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## Non-*Frankia* Actinomycetes Isolated from Surface-Sterilized Roots of *Casuarina equisetifolia* Fix Nitrogen†

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**Based on partial 16S sequences, we previously described a novel group of nonsymbiotic, acetylene reduction activity-positive actinomycetes which were isolated from surface-sterilized roots of *Casuarina equisetifolia* growing in Mexico. An amplified rRNA restriction analysis confirmed that these actinomycetes are distinct from *Frankia*, a finding substantiated by a 16S rRNA gene phylogenetic analysis of two of the Mexican isolates. Further support for these actinomycetes being separate from *Frankia* comes from the very low DNA-DNA homology that was found. Nevertheless, the Mexican isolates may be diazotrophs based not only on their ability to grow in N-free medium and reduce acetylene to ethylene but also on the results from <sup>15</sup>N isotope dilution analysis and the finding that a *nifH* gene was PCR amplified. A comparison of the *nifH* sequences from the various isolates showed that they are closely related to *nifH* from *Frankia*; the similarity was 84 to 98% depending on the host specificity group. An analysis of complete 16S rRNA gene sequences demonstrated that the two strains analyzed in detail are most closely related to actinobacteria in the *Thermomonosporaceae* and the *Micromonosporaceae*.**

In the course of isolating *Frankia* from surface-sterilized root nodules of *Casuarina equisetifolia* growing in Mexico, additional filamentous bacteria were obtained (13). The filaments of these bacteria are smaller in diameter than *Frankia* hyphae, the filaments do not develop typical nitrogen-fixing diazovesicles ex planta under aerobic conditions, and the strains are unable to reinfest *Casuarina* or other tested actinorhizal plants upon reinoculation (20). In addition, of 24 fatty acids examined, only 7 were shared by *Frankia* and the Mexican isolates (17). Based on an analysis of a partial sequence of the 16S rRNA gene, we proposed that these bacteria lie outside of the *Frankia* clade (20).

In this report, we examined the possibility that these non-*Frankia* Mexican actinomycetes have nitrogen-fixing genes based on their previously discovered abilities to reduce acetylene to ethylene, an indicator of nitrogenase activity, and to grow in N-free medium (13, 36). Nitrogenase, which catalyzes the reduction of dinitrogen to ammonia, is encoded by the genes of the *nifHDK* operon and is universally found in diazotrophs. Preliminary results with Southern blot analysis suggested that *nifH*- and *nifD*-like sequences were present in some of the Mexican isolates, but the bands weakly hybridized to a *Klebsiella pneumoniae nifHD* probe (unpublished results).

Weak hybridization to DNA isolated from two Mexican strains (L4 and 7702B) was also observed when a *Frankia* Cc13 *nifH* probe was used (19).

Because *nif* genes are conserved among a broad spectrum of bacteria, the use of universal primers has enabled the amplification and analysis of *nifH* sequences from very different microorganisms and environmental samples (21, 38, 40). However, when we used the universal degenerate primers Zf and Zr (39), the expected *nifH* gene products were not amplified. Instead, a PCR fragment with sequences encoding a hypothetical reductase with 40 to 43% gene sequence identity and only 19% amino acid similarity to *Frankia nifH* was amplified (22). Consequently, we developed new primers to determine whether the Mexican isolates have *nifH* sequences. We also undertook an analysis of the full-length 16S rRNA region to verify that these isolates are outside the *Frankia* clade and to establish their phylogenetic position in the order *Actinobacteria*. Our data show that the two isolates examined in detail cluster with the *Thermomonosporaceae* and the *Micromonosporaceae*, families that have not previously been described as diazotrophic. The possibility exists, however, that individual taxa fix nitrogen.

### MATERIALS AND METHODS

**Bacterial strains, cultivation, and microscopy.** We utilized *Frankia* belonging to different host specificity groups (HSG) as reference strains (Table 1). All the *Frankia* strains were cultured in DPM (3). The Mexican strains were isolated in DPM and grown in DPM supplemented with glucose instead of propionate as a carbon source (DGM), as described previously (36). *Micromonospora aurantiaca* (ATCC 27029) was routinely grown in ISP medium 2 (yeast maltose agar) (2).

The strains were grown for 1 month at 30°C, with the spent medium exchanged with fresh medium once a week. The bacteria were examined by using phase or Nomarski optics on a Zeiss Axiophot microscope or by staining aliquots with

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TABLE 1. Microbial strains, isolate designation, isolate origin and plant source, and presence or absence of aerial mycelia

| Isolate or strain      | Origin or source      | Aerial mycelium <sup>a</sup> | Plant source     |
|------------------------|-----------------------|------------------------------|------------------|
| <i>Frankia</i> BR      | Brazil                | –                            | <i>Casuarina</i> |
| <i>Frankia</i> IPNCe16 | Veracruz, México      | –                            | <i>Casuarina</i> |
| <i>Frankia</i> HFPCcI3 | Florida               | –                            | <i>Casuarina</i> |
| <i>Frankia</i> Mc3     | Veracruz, México      | –                            | <i>Myrica</i>    |
| <i>Frankia</i> Mc10    | Veracruz, México      | –                            | <i>Myrica</i>    |
| <i>Frankia</i> HFPCeI2 | Harvard Forest, Mass. | –                            | <i>Elaeagnus</i> |
| L3                     | Irapuato, México      | +                            | <i>Casuarina</i> |
| L5                     | Irapuato, México      | +                            | <i>Casuarina</i> |
| 7501                   | Veracruz, México      | +                            | <i>Casuarina</i> |
| 7702                   | Veracruz, México      | +                            | <i>Casuarina</i> |
| 8000                   | Veracruz, México      | +                            | <i>Casuarina</i> |
| <i>S. antibioticus</i> | ATCC 11891            | ND                           |                  |
| <i>M. aurantiaca</i>   | ATCC 27029            | +                            |                  |

<sup>a</sup> A – indicates the absence of aerial mycelia, and a + indicates the presence of aerial mycelia. ND, not determined.

acidine orange (0.05 mg ml<sup>-1</sup> in 50 mM H<sub>2</sub>PO<sub>4</sub>, pH 6.8) followed by epifluorescence microscope observation (31). Some strains were also examined by utilizing scanning electron microscopy. The cells were fixed as described previously (19), mounted on stubs, coated with gold-palladium, and examined with an ETEC scanning electron microscope at 10 kV.

Phenotypic characters were assessed by growing the bacteria in modified BAP medium (18) supplemented with phosphatidylcholine (31) or in DPM-DGM broth or medium solidified with 1.5% (wt/vol) agar. The solid medium was supplemented with filter-sterilized propionate or glucose as a C source. Nitrate and ammonia assimilation were assessed by growing the isolates in media containing either NH<sub>4</sub>NO<sub>3</sub> (0.01%) or (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (0.1%) or totally lacking combined nitrogen. Other growth responses were assayed by culturing the actinobacteria in ISP medium 2, ISP medium 3 (oatmeal agar), and a modified (L. S. Tisa, personal communication) Czapek agar medium (2). *Frankia* strain HFPCcI3 (41) and *M. aurantiaca* were also grown in these media.

Cultures were scored for growth responses after the plates were incubated for 2 to 3 weeks at 28°C.

<sup>15</sup>N isotope dilution. Strains L5, 7501, and 7702 were grown in DGM medium with 0.4 mM <sup>15</sup>NH<sub>4</sub>SO<sub>4</sub> (enriched to 10% of the total NH<sub>4</sub>SO<sub>4</sub>) added as the sole nitrogen source; the natural occurrence of <sup>15</sup>N is 0.366%. *Frankia* IPNCe16 and *Burkholderia vietnamiensis* TVV75<sup>1</sup> were grown as positive controls, and *Streptomyces antibioticus* and *Escherichia coli* DH5α were used as negative controls. The inoculum size was standardized at 4 μg of protein/ml of culture medium by using the Bradford technique. The flasks were incubated at 28°C for 40 days, after which time the cultures were harvested by centrifugation and washed with deionized water and their dry weights were determined.

Total nitrogen in the dry samples was determined by using the Kjeldahl method. After titration, the solution was brought to an alkaline pH and subsequently evaporated to dryness. The residue was suspended in water to a concentration of 1 μg of NH<sub>4</sub>Cl/μl.

<sup>15</sup>N enrichment was determined by using an atomic absorption spectrophotometer (model NOI-6E; Fischer Analysen Instrumente, Leipzig, Germany).

**DNA isolation.** Total genomic DNA from the various bacteria was isolated by using a modified cetyltrimethylammonium bromide protocol described previously (23). The concentration and purity of the DNA were evaluated with a spectrophotometer (Gen Quant; Pharmacia, Uppsala, Sweden).

**PCR amplification of *nifH* genes and sequence analysis.** We utilized a two-step PCR strategy to amplify the *nifH* genes from the Mexican isolates. *S. antibioticus* (ATCC 11891) (Table 1) was used as a negative control. The first reaction amplified a 1.2-kb fragment comprising the entire *nifH* gene, the intergenic spacer region, and the 5' end of *nifD*. The amplification was done by using the primer IGK (5'-TAC GGY AAR GCB GGY ATC GG-3') (25) and the reverse primer NDR-1 (5'-TTG GAG CCG GCR TAN GCR CA-3') (present study). The PCR mix was comprised of 50 ng of genomic DNA, 1.5 mM Mg<sup>2+</sup>, 200 μM deoxynucleoside triphosphates, 0.1 μmol of each primer and 2.5 U of *Taq* polymerase. The amplification was done with an initial denaturation step at 95°C followed by 35 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min with a final renaturation step for 7 min. The 1.2-kb PCR products were separated on

a 1.5% (wt/vol) agarose gel and purified by using the Concert rapid gel extraction system (Gibco, BRL).

The second step amplified an internal *nifH* fragment of 360 bp and used the primers PolF (5'-TGC GAY CCS ARR GCB GGY ATC GG-3') and PolR (5'-ATS GCC ATC ATY TCR CCG GA-3') (25) using 50 ng of the first PCR product as a template. The PCR conditions were the same as those described previously by Poly et al. (25). The reproducibility of the PCR was examined by repeating the reactions at least twice using both the same DNA preparation and DNA from separate extracts of the same isolate or strain.

The 360-bp fragment was purified by using the GeneClean II protocol (Bio 101, Carlsbad, Calif.). The purified PCR fragments were cloned into pBluescript SK(+) by using a TA cloning method that converts the plasmid into linear molecules with T-cohesive ends. We utilized *E. coli* DH5α for transformation by electroporation (Bio-Rad). Transformants were selected by looking for white colonies, and plasmids were isolated by an alkaline lysis method (30). The insertion of fragments of the correct size was inferred by restriction digestion with PstI and XhoI.

After verifying that a fragment of the correct size was produced, the cloned fragments were sequenced in an ABI PRISM 310 automatic sequencer. Analysis of obtained sequences was done with the BLAST algorithm (1) and DNAMAN (Lynnon BioSoft, Quebec, Canada). Deduced amino acid sequences were aligned with other *nifH* sequences from different diazotrophs. The gene sequences were aligned with the Clustal X software (34). Indel-containing regions were excluded from the analysis. Matrix pairwise comparisons were corrected for multiple-base substitutions by the two-parameter method of Kimura. Phylogenetic trees were constructed by the neighbor-joining method (29). A bootstrap confidence analysis was performed on 1,000 replicates to determine the reliability of the distance tree topologies obtained (10). A graphic representation of the resulting tree was obtained by using the NJ plot software (24).

**DNA-DNA hybridization analysis.** Total DNA was digested with BamHI, and restriction fragments were subjected to electrophoresis, blotted, and hybridized (5). DNA-DNA homology was based on relative levels of hybridization with two <sup>32</sup>P-labeled probes: total DNA from *Frankia* strain Br and total DNA from the non-*Frankia* isolate 7702. DNA amounts in gels and radioactivity levels were quantified by densitometry and liquid scintillation, respectively, as described previously (5).

**Amplified rRNA gene restriction analysis.** The 16S rRNA gene was amplified by using the universal primers rD1 and rD1 (37) using PCR conditions described previously by Benson et al. (4). These primers target the almost complete 16S rRNA gene (1,500 bp). Aliquots (5 μl) of the 16S PCR amplification product were digested according to the procedures described by the manufacturer. The following enzymes were used: CfoI, NciI, MspI, Sau3A (Boehringer Mannheim, Ottweiler, Germany), RsaI, HinfI, TaqI, and HaeIII (Gibco, Los Angeles, Calif.). Restricted DNA was analyzed by electrophoresis in 3% (wt/vol) agarose gels. Sequence divergences between the 16S rRNA genes of pairs of strains were estimated from the proportion of shared restriction fragments by using the Jaccard method (28). A dendrogram was constructed from the distance matrix by using the unweighted-pair group method with arithmetic mean (UPGMA). Both procedures were done with the NTSYSpc 1.8 program (28).

**PCR amplification of the 16S gene and sequencing.** The PCR fragments were cloned into pCRIVTOPO+. Transformants containing inserts were screened by the blue-white phenotype, and plasmids were obtained by the alkaline lysis method. The insertion of fragments of the correct size was inferred by restriction digestion with EcoRI. DNA sequencing of the PCR fragments was performed by Genome Express (Grenoble, France). In order to obtain the whole sequence, internal primers were designed. The primers were 5'-GGTGAAATGCGCAGATAT-3' (corresponding to coordinate 646 of the *Micromonospora* 16S sequence) and 5'-GGGCGCTTAATGCGTTAG-3' (corresponding to coordinate 860 of the *Micromonospora* 16S sequence). The 16S rRNA gene sequences were analyzed as indicated above for *nifH*.

**Nucleotide sequence accession numbers.** The complete sequence of the PCR fragments is available from GenBank under accession numbers AY534920 for isolate L5 and AY534921 for isolate 7501.

## RESULTS

**Morphological and physiological characteristics.** The isolates (7501, L3, L5, 8000, and 7702) examined in this report form aerial hyphae in agar medium and are highly branched and filamentous when grown in liquid media (Fig. 1B to D). The Mexican isolates are significantly smaller than the refer-

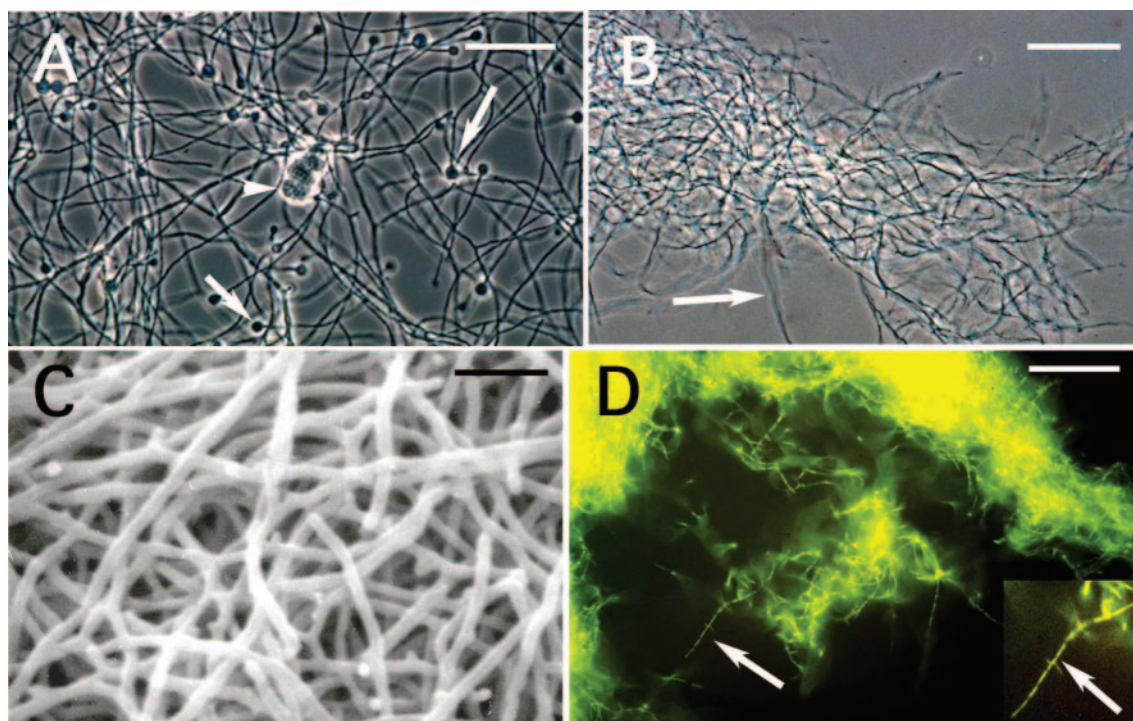


FIG. 1. Morphology of some of the actinobacteria analyzed in this report. (A) *Casuarina* strain CcI3 with multilocular sporangia (arrowhead) and diazovesicles (arrows) grown in liquid BAP minus N medium. Bar, 20  $\mu$ m. (B) Hyphae (arrow) of L5 from a culture grown in stirred BAP plus N. Bar, 40  $\mu$ m. (C) Scanning electron micrograph of hyphae from strain 8103 grown in stirred BAP plus N. Bar, 2.5  $\mu$ m. (D) Hyphae of 7702 stained with acridine orange. Segmented hyphae are indicated with the arrow and in the insert. Bar, 20  $\mu$ m.

ence *Frankia* strain CcI3 (Fig. 1A), measuring 0.25 to 0.4  $\mu$ m in diameter, compared to 0.5 to 0.6  $\mu$ m for strain CcI3. Although *Frankia* filaments are septate, partitions are more obvious in the filaments of the Mexican isolates, especially when stained with acridine orange (Fig. 1D). Under certain growth conditions, the hyphae appeared fragmented.

The cells of the Mexican isolates were gram positive and aerobic when grown with combined N. Changes in morphology and growth rate were observed when the bacteria were grown in media containing different C sources (data not shown). They formed numerous spores and produced raised colonies that were yellow-white in color on DGM agar plates. When grown in ISP medium 2, the colonies of the Mexican isolates ranged from light orange to maroon. *M. aurantiaca* was bright orange in this medium. All the strains, including *Frankia* CcI3, grew in ISP medium 3 (data not shown).

Unlike *Frankia* strains Br and CcI3, which produce numerous thick-walled, diazotrophic vesicles when grown under conditions of nitrogen limitation (Fig. 1A), the isolates lacked similar structures. When growing in DGM without N, the Mexican isolates frequently exhibited an altered morphology, consisting of hyphae of different diameters. The thin, highly branched filaments were localized toward the center of the colony, whereas the thicker hyphae were observed on the periphery (data not shown).

**$^{15}\text{N}$  isotope dilution analysis.** To confirm that the isolates were capable of fixing nitrogen, we performed a  $^{15}\text{N}$  isotope dilution analysis. We found that isolates L5, 7501, and 7702 and the positive controls *Frankia* IPNCe16 and *B. vietnamiensis*

acquired significant mass over the course of the experiment, while the negative controls grew poorly. As evaluated by total N determination using the Kjeldahl method, the negative controls accumulated very low levels of nitrogen. Both positive controls and the isolates L5, 7501, and 7702 exhibited considerable isotopic dilution, indicating active nitrogen fixation. *B. vietnamiensis* showed the highest level of isotopic dilution, whereas *Frankia* and isolates L5, 7501, and 7702 were similar to one another, exhibiting a ca. 5% dilution of  $^{15}\text{N}$  (Table 2). This result demonstrates that the Mexican isolates possess an active nitrogenase as was previously suggested from our acetylene reduction assay data (36) and from the ability of these strains to grow in N-free media (13).

TABLE 2. Biomass, total N, and excess of  $^{15}\text{N}$  obtained in the isotopic dilution assay<sup>a,b</sup>

| Isolate or strain                          | Biomass (dry weight) (mg) | Total N (Kjeldahl) (mg) | % Excess of $^{15}\text{N}$ <sup>a</sup> |
|--|---------------------------|-------------------------|--|
| <i>Frankia</i> IPNCe16                     | 6.7                       | 0.42                    | 4.69 $\pm$ 0.05                          |
| <i>B. vietnamiensis</i> TVV75 <sup>T</sup> | 14.4                      | 0.98                    | 1.07 $\pm$ 0.002                         |
| L5   | 8.6                       | 0.29                    | 5.43 $\pm$ 0.01                          |
| 7501                                       | 14.2                      | 0.45                    | 5.74 $\pm$ 0.003                         |
| 7702                                       | 14                        | 0.68                    | 5.29 $\pm$ 0.01                          |
| <i>S. antibioticus</i>                     | 2.5                       | 0.02                    | ND <sup>b</sup>                          |
| <i>E. coli</i>                             | 2.1                       | ND                      | ND                                       |

<sup>a</sup> Three replicates  $\pm$  standard deviations are shown.

<sup>b</sup> ND, not determined.

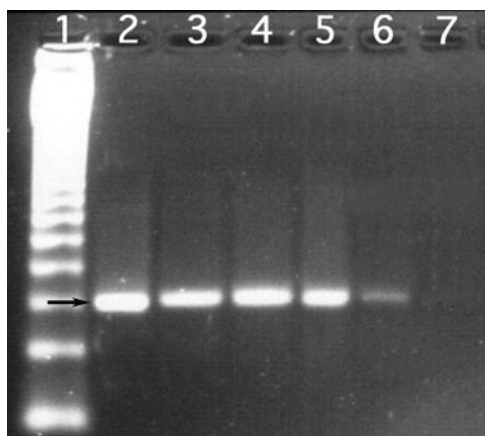


FIG. 2. PCR amplification of an internal *nifH* fragment of 360 bp (indicated by the arrow) using the universal primers PolF and PolR. Lane 1, ladder; lane 2, *Frankia* IPNCe16 (isolated from *C. equisetifolia* grown in Mexico); lanes 3 to 6, non-*Frankia* actinobacteria L3, L5, 7501, and 8000, respectively; lane 7, no-DNA control.

**PCR amplification of *nifH* genes and sequence analysis.**

After the first PCR for the *nifH* gene amplification using primers IGK and NDR-1, several bands were observed in the lanes containing DNA from the Mexican isolates L3, L5, 7501, and 8000 and from the positive controls, which include *Frankia* strain IPNCe16, one of several actinobacteria isolated from nodules of *C. equisetifolia* trees growing in Veracruz, Mexico (23, 35). No bands were observed in the lane containing DNA from *S. antibioticus*, the negative control (data not shown). Bands with a molecular mass similar to the expected size of the *nifH-nifD* region (1.2 kb) were purified and used as a template for the second PCR with primers PolR and PolF. All the PCR products were of the expected size of 360 bp (Fig. 2). When the fragments were sequenced, they were found to be very similar to *nifH* sequences from *Frankia* (Table 3). The isolates showed 97 to 98% similarity to *nifH* from *Casuarina*-nodulating *Frankia* and 84 to 85% similarity to unculturable *Frankia* from

TABLE 3. Percent similarity between the sequences of a 315-bp fragment of *nifH* from the Mexican isolates and different diazotrophic actinomycetes

| Reference strain (Genbank accession no.)               | % Similarity to Mexican isolate (GenBank accession no.) |               |                 |
|--|---|---------------|-----------------|
|  | L3 (AY714296)   | L5 (AY714297) | 7501 (AY714295) |
| <i>Frankia</i> IPNCe16 ( <i>Casuarina</i> ) (AY714294) | 97  | 98            | 97              |
| <i>Frankia</i> FaC1 ( <i>Alnus</i> ) (X73983)          | 92  | 93            | 92              |
| <i>Frankia</i> EuIK1 ( <i>Elaeagnus</i> ) (U53362)     | 92  | 93            | 92              |
| <i>Frankia</i> HRN18a ( <i>Alnus</i> ) (X17522)        | 92  | 92            | 91              |
| <i>Frankia</i> ArI3 ( <i>Alnus</i> ) (L41344)          | 91  | 91            | 90              |
| Uncultured <i>Datisca</i> endophyte (X76398)           | 87  | 88            | 87              |
| Uncultured <i>Coriaria</i> endophyte (X76399)          | 86  | 86            | 85              |
| Uncultured <i>Ceanothus</i> endophyte (U78306)         | 84  | 85            | 84              |

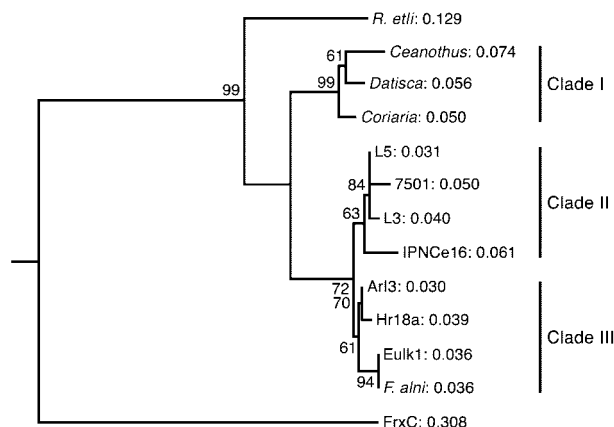


FIG. 3. Neighbor-joining tree based on the deduced amino acid sequences derived from partial *nifH* gene nucleotide sequences of *Frankia* strains and a new group of putative nitrogen-fixing actinomycetes. Numbers on branches are *P* values for bootstrap replicates (100). *frxC* (GenBank accession number X60490) is a *Chlamydomonas reinhardtii* *nifH*-like gene, namely the chloroplast *chlL* gene for light-independent protochlorophyllide reductase. It was used as an outgroup.

*Ceanothus* nodules. This result confirms our earlier findings using Southern blot analysis that these isolates carry at least one gene, *nifH*, from the *nif* operon (19).

From the partial *nifH* nucleotide sequences, the deduced amino acid sequences were aligned with homologous regions from various *Frankia* strains (see the supplemental material), and a phylogenetic tree was constructed. Figure 3 shows the consensus tree based on 100 bootstrap analyses. The *nifH* gene-containing actinobacteria were grouped into three different clades. Clade I includes unculturable microsymbionts detected in nodules from plants belonging to the Rhamnaceae, Coriariaceae, and Datisceae. Clade II includes DNA sequences from the Mexican isolates as well as from *Frankia* strain IPNCe16 (*Casuarina* HSG). Clade III encompasses *nifH* sequences from *Frankia* strains belonging to the *Alnus* and *Elaeagnus* HSG. Another neighbor-joining tree, which included the *Casuarina cunninghamiana*-isolated strain CcI3 (Table 1), was generated. In this tree, L5, L3, and 7501 clustered with CcI3 and not with the *Alnus Frankia* strains, further supporting the close relationship between *nifH* from *Casuarina Frankia* strains and the Mexican isolates (see the supplemental material).

Based on *nifH* gene analysis, the Mexican isolates lie outside the *Alnus-Elaeagnus Frankia* group but are in the same clade as *Frankia* strains isolated from nodules of *Casuarina*.

**DNA-DNA hybridization.** DNA relatedness has been used as a genotypic parameter to delineate species (5). Values below 70% are considered to show that the organisms belong to different species (32).

Levels of relatedness were between 43 and 48% among the Mexican isolates when DNA from the non-*Frankia* strain 7702 was used as a probe (Table 4). The relatively low level of DNA relatedness among the Mexican isolates, ca. 44% relatedness to L5 and 48% relatedness to 7501, suggests that these strains are distinct from isolate 7702. By contrast, DNA from *Frankia* Br (*Casuarina* HSG) showed lower levels of DNA relatedness

TABLE 4. Percent DNA-DNA relatedness between the Mexican actinobacteria and different *Frankia* species

| Strain or isolate      | % DNA-DNA relatedness with: |              |
|------------------------|-----------------------------|--------------|
|                        | <i>Frankia</i> Br           | Isolate 7702 |
| <i>Frankia</i> Br      | 100                         | 39.21        |
| <i>Frankia</i> IPNCe16 | 98.5                        | 39.55        |
| L5                     | 39.59                       | 43.60        |
| 7501                   | 26.06                       | 48.43        |
| 7702                   | 37.20                       | 100          |

to the isolates, from 27 to 40%. These data give additional support to the conclusion that the Mexican isolates are completely distinct from *Frankia*.

**Amplified restriction analysis of 16S rRNA genes.** The 16S rRNA PCR products were digested with nine different restriction enzymes (see Materials and Methods) providing 52 different markers. These markers were treated as binary data and transformed into a distance matrix using the Jaccard coefficient. The constructed dendrogram using UPGMA is shown in Fig. 4. The dendrogram shows that the *Frankia* clade is clearly separated from the new group of *nifH*-containing actinobacteria. Moreover, the non-*Frankia* actinobacteria formed two different clades, one consisting of isolates L5, 8000, and 7702 and the other including isolates L3 and 7501.

**Phylogenetic analysis based on 16S rRNA.** For the phylogenetic analysis, we amplified the complete 16S rRNA regions of two of the isolates, one of each clade determined by amplified rRNA restriction analysis (7501 and L5), and compared them to a broad range of actinomycetes. The DNA amplified from the isolates was a single band of about 1,500 bp, the expected size of the 16S rRNA gene among eubacteria, using the universal 16S rRNA primers. The 16S rRNA nucleotide sequences of the isolates L5 and 7501, representing the two clades, were aligned with homologous regions from various actinomycetes, and a phylogenetic tree was constructed. The dendrogram constructed (UPGMA tree) with the 16S rRNA sequence and compared with the members of the *Actinobacteria* showed four clades. Besides the *Cryptosporangium* and *Frankia-Geodermatophilus* phylogenetic clusters (clades 3 and 4, respectively), clade 1 includes *Actinomadura*, *Actinocorallia*, *Thermomonospora*, and *Spirillospora* as well as isolate 7501, whereas clade 2 comprises *Micromonospora*, isolate L5, and *Salinospora* (Fig. 5).

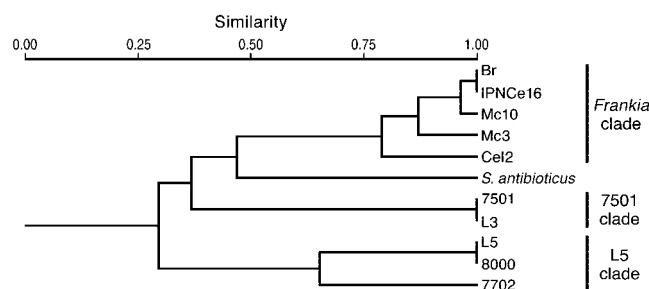


FIG. 4. Dendrogram (UPGMA) of genetic relationships among 16S rRNA genotypes identified by amplified rRNA restriction analysis. A matrix of genetic distances (Jaccard) was used to construct the dendrogram. *S. antibioticus* was used as the outgroup.

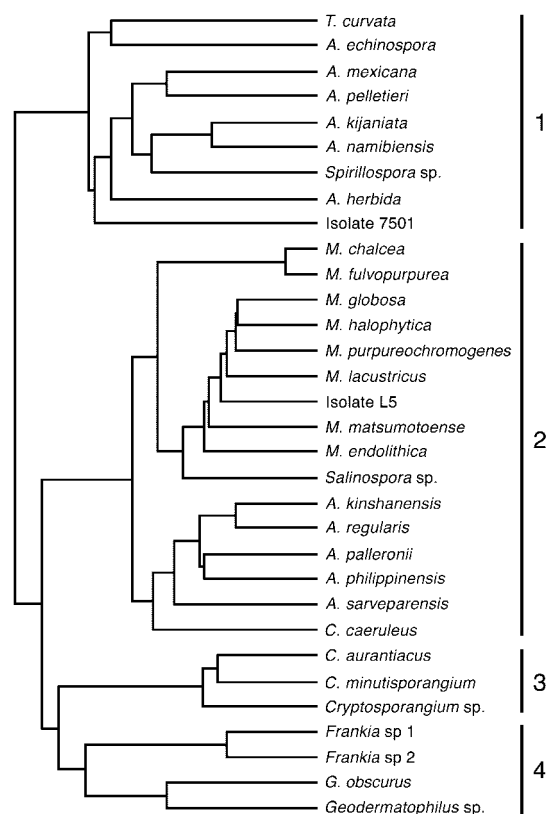


FIG. 5. Dendrogram (GCG Program Wisconsin version 10.3) of genetic relationships among 16S rRNA sequences. L5 and 7501 are compared with diverse members of the *Actinobacteria*. The genus names of the various actinobacteria and the accession numbers (in parentheses) for their 16S rRNA DNA sequences are as follows: *Thermomonospora curvata* (AF002262), *Actinomadura echinospora* (J420135), *Actinomadura mexicana* (AF277195), *Actinomadura pelletieri* (AF163119), *Actinomadura kijaniata* (X97890), *Actinomadura namibiensis* (AJ420134), *Spirillospora* sp. strain JCM3123 (AF163124), *Actinocorallia herbida* (D85473), *Micromonospora chalcea* (X92594), *Micromonospora fulvopurpurea* (X92595), *Micromonospora globosa* (X92600), *Micromonospora halophytica* (X92601), *Micromonospora purpureochromogenes* (X92611), *Micromonospora lacustricus* (X92622), *Micromonospora matsumotoense* (AF152109), *Micromonospora endolithica* (AJ560635), *Salinospora* sp. strain CNH643 (AY040619), *Actinoplanes kinshanensis* (AB047496), *Actinoplanes regularis* (AB047502), *Actinoplanes palleronii* (AB048216), *Actinoplanes philippinensis* (AB037007), *Actinoplanes sarveparensis* (AB047505), *Couchioplanes caeruleus* subsp. *caeruleus* (X93202), *Cryptosporangium* (formerly *Actinoplanes* [33]) *aurantiacus* (AB047490), *Cryptosporangium* (formerly *Actinoplanes* [33]) *minutisporangium* (AB037007), *Cryptosporangium* sp. (AB006168), *Frankia* sp. 1 (M55343), *Frankia* sp. 2 (L40622), *Geodermatophilus obscurus* (L40621), and *Geodermatophilus* sp. (X92358).

When L5 and 7501 were examined in a neighbor-joining tree using the GCG Program (Wisconsin ver. 10.3) with the complete 16S sequences of selected species in the genus *Micromonospora* (12), isolate L5 was found to cluster within this genus, with its closest relative being *M. aurantiaca* (X92604) (see the supplemental material). Isolate 7501 was found to cluster with members of the family *Thermomonosporaceae*.

## DISCUSSION

This study is the first to use molecular data to show that actinobacteria other than *Frankia* have *nifH* sequences. *Strep-*

*tomyces thermoautotrophicus* UBT1 fixes N<sub>2</sub> based on <sup>15</sup>N analysis and growth in N-free medium, but it does not reduce acetylene. It has an unusual nitrogen reduction system which is coupled to carbon monoxide reduction and is dependent on oxygen (27). This strain inhabits the soil covering burning charcoal piles and is not associated with plants. In contrast, the Mexican isolates reduce acetylene to ethylene and associate with actinorhizal plants as endophytes (13, 36). They were found in the outer cells of the nitrogen-fixing nodule, a location distinct from the *Frankia*-containing cells, which are found in the interior of the root nodule. When we tried to isolate *Frankia* from the nodules of *C. equisetifolia*, the non-*Frankia* actinobacteria overgrew the culture medium and masked the presence of the *Frankia* endosymbiont, which was later designated IPNCe16 (22, 35).

The Mexican isolates were originally obtained from nodules of *C. equisetifolia* trees growing on the dunes of the Gulf of Mexico near sea level and growing in altitudes of up to 2,300 m. They came from locations that included gardens, agricultural soils, and highly eroded sites (13). Observations of the frequency of endophyte isolation suggest that a medium lacking N was most effective for isolation (data not shown). These actinobacteria, especially members of the L5 clade, utilize several of the C sources recommended for the isolation and growth of *Frankia*, such as propionate and pyruvate as well as glucose (36). However, isolate 7501 grows better in medium containing glucose as a C source. The Mexican isolates also grew on several media used to support the growth of *Streptomyces* and other actinomycetes.

Previously, we determined that some of the Mexican isolates could grow on media lacking nitrogen and that they were capable of reducing acetylene to ethylene (13, 36). We also observed weak hybridization of a *nifHD* probe from *K. pneumoniae* (unpublished results) and a *nifH* probe from *Frankia* Cc13 to the DNA of the Mexican isolates (19). These results led us first to confirm by <sup>15</sup>N isotopic dilution assay that the isolates fix nitrogen (Table 2) and second to investigate whether one of the structural genes for nitrogenase, *nifH*, was present in these strains. Using newly developed primers, we found *nifH*-homologous sequences in four of the Mexican isolates: L3, L5, 7501, and 8000. The *nifH* genes of these strains were more fully characterized and found to lie in a clade that includes *nifH* from IPNCe16, the *Frankia* strain isolated from *C. equisetifolia* growing in Mexico.

We expect that the *nifDK* genes are also present in the Mexican isolates based on our preliminary Southern blots and possibly in the same operon as *nifH* based on the fact that one of the primers used in this study incorporated the 5' end of the *nifD* gene. A PCR-based approach to find the *nifDK* genes is under way, as is the search for any specialized hyphal structures or physiological adaptations that could potentially permit nitrogenase activity in a microaerophilic environment. If the *nifDK* genes show robust homology to the cognate genes in *Frankia* strain IPNCe16, as does *nifH*, this would strongly suggest that lateral gene transfer from *Frankia* has occurred. In any case, the combination of features—growth on an N-deficient medium, positive acetylene reduction and <sup>15</sup>N isotopic dilution assays, and the presence of the *nifH* gene—gives strong support to the conclusion that these actinobacteria fix atmospheric N to ammonia.

The relationship of these unusual isolates has been addressed by amplified rRNA restriction analysis, which has been successfully utilized to define phylogenetic relationships among microorganisms belonging to many different bacterial species (16). Recently, by using this approach, several unidentified N<sub>2</sub>-fixing *Burkholderia* groups were described (9), and three of these groups have been officially validated as novel *Burkholderia* species: *B. unamae* (6), *B. tropica* (26), and *B. xenovorans* (12). In this study, the *Casuarina* nodule-isolated actinobacteria were delineated into two clades by using amplified rRNA restriction analysis, and in addition, DNA-DNA hybridization assays were included to confirm their distinctness from *Frankia*. Based on the latter, their genetic similarity was 27 to 40%, whereas the DNA-DNA relatedness between the two clades was <50%. Taken together, these findings suggest that there are at least two separate groups of Mexican isolates.

The construction of a dendrogram based on the 16S rRNA sequence of two of the isolates and the published 16S rRNA sequences of a number of actinobacteria confirmed the results of the amplified rRNA restriction analysis and DNA-DNA hybridization assays (Fig. 5). These results showed that isolate 7501 is located in clade 1, forming a distinct phylogenetic cluster among the members of the family *Thermomonosporaceae*, whereas isolate L5 is included within the genus *Micromonospora* within clade 2, and may represent a novel species. A neighbor-joining phylogenetic tree of 16S rRNA sequence data of 14 selected species included in the genus *Micromonospora* (15) confirmed that isolate L5 is closely related to *M. aurantiaca* and that isolate 7501 clusters with *Thermomonosporaceae*.

It is noteworthy that isolates 7501 and L3, which have an identical amplified rRNA restriction analysis profile, have been recovered from nodules of *Casuarina* grown at a high altitude in Irapuato (2,300 m), whereas isolates L5 and 8000, whose amplified rRNA restriction analysis pattern is also identical, have been recovered from *Casuarina* trees growing in locations widely distant: at sea level, on dunes at Veracruz, 800 km away. This finding suggests that these bacterial species are widely distributed in geographical regions that exhibit very different environmental conditions. The presence of similar actinobacteria in surface-sterilized nodules has been noted by others trying to isolate *Frankia* from *Casuarina* nodules in South America (L. Wall, personal communication) and in Thailand (N. Boonkerd and A. Nuntagij, personal communication). Hence, these actinobacteria may be widespread.

*Micromonospora* has been isolated from soil and plants (14) and as an endophyte of healthy wheat plants (7). Several species of *Micromonospora* have the potential for biological control because they are able to parasitize the fungal phytopathogen *Pythium* (8). The isolation of a selected group of actinobacteria from within the tissue of nitrogen-fixing root nodules suggests that the host derives some benefit from harboring the endophyte. Whether these actinobacteria are involved in improving the life of the *Casuarina* plants by fixing nitrogen, by producing compounds that facilitate plant growth, or by competing with pathogenic bacteria or fungi in the rhizosphere is not known at this time. Actinobacteria are well known for their ability to produce a broad range of antibacterial, antifungal, and plant growth-regulatory metabolites

(11). The ability to fix nitrogen by a select group of actinobacteria in addition to *Frankia* may be added to this list.

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