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Frankia Genus-Specific Characterization by Polymerase Chain Reaction

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The polymerase chain reaction (PCR) is an in vitro procedure for primer-directed enzymatic amplification of specific template nucleic acid sequences. In order to determine whether a given actinomycete isolated from an actinorhiza (nodule) belongs to the genus *Frankia* or is a contaminant, we have developed a test based on the PCR. Primers complementary to sequences of two DNA regions corresponding to the *nif* genes (*nifH* and *nifD*) and the rRNA genes (16S and 23S) were specifically chosen to differentially amplify DNAs from *Frankia* strains but not those from other microorganisms. A series of positive and negative controls were set up by using universal or selective primers resulting in a discriminant amplification, which could be detected after agarose gel electrophoresis. In the *nif* region, degenerate oligonucleotide primers were used to amplify a target common to all the nitrogen-fixing microorganisms tested, while another set of primers amplified a target with a high specificity for *Frankia* strains. In the rRNA gene region, universal and specific primers were characterized and tested with DNAs from a wide range of microorganisms. The efficiency of this rapid and sensitive PCR assay was tested with an isolate obtained from *Alnus nepalensis* nodules, confirming results obtained by nodulation tests.

Investigations of symbiotic nitrogen fixation have so far concerned interactions between *Rhizobium* spp. and annual leguminous plants. Limited information has been generated on another important nitrogen-fixing symbiosis involving woody dicotyledonous plants and *Frankia* strains, filamentous prokaryotes belonging to the order Actinomycetales (for recent reviews, see Normand and Lalonde [31], Simonet et al. [43], and Mullin and An [25]). One of the reasons for this can be the problems associated with the isolation and identification of *Frankia* strains. Although an increasing number of *Frankia* strains have been isolated since 1978, problems of obtaining isolates in pure cultures and the definition of their growth requirements have restricted studies pertaining to the genetics, ecology, and physiology of this symbiosis. Despite considerable improvements in various culture media, *Frankia* strains continue to be slowly growing actinomycetes requiring months to obtain a biomass suitable for experimentation. Moreover, in spite of numerous attempts, no isolate from *Alnus* sp. nodules or from the host species belonging to the families *Datiscaceae*, *Coriariaceae*, *Rhamnaceae*, and most *Rosaceae* has yet been described.

The first classification within the genus *Frankia* was based on the ability to nodulate various host plants with crushed nodules as inocula. However, numerous problems were encountered (3), and improvement became possible only with the development of new criteria of classification, making use of biochemical and molecular biology techniques. For instance, isolates belonging to the genus *Frankia* can be characterized by criteria such as morphology (presence of hyphae, vesicles, and sporangia) (21), physiology (presence of a typical sugar) (24), cell wall components of type III (23), and genotype (G+C content in the range of 68 to 72%) (12). A typical *Frankia* strain also possesses the ability to nodulate and fix nitrogen, but the determination of such criteria is time-consuming and does not take into account strains deleted of their *nod* or *nif* genes. More recently, comparison of 16S rRNA sequences has allowed a redefinition of the family *Frankiaceae* by including in it the genera *Blastococcus* and *Geodermatophilus* (15). Moreover, genetic diversity within the genus *Frankia* was investigated by using DNA-DNA hybridization of the whole genome and DNA base composition which resulted in the delineation of nine genomic species among the strains tested (12). Finally, the development of rapid protocols to amplify and sequence segments of 16S rDNA confirmed these results and showed particular potential to classify nonisolatable strains by amplifying and sequencing *Frankia* DNA extracted from a nodule (27). Regarding other criteria, several authors have demonstrated that morphological characteristics are dependent on culture conditions and most of the physiological or genetic properties described for *Frankia* strains are also common to other actinomycetes. It appears that actinorhiza researchers need a fast and easy identification test based on more than one parameter which could be used for the first cells growing outside the crushed nodule in the in vitro isolation assay.

Nucleic acid hybridization probes show an increasing potential for the rapid identification of microorganisms, and they are particularly useful for screening large numbers of specimens. Detection of organism-specific DNA sequences by nucleic acid hybridization offers the possibility of working on crude material, thus eliminating the need to obtain the microorganism in pure culture. However, nucleic acid hybridization suffers from disadvantages, including the many precautions necessary when using radioactive labels (though nonradioactive labels have been developed and used successfully) and a lack of sensitivity, requiring large numbers of cells for detection (42). Improvement in the sensitivity of the hybridization assay can be achieved by the polymerase chain reaction (PCR) method. This process is capable of

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TABLE 1. Bacterial strains

Organism	Reference (source)
Proteobacteria	
Xanthomonas populi Spm	35
Escherichia coli ED8767	26
Micrococcus lysodeikticus	Unpublished results (Institut Pasteur, Lyon, France)
Streptococcus sanguis 480	20
N_2-fixing proteobacteria	
Pseudomonas paucimobilis 5AJ	2
Azospirillum brasilense Sp7	49
Klebsiella oxytoca 1ABI	2
Enterobacter cloacae 7ATR	2
Rhizobium strain IC-2018 (*Cicer arietinum*)	1
Agrobacterium tumefaciens C58	51
Azorhizobium caulinodans ORS571	10
Rhizobium meliloti	Unpublished results
Actinomycetes	
Streptomyces lividans TK24	16
Geodermatophilus obscurus subsp. *amargosae* G96	14
Geodermatophilus obscurus subsp. *dictyosporus* G97	14
Frankia alni Arl3	4
Frankia alni ACoN24d	40
Frankia strain EUN1f	19
Frankia strain Ccl2	54
Frankia strain ORS020609	27a
Frankia strain ARgP5AG	31
Frankia strain AVN17v	Unpublished results
Frankia strain AVN17s	Unpublished results
Frankia strain AVN17o	12
Frankia strain AVN17y	Unpublished results
Frankia strain ORS020602 (D11)	54

selectively amplifying target sequences of DNA by many orders of magnitude, providing a highly sensitive and specific nonradioactive detection test. The PCR process is known to be very sensitive since a single copy of a particular target sequence can be amplified manyfold. Moreover, specificity is high, as achieved by hybridization between oligonucleotide probes used as primers and target DNA.

In this study, we investigated the feasibility of using the PCR to specifically detect *Frankia* strains. In order to meet the requirements of testing many criteria, we decided to characterize PCR primers in different DNA regions, including *nif* genes which encode a function restricted to only a few prokaryotes and rDNA, by keeping in mind that the genus *Frankia* is related to the other actinomycetes.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1.

In vitro isolation from *Alnus nepalensis* nodules. Nodules were treated for *Frankia* isolation and growth with the OsO_4 technique as described by Lalonde et al. (19), with the exception that growth incubation was performed in the FTW culture medium (45).

DNA isolation. Total genomic DNAs from the different microorganisms used in this study were recovered and purified by previously described methods (7, 41). A simpler, direct lysis method was also used for recovery of DNA from cells of an isolate obtained from a nodule of *A. nepalensis*. Cells were collected in Eppendorf tubes, sedimented by centrifugation (5 min at 12,000 × *g*), washed with pure water, resuspended in 100 μl of 10 mM Tris–1 mM EDTA, and sonicated with a titanium horn operated at a power setting of 50 W for 5 min. After sonication, the homogenates were centrifuged at 12,000 × *g* for 5 min and the supernatant was used as such for DNA amplification. Finally, the nodule lobe collected from an *A. nepalensis* tree in India was treated for DNA recovery as described by Simonet et al. (44).

Oligonucleotide primers. The primers used in this study were chosen to correspond to conserved and variable regions in the *nifH-nifD* and in the 16S-23S rDNA regions. In order to characterize universal primers in the *nif* region, we compared published sequences of the *nifH* gene including those from *Azotobacter chroococcum* (36), *Anabaena* spp. (23), *Azotobacter vinelandii* (5, 8), *Clostridium pasteurianum* (52), *Frankia* spp. (30, 32), *Klebsiella pneumoniae* (48), *Methanococcus voltae* (46), *Rhodobacter capsulatus* (37), *Rhizobium phaseoli* (34), *Bradyrhizobium japonicum* (13), *Rhizobium meliloti* (50), *Azorhizobium caulinodans* (29), *Parasponia Rhizobium* (39), *Rhizobium trifolii* (38), and *Thiobacillus ferrooxidans* (33). *Frankia*-specific primers in the *nif* region were characterized according to sequences determined for *nifH* and *nifD* in Arl3 (32) and HRN18a (30). Prokaryote-specific primers were determined according to conserved portions of 16S rRNA, as determined by Embley et al. (11). In order to characterize *Frankia*-specific primers in this region, we compared published complete or partial sequences of 16S rRNAs including those of *Mycobacterium bovis* (28), *Streptomyces ambofaciens* (28), *Streptomyces lividans* (28), *Actinomyces bovis* (47), *Faenia rectivirgula* (11), *Saccharopolyspora hirsuta* (11), *Pseudonocardia thermophila* (11), *Propionibacterium freudenreichii* (9), *Saccharotrix australiensis* (6), *Neisseria gonorrhoeae* (28), *Kibdelosporangium aridum* (6), *Escherichia coli* (28), and *Thermophilus thermus* (28) with those obtained for some *Frankia* strains by Nazaret et al. (27).

PCR amplification. PCR amplification was performed in a total volume of 50 μl in 1.5-ml Eppendorf tubes under a layer of paraffin oil by using a DNA thermal cycler (Dri-Block PHC-2; Techne Inc., Princeton, N.J.). The classical PCR solution contained 1× PCR amplification buffer (10× buffer containing 100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, and 0.01% [wt/vol] gelatin), 200 μM deoxynucleoside triphosphate (dNTP), 1 μM primer, 100 ng to 1 μg of template DNA, and 2.5 U of Taq DNA polymerase (GIBCO BRL, Gaithersburg, Md.). In some tests, the concentration of total magnesium ions in the PCR was varied over the range 0.5 to 4 mM Mg^{2+} (other components in the 10× reaction mixture were maintained at the standard concentrations), the concentration of each dNTP was varied between 20 and 200 μM, and the concentration of each primer was varied over the range 0.02 to 1 μM. Template DNAs were initially denatured at 94°C for 3 min. A total of 35 to 40 PCR cycles were run during which DNAs were denatured at 94°C for 1 min, primers were annealed at 55°C for 1 min, and DNAs were extended at 72°C for 2 min. PCR-amplified DNAs were detected by using 2% (wt/vol) horizontal agarose gel electrophoresis in TBE buffer (0.089 M Tris borate, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) at 10 V cm^{-1} for 30 min. The gels were stained in an aqueous solution of 0.4 mg $liter^{-1}$ of ethidium bromide, destained in distilled water, and photographed with Ilford FP4 film with a 312-nm UV source.

Direct sequencing of PCR products. By using the primers

FGPS849 and FGPS1176′ and the Sequenase sequencing reagents (U.S. Biochemical Corp., Cleveland, Ohio), we determined the partial nucleotide sequence of the 16S rDNA of *Geodermatophilus obscurus* G96 and G97, in a region corresponding to the primer FGPS958, by using a method for sequencing double-stranded PCR products (53).

Nucleotide sequence accession number. The nucleotide sequence of primer FGPS958 is part of *Frankia* strain ORS020606 (GenBank accession no. M58598).

RESULTS AND DISCUSSION

The 16S rRNA gene is a patchwork of conserved and nonconserved regions, making it suitable for studying problems ranging from phylogenetic analyses to the characterization of specific probes. The conserved regions show high sequence conservation and are invariant and present in all kingdoms (eukaryotes, eubacteria, and archaebacteria), whereas others vary among but not within kingdoms. On the other hand, the most variable regions can be used to characterize species- or even subspecies-specific probes. Similar characteristics can be ascribed to the *nif* genes coding for the structure of the nitrogenase enzyme. However, *nif* genes are less ubiquitous; they are restricted to those prokaryotes which can fix nitrogen and are missing in other organisms. Because of the presence of conserved and variable regions, *nif* and rDNA genes are suitable targets for developing universal or specific PCR primers. The protocol for PCR is simple in execution and has the exquisite specificity characteristic of the nucleic acid hybridization methodology and a level of sensitivity higher than other detection techniques. *Frankia* sequences, including part of the 16S rDNA for more than 35 strains (27) and all of the *nifH* gene for two other strains (30, 32), are now available and provide enough information to characterize universal or *Frankia*-specific PCR primers or oligonucleotide probes. This makes the PCR technique suitable for studying the actinomycete *Frankia*, which is characterized among other things by its slow growing properties. Amplification of target DNA sequences present in nodules before probing with strain-specific oligonucleotides has already allowed for the identification of *Frankia* strains in competition studies (44). However, a genus-specific characterization based on selective amplification could also be suitable and would offer a sensitive alternative to confirm the status of newly isolated strains when little biomass is available. We, therefore, developed a *Frankia* genus-specific characterization test based on enzymatic amplification of target sequences in the *nif* region and in the rDNA region.

The ability to recognize and penetrate the roots of woody dicotyledonous plants to develop actinorhizae in which nitrogen fixation occurs is a common feature among typical *Frankia* strains. This means that the genus *Frankia* is characterized by the presence of *nod* and *nif* genes encoding nodulation and nitrogen fixation properties, respectively. The *nifH* gene, which encodes a subunit of the nitrogenase enzyme, has been found to be highly conserved among nitrogen-fixing species both at the amino acid and nucleic acid levels.

Specificity for nitrogen-fixing microorganisms of *nifH* amplification with universal primers. By comparing the DNA sequences of the *nifH* gene from the various nitrogen-fixing microorganisms studied, we found that a positive amplification signal could be expected for each of these microorganisms by the use of degenerate primers to compensate for the degeneracy of the genetic code. The sequence of these universal *nifH* primers, FGPH19 and FGPH273′, is given in Fig. 1, and a fragment of 254 bp could be expected in *Frankia* Arl3, as determined by the sequence published by Normand et al. (32). A PCR assay using FGPH19 and FGPH273′ as primers and standard conditions yielded negative results for bacteria which did not fix nitrogen, i.e., which did not possess *nif* genes (Fig. 2A). The test included *S. lividans*, *G. obscurus* G96 and G97, *Micrococcus lysodeikticus*, *Streptococcus sanguis*, *Xanthomonas populi*, and *E. coli*. Under the same conditions, we observed the 254-bp fragment with the *Frankia* strain tested (ACNI^{AG}) and also with other nitrogen-fixing microorganisms such as *Pseudomonas paucimobilis*, *Rhizobium loti*, *Azospirillum brasilense*, *A. caulinodans*, *Klebsiella oxytoca* (Fig. 2A), or *Bacillus polymixa* (results not shown). However, in *Enterobacter cloacae*, the amplified fragment was larger than the expected 254 bp, and nonspecific bands also appeared. Attempts to improve the yield of a single fragment in *E. cloacae* by raising the annealing temperature or carrying out the amplification at lower dNTP or primer concentrations did not meet with any success (results not shown). The amplification of part of the *nifH* gene was also successfully carried out for *Agrobacterium tumefaciens* C58, which displayed a band corresponding to the fragment observed for *E. cloacae*. These results confirm at the molecular level recent reports on the diazotrophic status of *A. tumefaciens* C58 (18).

According to recent reports (21a), some *Streptomyces* isolates also fix nitrogen and consequently should display positive signals with these universal primers. This means that the genus *Frankia* is not the only N-fixing actinomycete, indicating that more specific probes are necessary to discriminate between *Frankia* strains and these isolates. However, no further work on differential amplification among the actinomycetes has been possible since the above-mentioned strains are not yet available.

***Frankia*-specific amplification in the *nifH-nifD* region.** In

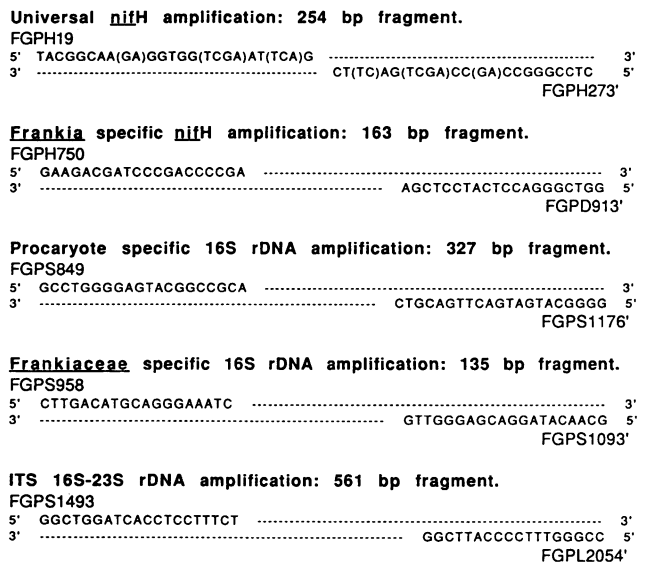

FIG. 1. Structure of oligonucleotide primers used for amplification. Numbering in primer designations refers to the position of the complementary sequence in the *nifH* or *nifD* gene for *Frankia* strain Arl3 (30) or in 16S or 23S rDNA for *Frankia* strain ORS020606 (32a). Degenerate primers FGPH19 and FGPH273′ consisted of a mixture of all possible combinations of the base sequences.

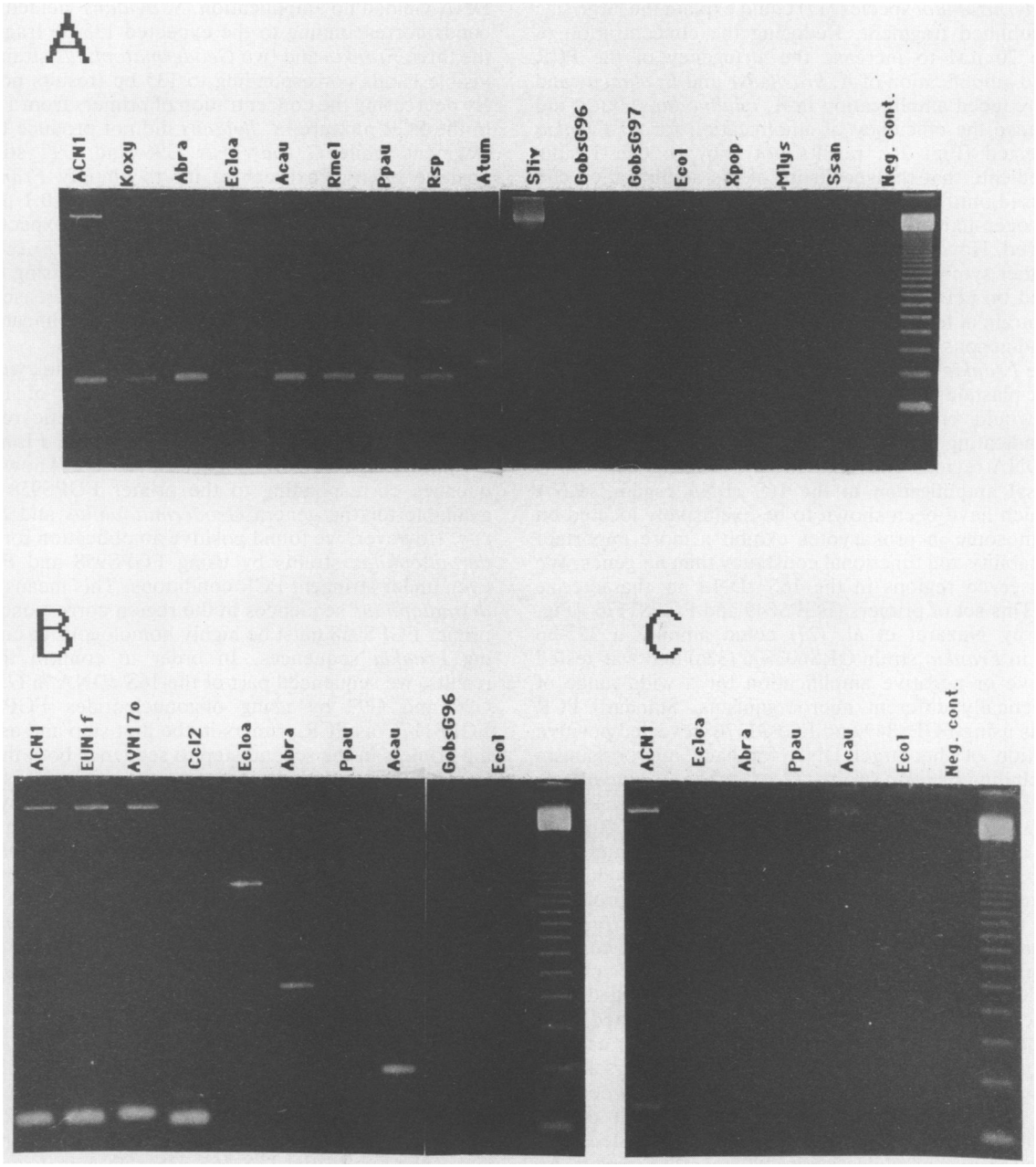

FIG. 2. Agarose (2%) gel electrophoresis of PCR-amplified DNAs from various prokaryotic strains. A 123-bp ladder standard is shown for each gel. Abbreviations: Ppau, *P. paucimobilis*; Abra, *A. brasilense*; Koxy, *K. oxytoca*; Ecloa, *E. cloacae*; Acau, *A. caulinodans*; Rmel, *R. meliloti*; Ppau, *P. paucimobilis*; Sliv, *S. lividans*; Xpop, *X. populi*; Ecol, *E. coli*; Atum, *A. tumefaciens*; GobsG96, *G. obscurus* subsp. *amargosae*; GobsG97, *G. obscurus* subsp. *dictyosporus*; Rsp, *Rhizobium* strain IC-2018 (*Cicer arietinum*); Mlys, *M. lysodeikticus*; Ssan, *S. sanguis*; Ccl2, *Frankia* strain Ccl2; AVN17o, *Frankia* strain AVN17o; ACN1, *Frankia* strain ACNI^{AG}; EUN1F, *Frankia* strain EUN1F; Neg. cont., negative control. (A) Universal *nifH* amplification with primers FGPH19 and FGPH273′ under standard PCR conditions (1 μM, primer concentration; 200 μM, dNTP concentration; 55°C, primer annealing temperature. (B and C) *Frankia*-specific *nifH* amplification with primers FGPH750 and FGPD913′. The PCR process was conducted under standard PCR conditions (B) or under more stringent PCR conditions (0.1 μM, primer concentration; 20 μM, dNTP concentration; 57°C, primer annealing temperature) (C).

order to increase the specificity of the PCR amplification test for *Frankia* strains, we characterized another set of primers (FGPH750 and FGPD913′) (Fig. 1) and tested them for amplification with various *Frankia* strains and other nitrogen-fixing microorganisms. The standard PCR conditions used with FGPH750 and FGPD913′ never amplified a DNA fragment from non-nitrogen-fixing microorganisms (Fig. 2B), but the expected 163-bp fragment was evident by ethidium bromide staining for each *Frankia* strain tested. Positively amplified DNA bands for *A. brasilense*, *E. cloacae*, and *A. caulinodans* were also observed. However, the sizes of the amplified DNAs were very different from the one obtained with *Frankia* DNAs as the template. Particularly with *E. cloacae*, a separation between *nifH* and *nifD* like that shown

with *Bradyrhizobium* species (17) could explain the large size of the amplified fragment. Reducing the concentration of dNTP to 20 μM to increase the stringency of the PCR eliminated amplification of *A. brasilense* and *E. cloacae* and strongly reduced amplification in *A. caulinodans*, but it did not decrease the efficiency of amplification for all *Frankia* strains tested (Fig. 2C; results not shown). Our results clearly indicate that the specificity of the amplification can be increased until nearly no significant cross-reactivity with other nitrogen-fixing species or other bacterial genera could be observed. However, in numerous nitrogen-fixing species, *nif* and other symbiotic genes have been shown very often to be located on plasmids. Recently, we reported that in one *Frankia* strain at least some of the *nif* genes were located on a large indigenous plasmid of 190 kb (41). This would mean that some *Frankia* strains could be naturally deleted of their symbiotic plasmids and consequently of their *nif* genes. Such isolates would provide negative amplification in the *nif* region, indicating the need for developing targets for the PCR in DNA regions not related to symbiotic genes.

Universal amplification in the 16S rDNA region. rRNA genes which have been shown to be exclusively located on the chromosome in prokaryotes exhibit a more important genetic stability and functional constancy than *nif* genes. We used conserved regions in the 16S rDNA to characterize primers. This set of primers (FGPS849 and FGPS1176′ [Fig. 1]) used by Nazaret et al. (27) could amplify a 327-bp fragment in *Frankia* strain ORS602606 (32a) and was tested for positive or negative amplification for a wide range of phylogenetically different microorganisms. Standard PCR conditions using FGPS849 and FGPS1176′ revealed positive amplification of the target DNA for each microorganism tested including *Frankia* strains, *G. obscurus*, *S. lividans*, *A. brasilense*, *P. paucimobilis*, *E. cloacae*, and *A. caulinodans* (Fig. 3A). Moreover, an electrophoretic analysis of the PCR products indicated no difference in the size of the amplified fragments among these microorganisms, with the production of relatively few nonspecific bands. Because of problems generally encountered in DNA extraction with *Frankia* strains, such a test would provide a positive PCR control, indicating that DNA extracted from nodules or pure cultures was not degraded and was pure enough to be amplified. Our results clearly indicate that the different DNAs tested were amplifiable, since a positive band was obtained for each of them. Moreover, these results confirmed the universality of the primers FGPS849 and FGPS1176′, since a positive fragment was obtained for the DNA from each of the gram-positive or proteobacterium DNA species tested.

***Frankia*-selective amplification in the 16S rDNA region.** As a target for PCR primers, we also selected a 16S rDNA highly variable region as defined by Embley et al. (11). The aim was to achieve primer characterization as well as PCR conditions to specifically amplify *Frankia* DNA. By comparing complete or partial 16S rRNA sequences published for different actinomycetes, we constructed primer FGPS958, corresponding to the *Frankia* sequence determined by Normand et al. (32a). The sequence of this primer showed low homology, particularly in the 3′ part, with published sequences from other microorganisms studied, including those that are gram positive or the actinomycetes (Table 2). Primers FGPS958 and FGPS1093′ (Fig. 1) were tested in the PCR with target DNA purified from six different isolates belonging to the actinomycetes, including three *Frankia* isolates (ACNI^{AG}, EUN1f, and ORS020606), two *G. obscurus* strains (G96 and G97), *S. lividans*, and *E. coli*. Under standard conditions, negative controls provided by *E. coli* DNA yielded no amplification. *S. lividans* yielded very faint bands corresponding to the expected 135-bp fragment, and the three *Frankia* and two *Geodermatophilus* strains all gave visible bands corresponding to 135 bp (results not shown). By decreasing the concentration of primers from 1 to 0.1 μM in the PCR mixture, *S. lividans* did not produce the 135-bp fragment while *G. obscurus* G96 and G97 still yielded positive bands. In order to try to amplify *Frankia* DNA specifically, PCR assays were run by using 0.1 μM primer and 20 μM dNTP. As seen in Fig. 3B, the expected 135-bp fragment was detected on 2% agarose gels in *Frankia* as well as in *Geodermatophilus* DNA. Moreover, raising the primer annealing temperature to 58°C to further increase the stringency of the PCR did not eliminate amplification in *G. obscurus* (results not shown).

Recent works (15, 43) using RNA sequencing with reverse transcriptase or oligonucleotide cataloging of 16S rRNA have demonstrated that a close phylogenetic relationship does exist between members of the genera *Frankia*, *Geodermatophilus*, and *Blastococcus*. To our knowledge, sequences corresponding to the primer FGPS958 were not available for the genera *Geodermatophilus* and *Blastococcus*. However, we found positive amplification for two *Geodermatophilus* strains by using FGPS958 and FGPS1093′ even under stringent PCR conditions. This means that *Geodermatophilus* sequences in the region corresponding to the primer FGPS958 must be highly homologous to corresponding *Frankia* sequences. In order to confirm these PCR results, we sequenced part of the 16S rDNA in *G. obscurus* G96 and G97 by using oligonucleotides FGPS849 and FGPS1176′ as PCR primers in the first step and as sequencing primers in the second step to sequence both the strands. Results (Table 2) indicated that the sequence similarities between *Frankia alni* and *G. obscurus* diverged by only one mismatch inside the primer FGPS958, confirming that PCR amplification could be carried out with either *G. obscurus* or *Frankia* strains under the same conditions. By using this set of primers, we concluded that PCR amplification could not achieve a specificity restricted to the genus *Frankia*. However, a specific amplification may be expected for the family *Frankiaceae*, which includes the genera *Frankia*, *Geodermatophilus*, and *Blastococcus*.

Amplification of ITS 16S-23S rDNA. In order to differentiate between the genera *Frankia* and *Geodermatophilus*, we based a test on the size of the ITS separating 16S and 23S rDNA genes. We characterized two other primers, one in the 16S rDNA (FGPS1493) and the second in the 23S rDNA (FGPL2054′) (Fig. 1), which could amplify a 561-bp fragment corresponding to the ITS 16S-23S rDNA in *Frankia* strain ORS020606 and tried to amplify DNAs from several *Frankia* strains, the two *Geodermatophilus* strains, and other prokaryotes including *S. lividans*, *B. polymixa*, *E. cloacae*, *A. brasilense*, and *A. caulinodans*. Our results (Fig. 3C) indicated that the size of the amplified fragment was well conserved among *Frankia* strains and that it corresponded to a 561-bp fragment for the great majority of *Frankia* strains or a little longer in strains AVN17y, AVN17v, and AVN17s isolated from the same alder stand. On the other hand, all non-*Frankia* DNAs yielded fragments whose sizes were found to be very different from the 561-bp fragment observed for *Frankia* DNAs. For instance, by using *G. obscurus* G96 or G97 as a template, the amplified DNAs were smaller and larger, respectively, than when *Frankia* DNA was the template, indicating differences in the sizes of the ITS 16S-23S rDNAs among microorganisms belonging to the family *Frankiaceae*. Finally, genomic DNAs from other microor-

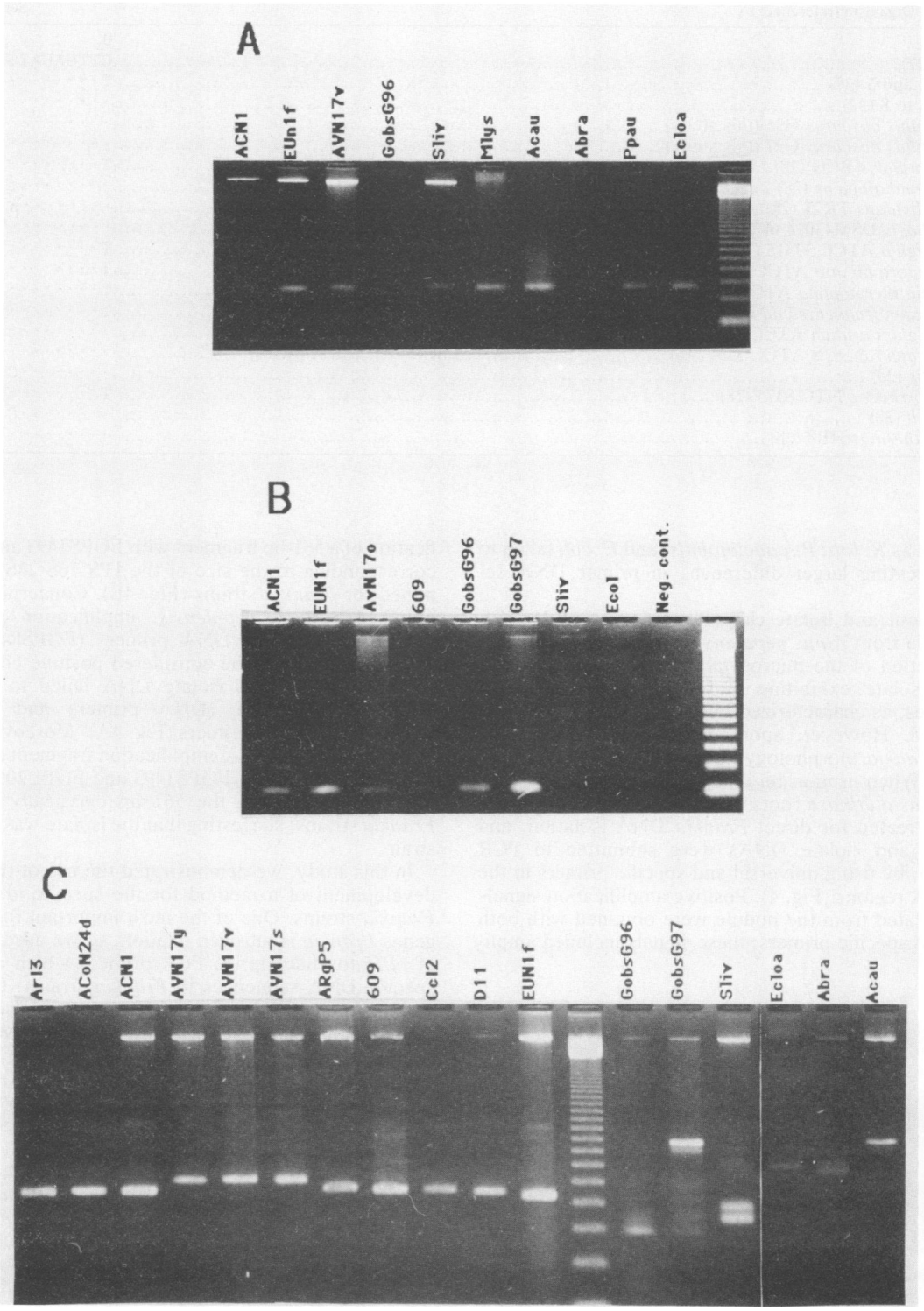

FIG. 3. Agarose (2%) gel electrophoresis of PCR-amplified DNAs from various prokaryotic strains. A 123-bp ladder is shown for each gel. (A) Prokaryote-specific 16S rDNA amplification with primers FGPS849 and FGPS1176′ under standard PCR conditions: (B) *Frankiaceae*-specific 16S rDNA amplification with primers FGPS958 and FGPS1093′ under standard PCR conditions. (C) ITS 16S-23S rDNA amplification with primers FGPS1493 and FGPL2054′ under the following PCR conditions: 0.1 μM, primer concentration; 20 μM, dNTP concentration; 57°C, primer annealing temperature. Abbreviations (in addition to those shown in the legend to Fig. 2): Arl3, *Frankia* strain Arl3; ACoN24d, Frankia strain ACoN24d; G09, *Frankia* strain ORS020609; D11, *Frankia* strain ORS020602 (D11); ARgP5, *Frankia* strain ARgP5^{AG}; AVN17v, *Frankia* strain AVN17v; AVN17s, *Frankia* AVN17s; AVN17y, *Frankia* strain AVN17y.

TABLE 2. Lineup of sequences homologous to the FGPS958 primer

Organism (reference)	Sequence
	0 19
Frankia alni (27)	CTTGACATGC AGGGAAATC
Frankia spp. 2 and 3 (27)	T····· ·····
Frankia spp. 4 to 8 (27)	····· ··A·····
Geodermatophilus obscurus G96 (this study)	····· ·C·····
Geodermatophilus obscurus G97 (this study)	····· ·C·····
Mycobacterium bovis BCG (28)	T····· ·CA·G·CG·
Streptomyces ambofaciens (28)	·····A· ·CC·G··AG
Streptomyces lividans TK21 (28)	·····A· ·CC·G··AG
Actinomyces bovis DSM43014 (47)	·····T G··TGTTGA
Faenia rectivirgula ATCC 33515 (11)	T····· ·CTNG·TCG
Saccharopolyspora hirsuta ATCC 27875 (11)	T····· ·CTAG·CAG
Pseudonocardia thermophila ATCC 19285 (11)	T····· ·CC·G·CG·
Propionibacterium freudenreichii DSM20271 (9)	T·····G ·CT·G··G·
Kibdelosporangium aridum ATCC 39323 (6)	····· GCCAG·CA·
Saccharothrix australiensis ATCC 31947 (6)	····· ·CC·G··A·
Bacillus subtilis (28)	·····C· TCT··CAAT
Neisseria gonorrhoeae NTC 8375 (28)	T·····T GC····TC·
Escherichia coli (28)	·····C· ·C····G·T
Thermophilus thermus HB8 (28)	····· TA··G··C·

ganisms such as *R. loti*, *P. paucimobilis*, and *E. coli* failed to amplify, indicating larger differences in primer DNA sequences.

Endosymbiont and isolate characterization. Nodules collected in India from *Alnus nepalensis* roots were treated for in vitro isolation of the microsymbiont by the OsO_4 technique. An isolate exhibiting morphology related to the actinomycetes, as characterized by the presence of hyphae, was obtained. However, sporangia, spores, or vesicles, typical of *Frankia* morphology, were not detected (results not shown). When used as an inoculum, this isolate did not nodulate *Alnus glutinosa* roots (results not shown). Another nodule was treated for direct *Frankia* DNA isolation, and both nodule and isolate DNAs were submitted to PCR amplification, by using universal and specific primers in the *nif* and rDNA regions (Fig. 4). Positive amplification signals for DNA isolated from the nodule were obtained with both universal and specific primers; these signals included amplification of a 561-bp fragment with FGPS1493 and FGPL2054′ corresponding to the size of the ITS 16S-23S rDNA determined for *Frankia* strains (Fig. 4B). Concerning the isolate obtained from *A. nepalensis*, amplification occurred only with universal 16S rDNA primers (FGPS849 and FGPS 1176′), which could be considered positive controls for the PCR. However, this isolate DNA failed to amplify with *Frankia*-specific 16S rDNA primers and universal or *Frankia*-specific *nif* primers (Fig. 4A). Moreover, the size of the ITS 16S-23S rDNA amplification fragment as determined by amplification with FGPS1493 and FGPL2054′ was found to be different from the 561 bp classically observed for *Frankia* strains, suggesting that the isolate was not a *Frankia* strain.

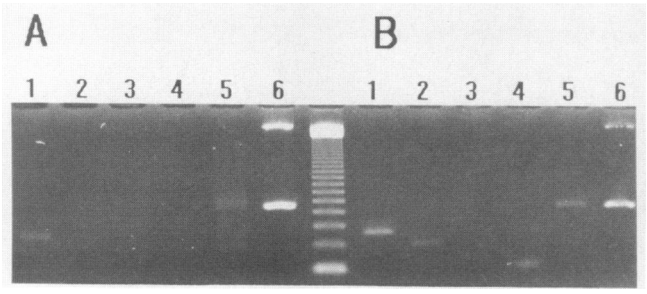

FIG. 4. Agarose (2%) gel electrophoresis of PCR-amplified DNA. (A) DNA extracted from an isolate cultivated from *A. nepalensis* nodules. (B) DNA used in the PCR process which was directly extracted and purified from one of the *A. nepalensis* nodules used for panel B. Lanes: 1, universal *nifH* amplification with primers FGPH19 and FGPH273′; 2, *Frankia*-specific *nifH* amplification with primers FGPH750 and FGPD913′; 3, prokaryote-specific 16S rDNA amplification with primers FGPS849 and FGPS1176′; 4, *Frankiaceae*-specific 16S rDNA amplification with primers FGPS 958 and FGPS1093′; 5, ITS 16S-23S rDNA amplification with primers FGPS1493 and FGPL2054′.

In this study, we demonstrated the use of the PCR in the development of a method for the specific identification of *Frankia* strains. One of the most important functions in the genus *Frankia* is nitrogen fixation, so we used the sequence of *nifH* to characterize PCR primers which could amplify specific DNA sequences in *Frankia* strains. In the case of strains deleted of their *nif* genes, primers based on the 16S rDNA sequence could differentiate between various actinomycete genera and actinomycetes belonging to the family *Frankiaceae*. A specific characterization of the genera *Frankia* and *Geodermatophilus* can be achieved by amplification of the 16S-23S ITS DNA. These experiments also demonstrated that DNA could be amplified directly from nodules or from a pure culture. This permitted us to determine that the isolate obtained from an *A. nepalensis* nodule was in fact a contaminant and not the *Frankia* strain involved in the symbiotic process. The PCR protocol we have described here permits rapid, accurate, sensitive, and inexpensive identification of microorganisms for which biochemical, physiological, or genetic markers are not currently available.

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