Nocardia spp. are gram-positive, aerobic actinomycetes with a worldwide distribution in soil. At least six species are pathogenic for both humans and animals and may enter the body via inhalation of contaminated dust particles or via wounds contaminated with dust or soil (18). They are responsible for several infections including pulmonary, central nervous system, and cutaneous infections (1). An increasing number of cases have been reported since 1980, which supports the view of many investigators that the incidence of nocardiosis is on the rise (2). Nocardiosis is diagnosed by isolation and culture identification. However, colonial characteristics and cellular morphology are variable, and Nocardia spp. may be misidentified and confused with members of closely related genera such as Dietzia (22), Gordona, Mycobacterium, Rhodococcus, Tsukamurella, and even Streptomyces. Conventional identification to the genus level is based on chemotaxonomic characteristics, such as the presence of a major amount of meso-diaminopimelic acid, arabinose, galactose, and mycolic acids with 46 to 60 carbons in cell walls (12). However, these techniques sometimes fail to fully differentiate some of the most closely related genera. Moreover, they are laborious, time-consuming, and expensive and can take 1 to 3 weeks to accomplish.

In this study, we developed an alternative PCR-based identification strategy targeted at the gene coding for 16S rRNA. Although the sequence for this gene is largely phylogenetically conserved, there may be variable sequences characteristic of particular organisms. The detection of these variable regions can therefore allow bacteria to be identified and differentiated from each other (13, 17, 26). The comparative analysis of the sequences of the complete 16S rRNA genes from 85 actinomycetes provided the basis for the design of diagnostic primers with a potential for the genus-specific detection of Nocardia by a PCR assay. We evaluated this approach for the fast, sensitive, and accurate identification of a wide range of Nocardia spp. isolated from pathogenic samples.

**RAPID IDENTIFICATION OF CLINICALLY RELEVANT NOCARDIA SPECIES TO GENUS LEVEL BY 16S rRNA GENE PCR**

**FREDERIC J. LAURENT,1,2* FREDERIQUE PROVOST,1 AND PATRICK BOIRON1**

Institut Pasteur, Unité de Mycologie, Centre National de Référence des Mycoses Humaines, des Antifongiques et des Actinomycètes, 75724 Paris Cedex 15,1 and Laboratoire de Bactériologie, C.H.U. Lyon Sud, 69495 Pierre Bénite Cedex,2 France

Received 15 May 1998/Returned for modification 8 July 1998/Accepted 25 September 1998

Two regions of the gene coding for 16S rRNA in Nocardia species were selected as genus-specific primer sequences for a PCR assay. The PCR protocol was tested with 60 strains of clinically relevant Nocardia isolates and type strains. It gave positive results for all strains tested. Conversely, the PCR assay was negative for all tested species belonging to the most closely related genera, including Dietzia, Gordona, Mycobacterium, Rhodococcus, Streptomyces, and Tsukamurella. Besides, unlike the latter group of isolates, all Nocardia strains exhibited one MluI recognition site but no SacI restriction site. This assay offers a specific and rapid alternative to chemotaxonomic methods for the identification of Nocardia spp. isolated from pathogenic samples.

**MATERIALS AND METHODS**

**Bacterial strains.** One hundred seventeen clinical strains and eighteen reference strains of actinomycetes have been cultured (Table 1). Cultures were obtained from the National Reference Center for Human Mycosis, Antifungal Agents, and Actinomycetes (Institut Pasteur, Paris, France) or from the American Type Culture Collection (ATCC; Rockville, Md.). All isolates were identified by conventional methods including biochemical tests, whole-cell composition, and enzymatic profiles (3). The isolates included 5 strains of Dietzia, 8 strains of Gordona, 17 strains of Mycobacterium, 60 strains of Nocardia belonging to 8 different species, 20 strains of Rhodococcus, 20 strains of Streptomyces, and 5 strains of Tsukamurella. Cultures were maintained on Bennett's agar medium at room temperature before being treated simultaneously.

**Primers selection.** The sequences of the 16S rRNA genes from a large number of isolates belonging to the genera Dietzia, Gordona, Mycobacterium, Nocardia, Rhodococcus, Streptomyces, and Tsukamurella were found in the DDBJ/EMBL/Genbank database. They were analyzed with the PILEUP program (9) of Genetics Computer Group software package (8). The existence of regions exhibiting possible sequence signatures to the genus level was investigated. The results allowed the design of two primers with 3′ extremities specific for the Nocardia genus (Fig. 1): forward primer NG1 (5′-ACCGACCACAAGGGG-3′) was complementary to positions 966 to 982 on the antisense strand, and reverse primer NG2 (5′-GGTGTAACCTCTCIGA-3′) was complementary to positions 386 to 405 on the sense strand. Both primers were custom synthesized by Appligene (Ailkirch, France). Thus, the amplified product was to be 596 bp in length.

**DNA extraction, PCR amplification, and detection of PCR products.** Isolated colonies were subcultured for 3 to 5 days at 34°C on sterile cellulose acetate membranes (Millipore, Bedford, Mass.) that had been deposited on blood agar (bioMérieux, Marcy l’Étoile, France). DNA was extracted as described previously (21). A colony was suspended in 650 μl of a reaction mixture containing 500 μl of sterile pyrolyzed water and 150 μl of Chelex solution (15% [wt/vol] Chelex 100 resin, 0.1% [wt/vol] sodium dodecyl sulfate, 1% [vol/vol] Nonidet P-40, 1% [vol/vol] Tween 80) and boiled for 30 min at 100°C. The samples were centrifuged for 5 min at 5,000 × g. The final supernatant containing the extracted DNA was recovered, and 15 μl of a 1:20 dilution aliquot was used for amplification. The 100-μl DNA amplification reaction mixture contained 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, each deoxynucleoside triphosphate at a concentration of 200 μM, 1 μM each primer, and 1 U of AmpliTaq Gold (Perkin-Elmer, Norwalk, Conn.). Amplified reactions were carried out in a DNA thermal cycler (Hybaid; Omni- gene, Teddington, United Kingdom). After initial denaturation at 94°C for 1 min, the reaction mixture was run through 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 20 s, and extension at 72°C for 1 min. Cycling was followed by a final extension incubation of 10 min at 72°C.

To ensure that no contaminating DNA would give positive results, one sample lacking a template was included in each series of reactions. After amplification, 12-μl samples were analyzed by electrophoresis for 2 h at 100 V in horizontal TAE (40 mM Tris-acetate, 2 mM Na2 EDTA; 2H2O) gels containing 2% agarose (Ultrapure; Bio-Rad Laboratories, Hercules, Calif.) and 0.5 μg of ethidium bromide per ml. The gels were then exposed to UV light to visualize the amplified products and photographed. The reproducibility of the reaction was tested at intervals of different numbers of days with freshly extracted DNA.

The identity of the amplified products was further investigated by restriction
Differences in base sequences are indicated with boldface characters.

Because of the slow growth of *Nocardia*, we used a direct DNA extraction method from a single colony (isolated in primary culture or from samples received by the reference laboratory) using the Chelex method in less than 40 min. This DNA extraction method from a single colony (isolated in primary culture or from samples received by the reference laboratory) using the Chelex method in less than 40 min. This method was found to be effective in recovering sufficiently purified DNA for amplification even if DNA cannot be visualized. By reducing the time and labor associated with the conventional DNA isolation procedure with the rapid-release method, the entire PCR assay and restriction endonuclease cleavage can be completed within 2 days.

PCR, which allows the specific and sensitive amplification of a preselected DNA region, has been intensively applied to the species identification of numerous organisms (7, 11, 14, 23, 27). Its use requires the detailed examination of the molecular genetics of organisms, especially the identification of the DNA sequences that are specific for the organism tested. The target regions usually are pathogenicity or virulence genes. On the basis of the homologies of a heat shock protein (Hsp65) gene from *Nocardia* strains and rapidly growing *Mycobacterium* strains, Lungu et al. (16) developed such an approach: an RFLP analysis of DNA amplified from the Hsp65 gene enabled them to distinguish these two genera. In this case, however, no extensive study concerning other actinomycetes was done. Moreover, because the primers were not specific, only endonuclease digestion gave specificity to this test. Finally, the profiles obtained were sometimes not easy to interpret since the number of patterns was large for rapidly growing *Mycobacterium* and since fragment sizes were difficult to analyze.

Little is known about DNA from the *Nocardia* genus. The 16S rDNA gene (rDNA) sequences are the only extensive sequences that are specific for the organism tested. The target sequences that have been documented (6, 24). Thus, it is now established that the primary structure of rRNA is composed of regions with highly conserved sequences together with other regions composed of variable or hypervariable sequences, i.e., signatures, that are of special interest for identification to the genus or species level (4, 5, 10). The similarities in nucleotides of rRNA preparations provide a means of establishing relationships among representatives of diverse bacterial taxa. In the late 1970s, Mordarski et al. (19, 20) used DNA-rDNA pairing experiments to show the phylogenetic relationship between a genus belonging to the actinomycetes and the clustering of each genus. Recently, a comprehensive phylogenetic analysis of the genus *Nocardia* by 16S rDNA gene sequencing (6, 24) confirmed these results and indicated that the genus *Nocardia* (after several taxonomic modifications) constitutes a homogeneous taxon that can be distinguished from the other mycolic acid-containing organisms. In addition, ribotyping with a probe coding for a part of 16S RNA had demonstrated a species-level specificity for isolates belonging to the *N. asteroides* complex (15). Therefore, 16S rDNA appeared to be a potential interesting target in the development of new molecular techniques for *Nocardia* genus identification.

Thus, the presence of regions within 16S rDNA sequences of actinomycetes exhibiting possible sequence signatures at the genus level for *Nocardia* was investigated. The comparative analysis of the 16S rDNA sequences from 85 strains belonging to various *Nocardia* species, lungu mycolic acid-containing strains, and non-*Nocardia* actinomycetes was done. Moreover, because the primers were not specific, only endonuclease digestion gave specificity to this test. Finally, the profiles obtained were sometimes not easy to interpret since the number of patterns was large for rapidly growing *Mycobacterium* and since fragment sizes were difficult to analyze.

RESULTS AND DISCUSSION

Because of the slow growth of *Nocardia*, we used a direct DNA extraction method from a single colony (isolated in primary culture or from samples received by the reference laboratory) using the Chelex method in less than 40 min. This method was found to be effective in recovering sufficiently purified DNA for amplification even if DNA cannot be visualized. By reducing the time and labor associated with the conventional DNA isolation procedure with the rapid-release method, the entire PCR assay and restriction endonuclease cleavage can be completed within 2 days.

PCR, which allows the specific and sensitive amplification of a preselected DNA region, has been intensively applied to the species identification of numerous organisms (7, 11, 14, 23, 27). Its use requires the detailed examination of the molecular genetics of organisms, especially the identification of the DNA sequences that are specific for the organism tested. The target regions usually are pathogenicity or virulence genes. On the basis of the homologies of a heat shock protein (Hsp65) gene from *Nocardia* strains and rapidly growing *Mycobacterium* strains, Lungu et al. (16) developed such an approach: an RFLP analysis of DNA amplified from the Hsp65 gene enabled them to distinguish these two genera. In this case, however, no extensive study concerning other actinomycetes was done. Moreover, because the primers were not specific, only endonuclease digestion gave specificity to this test. Finally, the profiles obtained were sometimes not easy to interpret since the number of patterns was large for rapidly growing *Mycobacterium* and since fragment sizes were difficult to analyze.

Little is known about DNA from the *Nocardia* genus. The 16S rDNA gene (rDNA) sequences are the only extensive sequences that are specific for the organism tested. The target sequences that have been documented (6, 24). Thus, it is now established that the primary structure of rRNA is composed of regions with highly conserved sequences together with other regions composed of variable or hypervariable sequences, i.e., signatures, that are of special interest for identification to the genus or species level (4, 5, 10). The similarities in nucleotides of rRNA preparations provide a means of establishing relationships among representatives of diverse bacterial taxa. In the late 1970s, Mordarski et al. (19, 20) used DNA-rDNA pairing experiments to show the phylogenetic relationship between a genus belonging to the actinomycetes and the clustering of each genus. Recently, a comprehensive phylogenetic analysis of the genus *Nocardia* by 16S rDNA gene sequencing (6, 24) confirmed these results and indicated that the genus *Nocardia* (after several taxonomic modifications) constitutes a homogeneous taxon that can be distinguished from the other mycolic acid-containing organisms. In addition, ribotyping with a probe coding for a part of 16S RNA had demonstrated a species-level specificity for isolates belonging to the *N. asteroides* complex (15). Therefore, 16S rDNA appeared to be a potential interesting target in the development of new molecular techniques for *Nocardia* genus identification.

Thus, the presence of regions within 16S rDNA sequences of actinomycetes exhibiting possible sequence signatures at the genus level for *Nocardia* was investigated. The comparative analysis of the 16S rDNA sequences from 85 strains belonging to various *Nocardia* species, lungu mycolic acid-containing strains, and non-*Nocardia* actinomycetes was done. Moreover, because the primers were not specific, only endonuclease digestion gave specificity to this test. Finally, the profiles obtained were sometimes not easy to interpret since the number of patterns was large for rapidly growing *Mycobacterium* and since fragment sizes were difficult to analyze.
to actinomycetes (including data for 25 Nocardia strains) revealed a high degree of homology. However, all Nocardia strains examined exhibited the genus-specific GA at nucleotide positions 404 and 405 and CC at nucleotide positions 966 and 967. Primers NG1 and NG2 were constructed by using these sequences containing the Nocardia-specific signature and the corresponding preceding S′ area (Fig. 1). Although this area was highly conserved among actinomycetes, the sequence of two nucleotides specific for Nocardia was sufficient for the design of diagnostic primers.

Figure 2 shows an example of the results of a PCR assay with the primers that were designed. The general applicability of the primers was tested against a diverse array of Nocardia strains (including type strains). A 596-bp amplimer was observed in all Nocardia strains, whereas no amplification product was obtained in control experiments with DNA from any of the other actinomycetes strains (Fig. 2). These results demonstrated that all strains of Nocardia spp. tested can be distinguished from representative members of the genera Dietzia, Gordona, Mycobacterium, Rhodococcus, Streptomyces, and Tsukamurella by using the selected primer combination.

Restriction endonuclease analysis was performed as a second level of control to verify the specificity of the 596-bp amplified fragment. If a non-Nocardia isolate shows an amplified product despite theoretical and observed primer specificity, a second level of check exists. Two recognition sites at nucleotide positions 704 (MnlI) and 523 (SacI) on the amplified fragment allowed the clear differentiation of members of the Nocardia genus from other genera of the aerobic actinomycetes (Fig. 1). As shown in Fig. 3, all Nocardia strains exhibited one MnlI recognition site, producing two DNA fragments of 278 and 318 bp. On the other hand, no SacI restriction site was observed in Nocardia strains, and as expected, no restriction fragments were produced following endonuclease analysis; conversely, a potential SacI restriction site was noted at position 523 of the DNA sequences of non-Nocardia strains. In case of nonspecific amplification of the 16S rRNA gene fragment, the SacI restriction site may produce two fragments of 137 and 459 bp, and these differentiate an isolate from Nocardia species. In fact, even though we never observed nonspecific amplification, we retained the restriction endonuclease analysis step to increase the security of genus identification.

On the basis of our results, we could establish a simple and practical scheme for the identification of genus Nocardia through actinomycetes: (i) 596-bp fragment amplification with an MnlI restriction site but no SacI restriction site indicates the DNA of a strain belonging to the genus Nocardia; (ii) no amplification fragment indicates the DNA of a strain not belonging to the genus Nocardia; and (iii) amplification of a 596-bp fragment with a SacI restriction site and/or without an MnlI restriction site indicates the possible nonspecific amplification, requiring confirmation of the identification by conventional methods.

PCR amplification with two primers (primers TB11 and TB12) enabled amplification of a 440-bp fragment coding for a part of a 65-kDa heat shock protein. This fragment was digested for restriction endonuclease analysis with five enzymes (BstEII, HaeIII, MspI, HinII, and BsaHI). The results allowed establishment of a practical identification scheme according to the numbers and sizes of the bands generated with each enzyme. It has been used for the identification of different species of aerobic actinomycetes, including the Nocardia species, with an accuracy of 98.6% (25, 28). However, since the scheme described for the identification of actinomycete species presented several subgroups (several profiles with several bands for which sizes were not easy to estimate), the interpretation of the result for a clinical strain was difficult within the actinomycete group. On the contrary, if we knew in advance whether the strains belonged to the Nocardia genus, use of interpretation by the technique of Steingrube et al. (25) made identification of the different species easier. Therefore, the technique de-
scribed here complements the technique of Steingrube et al. (25). The genus- and species-specific techniques could be combined to accomplish the identification of Nocardia clinical isolates within 48 h after receipt of cultures in the reference laboratories. Further studies are needed to develop different systems of identification of all genera belonging to the pathogenic aerobic actinomycetes after the selection of primers specific for each one.

In the future, the PCR method that we developed could be performed directly with clinical samples such as skin biopsy, blood, sputum, or bronchoalveolar lavage specimens. The direct diagnosis of nocardiosis by PCR of cerebrospinal fluid could replace the use of cerebral biopsy, which requires the use of an aggressive technique. It would improve the speed of diagnosis that is closely related to the favorable course for the patients (in terms of mortality, relapses, and aftereffects).

In short, this study showed that the enzymatic amplification of 16S rDNA combined with restriction analysis of the amplimer can be applied to the identification of strains of the genus Nocardia. The technique, as it is described here, is conceived to enable the rapid identification of isolates from pure cultures. The development of such a rapid, simple, and valid assay for detection of members of the genus Nocardia combined with the technique described by Steingrube et al. (25) for the Nocardia species identification will facilitate rapid diagnosis and prompt the initiation of the appropriate chemotherapy. Moreover, it will facilitate epidemiological studies of the human carrier, environmental contamination, and/ or soil distribution of these bacteria.

ACKNOWLEDGMENT

We are grateful to Claude de Bievre for help in using the GCG software package.

REFERENCES