18] and cluster together in subcluster 1U [32].

β-Proteobacteria were represented by three members of Burkholderiales, viz., *Ralstonia* sp., *V. paradoxus*, and *B. petrii*. Members of the genus *Ralstonia* are well known as soil bacteria often found to develop close associations with plants. *Variovorax paradoxus* has been isolated as an endophyte from pepper and tomato plants. They are thought to metabolize trace metabolites produced by other biota and play integral roles in sustaining nutritional consortia [14]. Although *Bordetella* has been isolated from environmental and soil samples [29], its association with plants has not been reported so far.

Bacterial isolates belonging to the α-proteobacteria showed maximum similarity to *Azospirillum* sp., *Rhizobium* sp., *A. tumefaciens*, and *I. limosus*. Although *I. limosus* has previously been isolated from the lungs of cystic fibrosis patients [11], we, for the first time, report its association with a plant. As several pathogenic bacteria have been isolated from the rhizospheres of different plants, some of the mechanisms involved in the interaction between antagonistic plant-associated bacteria and their host plants might be similar to those responsible for their pathogenicity [6]. More research on the ecology and pathogenicity of pathogens may elucidate its mechanism of survival inside plants or in the rhizosphere.

Two of the isolates showed very close similarity with *A. tumefaciens* (*Rhizobium radiobacter*), soil bacteria known for its phytopathogenicity, causing crown gall disease in a wide range of dicotyledonous plants. They have been isolated as endophytes from many members of Gramineae [34]. The two representatives of *Rhizobium* isolated from *L. sindicus* showed maximum similarity with *Rhizobium* sp. ORS 1465, an endophyte that nodulates *Anthyllis henoniana*, a legume growing in the infra-arid zone of Tunisia [31]. PCR-RFLP of 16S rDNA of the two strains was identical when digested with *Alu*I but differed with *Sau*3a. The 16S rDNA of the strain ORS 1465 is closest to *Rhizobium galegae*, suggesting this species could be a biovar of *R. galegae*. Both of the *Rhizobium* isolates did not show any ARA under free-living condition. Although some strains of *Rhizobium* show N_2-fixation in free-living conditions, the metabolic significance of this is unclear [23]. A specific *nif*H amplicon was amplified from the *Rhizobium* sp. (isolate C6). However, the absence of *nif*H amplicon in the other isolate (i.e., C7) of *Rhizobium* sp. could be due to the loss of symbiotic plasmid that harbor *nif* and *nod* genes. Nonsymbiotic rhizobia occur naturally and can be more numerous than their symbiotic counterparts [22]. The endophytic association of rhizobia with rice [8] and maize [15] has aroused considerable interest in recent years in view of their potential for plant growth promotion in sustainable agriculture.

The isolate C3 showed closest identity with *Azospirillum* sp. and significant ARA, as well as the presence of *nif*H. Phylogenetic analysis of partial NifH amino acid sequence placed the *nif*H sequences from C3 and C6 close to sequences from *Azospirillum* and α-rhizobia in the subcluster 1J of the *nif*H phylogenetic cluster I [32] (Fig. 1). Bacteria of the genus *Azospirillum* are versatile plant-growth-promoting bacteria that fix atmospheric nitrogen and produce phytohormones [26]. Despite their nitrogen fixation ability, increases in crop yield due to inoculation with *Azospirillum* are mainly attributed to the improvement in root system, which increases the rates of water and mineral uptakes by the plant. This is especially effective when the minerals are scarce, as found in desert soils where nitrogen and soluble phosphate ions are limiting [7]. Although there are numerous reports on the beneficial effects of inoculation of cereals and grasses with *Azospirillum* [26], they have been more consistent in arid and desert areas [3]. For example, *A. brasilense* has been reported to show significant positive effects on the survival, development, and growth of seedlings and transplants of Cordon cactus of the Sonoran Desert [7].

Natural regeneration of grasses in the Thar Desert is very slow; hence, extensive efforts have been undertaken for the ecological restoration and regeneration of the Thar Desert, which include sand dune stabilization with effective "sand binders" like *L. sindicus*, which has a massive network of underground roots. Under the rangeland development program, some areas have been seeded with this perennial drought-tolerant grass. Although significant achievements have been made in the mass production of this nutritive fodder for desert livestock [9], the inoculation of seedlings of this grass with endogenous *Azospirillum* and other plant-growth-

promoting strains isolated originally from *L. sindicus* may bring about more benefits due to factors related to host-specificity [3]. The occurrence of *Rhizobium*, *Azospirillum* and *Pseudomonas pseudoalcaligenes* as endophytes inside *L. sindicus* suggests a possibility of meeting nitrogen requirements of this grass through nitrogen-fixing endophytes. They can also significantly contribute to the management of rangelands by improving root system in desert grasses and in rehabilitating the desert. Studies using ^{15}N isotope can reveal the quantitative estimates of biological nitrogen fixation carried out by *L. sindicus*-associated bacteria in the desert ecosystem.

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