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Analysis of Partial Sequences of Genes Coding for 16S rRNA of Actinomycetes Isolated from *Casuarina equisetifolia* Nodules in Mexico

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Filamentous bacteria isolated from surface-sterilized nodules of *Casuarina equisetifolia* trees in México were capable of reducing acetylene, a diagnostic test for nitrogenase, but were unable to nodulate their host. Analysis of partial 16S rRNA gene sequences suggests that the Mexican isolates are not *Frankia* strains but members of a novel clade.

Frankia strains, filamentous bacteria capable of fixing atmospheric nitrogen in culture and in symbiotic association, have been isolated from root nodules of actinorhizal plants worldwide. Attempts to classify *Frankia* isolates have been based on host specificity of infection (4), physiological and growth characteristics (14), and 16S rRNA relatedness (18). None of these criteria, however, enable one to identify all isolates from actinorhizal nodules unequivocally as *Frankia* strains.

Isolation and growth. Several years ago, we isolated a number of filamentous bacteria from nodules of *Casuarina equisetifolia* trees growing in México (9). In this study, five of these nodule isolates, 6201 (from Miraflores, Estado de México), 8103 and 7702B (from Isla del Amor, Veracruz), and L4 and L5 (from Irapuato, Guanajuato; from different lobes of one nodule) were characterized by several different approaches. Nodules were surface sterilized with 0.2% $HgCl_2$, rinsed with sterile water (six times), and incubated for 10 days in YD liquid medium (5a); sterile nodule lobes were then plated on various media (25). Isolate 6201 was isolated by sucrose density fractionation (3).

When grown at 30°C in media developed for *Frankia* culture—defined propionate medium without a nitrogen source (DPM minus N) (3) or another propionate-based medium, BAP (17), supplemented with 10 mM morpholineethanesulfonic acid (MES)-Tris and 2 μg of crude soybean phosphatidylcholine per ml (22)—with stirring, the Mexican isolates grew as highly branched filaments (data not shown). They are considerably smaller in diameter than our reference strain HFPCcI3 (25) (0.25 versus 0.5 μm).

Acetylene reduction ability. Preliminary studies demonstrated that the Mexican isolates reduce acetylene to ethylene in culture (9). Strains L4, 7702B, and 8103, when grown with stirring in BAP with propionate as a carbon source, reduced acetylene to levels nearly 50% of HFPCcI3 levels after 48 h (Table 1). In glucose-containing BAP, the acetylene reduction ability of strain 7702B increased.

Nodulation studies. Nodulation tests with *Casuarina* plants or plants from different host specificity groups were performed in the Los Angeles and México City laboratories. In the first test, 10 nonsterile seedlings of *Casuarina cunninghamiana*, *C. equisetifolia*, and *Elaeagnus angustifolia* were each grown in sterile 50-ml centrifuge (Falcon) tubes (13) containing one-quarter-strength Hoagland's medium without nitrogen (HM minus N) (15) and inoculated with the different isolates. For the second test, plastic dish pans containing sterile vermiculite moistened with sterile one-quarter-strength HM minus N were planted with *C. equisetifolia* and *E. angustifolia* seedlings or rooted cuttings of *Myrica gale* and *Myrica cerifera* (10 of each species). To each of these pans was added a dense pellet of bacteria resuspended in 100 ml of sterile water. In the third nodulation assay, roots of *Casuarina* or *Elaeagnus* plants (30 of each) grown in an aeroponics box (26) were directly inoculated with 250 to 500 μl of a dense bacterial pellet. Lastly, surface-sterilized seeds of *Alnus accuminata* were germinated in test tubes (20 by 200 mm) containing 20 ml of a 2:1 ratio of vermiculite and sand wetted with one-quarter-strength HM with N but, after 1 week, were starved of nitrogen and inoculated as described previously (9). These plants were maintained in a greenhouse at 30°C (daytime) and 18°C (nighttime) with a 14-h photoperiod for 10 weeks. Watering was with either sterile water or one-quarter-strength HM minus N. In none of the assays did the Mexican isolates invade or nodulate either the *Casuarina* species or the other actinorhizal plants. The experiments were carried out for up to 3 months, at which time the plants were severely nitrogen stressed. In contrast, *Frankia* strains HFPCcI3, CeI2 (5), ArI3 (6), and AvsI3 (2), controls for *Casuarina*, *Elaeagnus*, *Myrica*, and *Alnus* experiments, respectively, nodulated their hosts.

16S rDNA phylogenetic analysis. We isolated genomic DNA from the Mexican isolates and HFPCcI3 essentially as described by Guillén et al. (9). By using ~0.5 μg of genomic DNA, 1.0-kb fragments of the 16S rRNA gene (rDNA) were amplified with *Taq* polymerase (Gibco BRL, Grand Island, N.Y.) and primers P124 (5′-CACGGATCCGGACGGGTGA GTAACACG-3′) and P1115 (5′-GTGAAGCTTAGGGTTGC GCTCGTTG-3′) (Integrated DNA Technologies, Inc., Coralville, Iowa) (7, 16) for 35 cycles of 95°C for 1 min, 45°C for 2 min, and 72°C for 3 min on an Ericomp Easy-Cycler thermal

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TABLE 1. Acetylene reduction abilities[a] of the Mexican isolates grown with stirring in minimal medium with either propionate or glucose

Strain	Ethylene produced (nmol/mg of protein) with:	
	Propionate	Glucose
L4	80	75
7702B	80	200
8103	60	75
HFPCcI3	160	140

[a] Acetylene reduction ability was measured 48 h after removal of NH_4^+ from the culture medium.

cycler. Upon ligation into pGEM3Z (Promega, Madison, Wis.), the fragments were sequenced by using [^{35}S]dATP (1,000 to 1,500 Ci/mM; Dupont NEN, Wilmington, Del.) and the Sequenase kit (U.S. Biochemicals, Cleveland, Ohio) with T7 (Gold Biotechnologies, Inc., St. Louis, Mo.) and Sp6 (Gibco BRL) primers. One strand derived from each isolate was sequenced from three independently generated clones.

A region of 578 nucleotides (nt) of Mexican-isolate and HFPCcI3 16S rDNA clones, consisting of 285 positions read from P124 and 293 positions 3′ from P1115, was analyzed. These sequences were aligned with other *Frankia* and actinomycete 16S rDNA sequences by using CLUSTAL V (12). The *Frankia* sequences used were from CeD, ArI4, Cn7, and Ag45/Mut15. The 16S rDNA CeD sequence is identical to the partial sequence used by Nazaret et al. (18) and Normand et al. (19) for genomic species 9, which includes a number of *Casuarina* isolates that cannot reinfect their hosts. Cn7 is a noninfective *Frankia* strain isolated from *Coriaria* nodules that has been identified as a *Frankia* sp. on the basis of 16S rDNA phylogeny and the production of sporangia typical of members of the family *Frankiaceae* (16). While these nonreinfective isolates have been termed atypical frankiae, their relationship to other *Frankia* strains still needs to be resolved (1). Also included in the analysis were sequences from filamentous isolates of *Podocarpus* root outgrowths, L27 and G48, that have been described as *Frankia*-like organisms on the basis of 16S rDNA sequence analysis (24).

Two regions within the 578 nt, consisting of 180 positions, are shown in Fig. 1. Within these regions, two *Frankia*-specific oligonucleotide sequences described by Hahn et al. (11) were found only among the *Frankia* isolates (Fig. 1, underlining). In addition, a 22-nt-long *Frankia* genus-specific sequence was found (Fig. 1, box) (10). In Ag45/Mut15, ArI4, CeD, and CcI3, at least 19 of 22 nt of this sequence matched. However, for the Mexican isolates, 16 to 19 of 22 matches were observed, and then only when several gaps were introduced to align the sequences.

A PAUP (version 3.0s) (23) analysis of the DNA regions from these actinomycetes revealed six equally parsimonious trees. Figure 2 shows the 50% majority rule consensus tree based on 100 bootstrap analyses. A trichotomy consisting of the *Frankiaceae* (*Frankia* strains and members of the genus

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          148                                                                                                  233
CeD       CGGGGCTAAT GCCGGATATG -ACATTGCCG GGCATCTGGT --GGTG-TGG AAAGATTTAT CGGCTCGGGA TGGGCCCGCG GCCTATCAGC
CcI3      .......... .......... -.......... ....A..... --....-... .......... .......... .......N.. ..-.......
Cn7       .......... .........T C...CCTT.. .......... --....-... .......... T....T..... .......... ..........
ArI4      .......... .......... -...C..... .......... --....-... .......... ........N. ...--..... ..........
Ag/Mut    .......... .........- C......... .T.......-- -T....-... .......... .......... ...-...... ..........
7702B     T...T..... A......... ---.CCA.T. AA.GCA..T. CG....G... ....-...T. .....G.... ...A.T.... .T........
6201      T...T..... A......... ---.C...T. .C.GCA.... -T....G... ....-...T. .....G.... ...A.T.N.. .T........
8103      T...T..... A......... ---.CACA.T CC.GCA...G -AT..TG... ....-...T. .....G.... .....T.... .T........
L4        T...T..... A......... ---.C.CTTC ACTGCA.... -G.AG.G... ....-...T. .....G.... ...A.T.... .T........
L5        T...T..... A......... ---.C.CTTC ACTGCA.... -GAAG.G... ....-...T. .....G.... ...A.T.... .T........
L27       ...NN..... A...A...A. ---.C.C.T. AN.GCA.... CG-.GAG... ....-...T. ....CT.... .....T.... ..........
G48       .......... A...A..... ---.C.T.T. .T.GCA..AC CG-...G... ....-...T. ....CT.... .....T.... ..........
Geo       T......... A......G.. -..--C..T. .C.GCA.... CT....G... ....GG..TC .....G.... .......... ..........
Amycol    T...T..... A.......G. AC..----T. AT.GCA.... T-....G... ....T...T. .....G.... ...-...... .........-
Strep     G...T..... A.......CT G.TCCGCTT. ......CA.G C-...--.C. ....C.CCGG ...TG.A... ..A....... ..........

          925                                                                                                  1013
CeD       AATTCGATGC AACGCGAAGA ACCTTACCAG GGCTTGACAT GCAGGGAAAT CTCGTAGAGA TACGGGGTCC GTAAGGG-TC CTGCACAGGT
CcI3      .......... .......... .......... .......... .......... .......... .......... .......-.. ..........
Cn7       .......... .......... .......... .......... ..T....... .CTCC..... .GG....... T.N....G.. .A........
ArI4      .......... .......... .......... .......... .......... .......... .......... .......-.. ..........
Ag/Mut    .......... .......... .......... .ATA...... .....A..T. ...-C..... .GG...A... .......--T ...T......
7702B     .......C.. .......... .........A ..T..... CACC.....A ....C..... .G........ T.TT...CCG G..-......
6201      .......C.. .......... .........A ..T..... CACC.....A ....C..... .G........ T.TT...CCG G..-......
8103      .......C.. .......... .........A ..T..... CACC.....A .....C.... .G........ T.TT...CCG G..-......
L4        .......C.. .......... .........A ..T..... CACC.....A ....C..... .G........ T.TT...CCG G..-......
L5        .......C.. .......... .........A ..T..... CACC.....A ....C..... .G........ T.TT...CCG G..-......
L27       ........NN .-........ ........T. ..T..... .GCC.C...A ..GTC..... .GGCAN.... T.CG...GCG G.C-......
G48       ........CN .......... ........T. ..T..... .GCC.C...A ....C..... .GT.A..... T.CG...GCG G.C-......
Geo       .......... .......... ........TA .......... ...C...... ....C..... .G........ ........C. G.........
Amycol    N......... .......... ........-. ..T..... ...CCAG.CG .GTCC..... .GG.C.T... C.NGT..C.N G..TG...-.
Strep     .......C.. .......... .........A .......... A..CC.G..A GCAT...... ..GT.CCC.. C.TGT..TCG G..T......
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FIG. 1. Alignment of two fragments of 16S rDNA from the Mexican isolates and other actinomycetes. Positions 148 to 233 and 925 to 1013 refer to the 16S rDNA sequence of CeD (18, 19) (GenBank accession no. M55343). *Frankia*-specific sequences are underlined (11); the *Frankia* genus probe of 22 nucleotides is boxed (10). The GenBank accession no. for ArI4 is L11307, that for Ag45/Mut15 (11) is X53209, that for Cn7 (16) is L18982, that for L27 is M59075, that for G48 is L11306, that for *Geodermatophilus obscurus obscurus* (Geo) is L40620, that for *Amycolata alni* (Amycol) is X76954, and that for *Streptomyces ambofaciens* (Strep) (21) is M27242. The 5′ and 3′ ends of the PCR fragment of the Mexican isolates and HFPCcI3 are given individual GenBank accession numbers: 7702B, accession no. U35702 (5′) and U35703 (3′); 6201, U35700 and U35701; L4, U35708 and U35709; L5, U35710 and U35711; 8103, U35704 and U35705; HFPCcI3, U35706 and U35707. –, gap introduced during alignment; ., nucleotide identical to that in CeD sequence; N, nucleotide identity not determined.

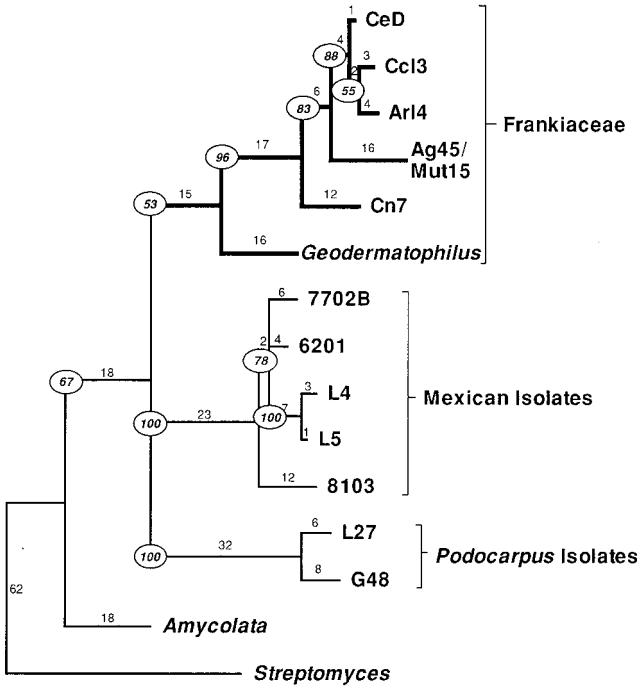

FIG. 2. 50% majority rule consensus tree based on 100 bootstrap pseudoreplicates, constructed by using a branch-and-bound analysis with maximum parsimony. Bootstrap values (8) are encircled at the bases of branches; branch lengths are given over the horizontal lines. The tree is rooted with the *Streptomyces ambofaciens* 16S rDNA sequence.

Geodermatophilus), the Mexican-isolate clade, and the *Podocarpus* isolate clade is separated from *Amycolata alni*, a non-*Frankia Alnus* nodule isolate, with 67% bootstrap support. The low bootstrap value for the *Geodermatophilus* branch (53%) arises because, in two of the six trees, the genus *Geodermatophilus* was allied either to a *Frankia-Podocarpus* isolate clade or to the Mexican-isolate clade, indicating that the position of the genus *Geodermatophilus* is not firmly established. In a recent report, Normand et al. (20) determined that there is no coherent grouping between the genera *Geodermatophilus* and *Frankia* and proposed to exclude the genus *Geodermatophilus* from the family *Frankiaceae*.

Figure 2 shows that the Mexican isolates lie outside the *Frankia* clade. Together with the morphological and nodulation capability differences, our results suggest that the Mexican isolates are distinct from *Frankia* strains and are members of a separate clade. The precise phylogenetic relationship of the Mexican isolates to *Frankia* strains and other *Frankia*-like isolates cannot be determined at present, however. The ability of the Mexican isolates to reduce acetylene and grow in nitrogen-depleted media suggests the presence of nitrogenase. Identification and characterization of nitrogenase-encoding genes from these isolates are currently under way.

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