Identification of *Nocardia* Species by Restriction Endonuclease Analysis of an Amplified Portion of the 16S rRNA Gene

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Identification of clinical isolates of *Nocardia* to the species level is important for defining the spectrum of disease produced by each species and for predicting antimicrobial susceptibility. We evaluated the usefulness of PCR amplification of a portion of the *Nocardia* 16S rRNA gene and subsequent restriction endonuclease analysis (REA) for species identification. Unique restriction fragment length polymorphism (RFLP) patterns were found for *Nocardia* sp. type strains (except for the *N. asteroides* type strain) and representative isolates of the drug pattern types of *Nocardia asteroides* (except for *N. asteroides* drug pattern type IV, which gave inconsistent amplification). A variant RFLP pattern for *Nocardia nova* was also observed. Twenty-eight clinical isolates were evaluated both by traditional biochemical identification and by amplification and REA of portions of the 16S rRNA gene and the 65-kDa heat shock protein (HSP) gene. There was complete agreement among the three methods on identification of 24 of these isolates. One isolate gave a 16S rRNA RFLP pattern consistent with the biochemical identification but was not identifiable by its HSP gene RFLP patterns. Three isolates gave 16S rRNA RFLP patterns which were inconsistent with the identification obtained by both biochemical tests and HSP gene RFLP; sequence analysis suggested that two of these isolates may belong to undefined species. The PCR and REA technique described appears useful both for the identification of clinical isolates of *Nocardia* and for the detection of new or unusual species.

The genus *Nocardia* contains several species that are well-recognized human pathogens; disease may be caused in normal hosts particularly by traumatic inoculation of the organism, while in immunocompromised individuals, the respiratory tract is often the initial site of infection (7). Although the majority of infections have been treated with sulfonamides, there are in vitro differences among the species in antimicrobial susceptibility, so accurate species assignment of clinical isolates may be important for predicting drug responsiveness if sulfonamides cannot be used or prove ineffective (7). Determining the species of an infecting organism may also be useful to help define the spectrum of disease caused by each species and the relative pathogenicities of the various species for different patient populations. Accurate and timely identification of these organisms by conventional methods is becoming more difficult due to the increasing number of recognized species (B. A. Brown, R. W. Wilson, V. A. Steingrube, Z. Blacklock, and R. J. Wallace, Jr., Abstr. 97th Gen. Meet. Am. Soc. Microbiol., abstr. C-65, p. 131, 1997) (13, 14, 17, 18), the limited number of conventional tests available, and the length of time required to complete the tests. Differences in in vitro susceptibility test results have been found helpful in identifying some of the species (16), but no standardized susceptibility testing method exists, and in any event few laboratories have the opportunity to develop expertise in such testing. A molecular method for identifying these organisms, based on restriction enzyme analysis (REA) of a portion of the 65-kDa heat shock protein (HSP) gene, has been demonstrated to be very useful in the identification of species

within the genus *Nocardia* (10, 11). However, little published information exists on the base pair sequence of the HSP gene region, making it difficult to utilize this region for further analysis of the significance of REA pattern differences. We report here the results of our examination of the utility of an REA procedure for the identification of these organisms based on a portion of the 16S rRNA gene, a region for which extensive base pair sequence information exists for many microorganisms, including many *Nocardia* species.

MATERIALS AND METHODS

Organisms. (i) Reference strains. Ten American Type Culture Collection (ATCC) strains were used as reference strains for restriction fragment length polymorphism (RFLP) analysis. These included seven strains which are designated as type strains by the ATCC (with GenBank accession numbers in parentheses): *Nocardia asteroides* ATCC 19247^T (Z36934), *Nocardia brasiliensis* ATCC 19296^T (Z36935), *Nocardia farcinica* ATCC 3318^T (Z36936), *Nocardia nova* ATCC 33726^T (X80593), *Nocardia otitidiscavium* ATCC 14629^T (X80599), *Nocardia pseudobrasiliensis* ATCC 51512^T (X84857), and *Nocardia transvalensis* ATCC 6865^T (X80598). Three additional *N. asteroides* ATCC strains which are designated as drug pattern types (10) were also included: *N. asteroides* drug pattern type I ATCC 23824 (X84851), *N. asteroides* drug pattern type IV ATCC 49872, and *N. asteroides* drug pattern type VI ATCC 14759. In addition, an isolate representative of *N. asteroides* drug pattern type II was also included.

(ii) Patient isolates. Twenty-eight patient isolates were categorized based on RFLP patterns. These isolates were obtained from patients being treated at The Warren Grant Magnuson Clinical Center of the National Institutes of Health, Bethesda, Md. (18 isolates), the George Washington University Hospital, Washington, D.C. (2 isolates), the Shady Grove Adventist Hospital, Gaithersburg, Md. (1 isolate), and the Hennepin County Medical Center, Minneapolis, Minn. (1 isolate). Six isolates had been referred for identification to the Maryland State Health Department, Baltimore, Md.

(iii) Other isolates. Thirty-nine additional ATCC and patient isolates belonging to the genera *Actinomyces* (2 isolates), *Corynebacterium* (2 isolates), *Mycobacterium* (16 isolates), *Nocardiopsis* (1 isolate), *Oerskovia* (2 isolates), *Rhodococcus* (6 isolates), *Rothia* (1 isolate), and *Streptomyces* (9 isolates) were also tested for amplification and RFLP patterns.

Phenotypic identification. Isolates were initially characterized as *Nocardia* species by their weakly positive acid-fast staining reaction and their microscopic and colonial morphologies. Isolates were biochemically identified to the species level by the following tests (4, 17): acid production from rhamnose, using cystine

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TABLE 1. Amplification and sequencing primers

Primer	Sequence ^a
Amplification of 16S rRNA gene	
Upstream	5'-CGA-ACG-CTG-GCG-GCG-TGC-TTA-AC-3'
Downstream 1	5'-CCT-GTA-CAC-CGA-CCA-CAA-GGG-GG-3'
Downstream 2	5'-ACC-TGT-ACA-CCA-ACC-ACA-AGG-GGG-3'
Amplification of 16S rRNA template for sequencing	
Reaction 1	
Upstream	5'-GTT-TTC-CCA-GTC-ACG-ACC-GAA-CGC-TGG-CGG-CGT-GCT-TAA-C-3'
Downstream ^b	5'-CAG-GAA-ACA-GCT-ATG-ACA-CCG-CCT-ACA-AGC-TCT-TTA-CGC-C-3'
Reaction 2	
Upstream ^b	5'-GTA-AAA-CGA-CGG-CCA-GGC-GCA-AGT-GAC-GGT-ACC-TGT-AG-3'
Downstream	5'-CAG-GAA-ACA-GCT-ATG-ACC-CTG-TAC-ACC-RAC-CAC-AAG-GGG-G-3' ^c
Amplification of HSP gene ^d	
Upstream	5'-ACC-AAC-GAT-GGT-GTG-TCC-AT-3'
Downstream	5'-CTT-GTC-GAA-CCG-CAT-ACC-CT-3'

^a Bases in boldface indicate tails complementary to the M13 primer.

^b Interior primer.

^c The "R" in position 28 of this primer is an IUPAC (International Union of Pure and Applied Chemistry) code indicating that this degenerate oligonucleotide was prepared with approximately 50% of strands with an adenine in this position and approximately 50% of strands with a guanine in this position.

^d Primers as specified in reference 12.

Trypticase agar with rhamnose, with phenol red as the indicator (BBL, Cockeysville, Md.); utilization of acetamide, using acetamide agar slants (BBL); decomposition of casein, xanthine, and tyrosine, using the Nocardia Quad Agar Plate (Remel, Lenexa, Kans.); decomposition of hypoxanthine, using hypoxanthine agar (Remel); decomposition of adenine, using adenine agar (Carr Scarborough, Stone Mountain, Ga. [now available by special order from Remel]); and susceptibility to erythromycin (15). Several isolates were also tested for susceptibility to a variety of agents by a broth microdilution procedure.

Molecular analysis. (i) DNA extraction. Organisms were grown on either Middlebrook 7H11 agar plates, Lowenstein Jensen agar slants, or Sabouraud dextrose agar slants (all from Remel). A loopful of growth was suspended in 3 ml of sterile distilled water with 3-mm-diameter glass beads and vortexed vigorously. The suspension was transferred to a microcentrifuge tube and centrifuged for 10 min at 16,000 × g. The pellet was lysed in 500 µl of guanidinium thiocyanide buffer (1) for 10 min at room temperature with frequent vortexing. Lysates were extracted in 500 µl of phenol-chloroform-isoamyl alcohol (25:24:1) (Amersham Pharmacia Biotech, Piscataway, N.J.), and DNA was purified with the Gene Clean II Kit (Bio 101, Inc., La Jolla, Calif.). DNA was eluted with Tris-EDTA buffer (pH 8.0) (Biofluids, Inc., Rockville, Md.) and frozen at -20°C until amplification.

(ii) Amplification of a portion of the 16S rRNA gene. A 999-bp fragment of the 16S rRNA gene was amplified by using a set of three primers (synthesized at Research Genetics, Huntsville, Ala.) designed to amplify all species of *Nocardia* for which sequence information was available (Table 1). Two downstream primers were used simultaneously with one upstream primer to allow amplification of nearly all of the commonly isolated *Nocardia* species. Five microliters of the extracted DNA was used in the 50-µl PCR mixture. The PCR mixture contained 1× PCR buffer, 1.5 mM MgCl₂, 0.2 mM each deoxynucleoside triphosphate (all from Perkin-Elmer, Norwalk, Conn.), 0.25 µM each primer, and 1.5 U of *Taq* polymerase (Perkin-Elmer). The DNA was denatured for 5 min at 94°C and then subjected to 40 cycles of amplification (94°C for 60 s, 68°C for 45 s, and 72°C for 60 s) followed by a 10-min extension at 72°C.

(iii) Amplification of a portion of the HSP gene. A 439-bp fragment of the HSP gene encoding the 65-kDa heat shock protein was amplified by using primers described by Telenti et al. (12) (Table 1) and amplification conditions described by Steingrube et al. (11). Five microliters of extracted DNA from each of the 28 patient isolates was used in the 50-µl PCR mixture. The PCR mixture contained 9% dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Mo.), 1× PCR buffer (Perkin-Elmer), 1.5 mM MgCl₂ (Perkin-Elmer), 0.2 mM each deoxynucleoside triphosphate (Perkin-Elmer), 0.3 µM each primer (Research Genetics), and 1.5 U of *Taq* polymerase (Perkin-Elmer). The DNA was denatured for 5 min at 94°C and then subjected to 45 cycles of amplification (94°C for 60 s, 55°C for 60 s, and 72°C for 60 s) followed by a 10-min extension at 72°C.

(iv) REA. PCR products from the amplification of the 16S rRNA gene were subjected to digestion using *Hin*P1I and *Dpn*II (New England Biolabs, Beverly, Mass.). Extracts from isolates which gave the RFLP pattern consistent with that of the *N. asteroides* type strain, *N. asteroides* drug pattern type I, and *N. brasiliensis* were subjected to subsequent REA using *Bst*EII and *Sph*I (New England Biolabs). PCR products from the amplification of the HSP gene were subjected to digestion using *Msp*I, *Hin*FI, and *Bsa*HI (New England Biolabs). All digestions were performed under conditions defined by the manufacturer.

Digestion reactions were stopped by the addition of loading buffer, and products were electrophoresed on a 2% MetaPhor agarose minigel (FMC Bioproducts, Rockland, Maine) containing 0.5 µg of ethidium bromide (Amresco, Solon,

Ohio)/ml. RFLP patterns of patient isolates were compared to those obtained with reference strains by using Molecular Analyst Software, PC Fingerprinting Plus (Bio-Rad Laboratories, Hercules, Calif.). Fragment sizes were calculated by the same software by using Gelmarker (Research Genetics) as the DNA size reference.

PCR and REA were repeated for all isolates which gave discrepant results by other methods.

(v) Sequence determination. Fluorescence-based cycle sequencing was performed on two PCR-amplified segments of the 16S rRNA gene for selected patient and reference strains. The two segments were designed to overlap with each other. Interior primers were designed with tails containing M13 forward or M13 reverse binding sites (Table 1). The exterior PCR primer sequences were also adapted to contain tails with the M13 binding sites (Table 1). One PCR amplification produced the template for sequencing the 16S rRNA gene target area from base 1 to base 537, while the second amplification produced the template for sequencing the area from base 419 to base 999. The PCR mixture for template amplification for both reactions contained 1× PCR buffer, 1.5 mM MgCl₂, 0.2 mM each deoxynucleoside triphosphate (all from Perkin-Elmer), 0.25 µM each primer (Table 1), and 1.5 U of *Taq* polymerase (Perkin-Elmer). For reaction 1, the DNA was denatured for 5 min at 94°C and then subjected to 5 cycles of amplification (94°C for 1 min, 68°C for 45 s, and 72°C for 1 min) and then 34 cycles of amplification (94°C for 1 min and 72°C for 1 min) followed by a 10-min extension at 72°C. PCR conditions for reaction 2 were identical to those (described above) for amplification of a portion of the 16S rRNA gene.

Following amplification of the two overlapping regions, the products were processed with Microcon microconcentrators (Amicon, Inc., Beverly, Mass.) to remove deoxynucleoside triphosphates, primers, and salts. One hundred microliters of PCR product was added to 400 µl of Tris-EDTA buffer (pH 8) (TE) in the column insert and spun at 3,000 rpm for 15 min. The flowthrough was discarded, the column insert was inverted, and the DNA was eluted with 50 µl of TE by spinning for 2 min at 3,000 rpm.

One-microliter aliquots of each of the PCR products were used in the fluorescence-based cycle sequencing reaction (ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit; Perkin-Elmer Applied Biosystems, Foster City, Calif.). M13 forward -40 and M13 reverse primers (Invitrogen, Carlsbad, Calif.) were used for chain termination sequencing reaction 1, and M13 forward -20 and M13 reverse primers (Invitrogen) were used for chain termination sequencing reaction 2. Cycle sequencing was performed according to manufacturer directions. After the sequencing reaction, excess dye terminators were removed by ethanol-sodium acetate precipitation according to manufacturer guidelines. Fluorescence-based sequence analysis of the extension products was performed with the ABI 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems).

(vi) Sequence analysis and comparison. Sequences were analyzed with MacVector and AssemblyLIGN (both from Oxford Molecular Group, Campbell, Calif.). Related sequences were identified by using the Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information, National Institutes of Health, Bethesda, Md.).

Sequence similarity was determined by aligning two sequences with the AssemblyLIGN software and determining the number of base differences. Ambiguous and skipped bases were disregarded. Percent similarity was determined by computing the number of base differences for the total length of the gene sequence.

TABLE 2. Comparison of identifications obtained by biochemical and molecular methods for discrepant isolates

Isolate (source)	RFLP 16S rRNA gene	Biochemical identification	RFLP HSP gene	Variable region of 16S gene sequence	BLAST results	
					Closest matches	% Similarity (no. of base differences)
A (sputum)	<i>N. farcinica</i>	<i>N. asteroides</i>	<i>N. asteroides</i> drug pattern type VI	<i>N. asteroides</i> drug pattern type I	<i>N. asteroides</i> drug pattern type I <i>N. farcinica</i> <i>N. brasiliensis</i> <i>N. otitidiscaviarum</i>	99.5 (5) 98.7 (13) 97.7 (23) 95.1 (49)
B (lung biopsy)	Unknown pattern	<i>Nocardia</i> sp.	<i>N. nova</i>	<i>N. nova</i> variant, <i>N. otitidiscaviarum</i>	<i>N. vaccinii</i> <i>N. nova</i> <i>N. pseudosporangifera</i> <i>N. otitidiscaviarum</i>	98.0 (20) 98.0 (20) 97.9 (21) 97.5 (25)
C (biopsy)	<i>N. asteroides</i> drug pattern type I	<i>N. otitidiscaviarum</i>	<i>N. otitidiscaviarum</i>	Ambiguous sequence	<i>N. seriolae</i> <i>N. otitidiscaviarum</i> <i>N. uniformis</i> <i>N. crassostreae</i>	98.2 (17) 97.7 (22) 96.9 (30) 96.7 (32)
D (wound)	<i>N. otitidiscaviarum</i>	<i>N. otitidiscaviarum</i>	Unknown pattern	<i>N. otitidiscaviarum</i> , <i>N. nova</i> variant	<i>N. otitidiscaviarum</i> <i>N. nova</i> <i>N. pseudosporangifera</i>	99.8 (2) 98.0 (20) 98.0 (20)

Nucleotide sequence accession numbers. The nucleotide sequences which we determined and submitted to GenBank have been assigned the following accession numbers: *N. asteroides* drug pattern type II (University of Texas Health Center at Tyler isolate N-565), AF163818; *N. asteroides* drug pattern type VI (ATCC 14759), AF162772; and the *N. nova* variant (NIH isolate 4793-2), AF162773.

RESULTS

Twenty-four of the 28 isolates studied showed complete agreement in identifications obtained by conventional biochemical reactions, 16S rRNA RFLP, and HSP gene RFLP. These 24 included *N. asteroides* drug pattern type I (one isolate), *N. asteroides* drug pattern type VI (seven isolates), *N. brasiliensis* (one isolate), *N. farcinica* (three isolates), *N. nova* (four isolates), the *N. nova* variant (six isolates; see below), *N. otitidiscaviarum* (one isolate), and *N. pseudobrasiliensis* (one isolate). Isolates identified biochemically as *N. asteroides* and molecularly as an *N. asteroides* drug pattern type, and isolates identified biochemically as *N. nova* and molecularly as the *N. nova* variant, were considered to be in agreement. The four isolates (A, B, C, and D) with discrepant results were further characterized as noted below (Table 2).

The 16S rRNA gene primers gave good amplification for all 28 clinical isolates of *Nocardia* and for all tested reference strains except *N. asteroides* drug pattern type IV (11, 16), which did not consistently amplify. Amplification was also obtained with all of the 39 *Actinomyces*, *Corynebacterium*, *Mycobacterium*, *Nocardia*, *Oerskovia*, *Rhodococcus*, *Rothia*, and *Streptomyces* isolates tested. RFLP patterns obtained on PCR products from these organisms were unlike those obtained for type strains of any of the *Nocardia* species.

By using *Hin*PII digests alone, unique RFLP patterns were obtained for the type strains of *N. farcinica* and *N. otitidiscaviarum* (Table 3; Fig. 1). By using a combination of *Hin*PII and *Dpn*II digests, unique RFLP patterns were obtained for *N. asteroides* drug pattern types II and VI and for the type strains of *N. nova*, *N. pseudobrasiliensis*, and *N. transvalensis*. *N. asteroides* drug pattern type I and the type strain of *N. brasiliensis* gave identical patterns with *Hin*PII and *Dpn*II but were easily differentiated with either *Bst*EII or *Sph*I digests (Table 3).

With all enzymes, the *N. asteroides* type strain gave RFLP patterns identical to those of *N. asteroides* drug pattern type I.

Preliminary studies showed that amplification of the 16S rRNA and subsequent REA may be able to distinguish at least two of the three members of the recently described "*N. brevicatena* complex" (Brown et al., Abstr. 97th Gen. Meet. Am. Soc. Microbiol.) (data not shown).

Six patient isolates which biochemically resembled *N. nova* gave RFLP patterns with *Dpn*II unlike those obtained for the *N. nova* ATCC type strain but similar to the patterns obtained for the *N. asteroides* type strain, *N. asteroides* drug pattern type I, and *N. brasiliensis*. Analysis of the sequence of the amplified area of the 16S rRNA gene for three of these *N. nova*-like isolates showed them to be similar to the sequence of the *N. nova* type strain (99.4 to 99.8% similarity), with one base change occurring within a *Dpn*II site. Digestion of the PCR products with *Bst*EII and *Sph*I gave a pattern which was easily distinguished from those obtained for the *N. asteroides* type strain, *N. asteroides* drug pattern type I, and *N. brasiliensis*. We have designated these isolates "*N. nova* variant." When compared to one another, the sequences of three *N. nova* variant isolates showed 99.6 to 100% similarity.

By using the RFLP patterns from digestion of a portion of the HSP gene and the identification scheme outlined by Steingrube et al. (11), identifications of 26 of 28 patient isolates were identical to those obtained biochemically. The two isolates with discrepant results, isolates B and D, were further characterized as described below.

An incidental finding with potential usefulness was also noted during the course of this work. Alignment of the 16S rRNA sequences of patient isolates with sequences of *Nocardia* type strains and *N. asteroides* drug pattern types (obtained from GenBank or experimentally determined) showed an area of base variability, with sequences which are unique for 6 of the 10 clinically important *Nocardia* species studied (Table 4). The site is located between base 149 and base 155 of *N. asteroides* ATCC 19247 (GenBank accession no. Z36934) (2).

GenBank and experimentally determined (*N. asteroides* drug pattern types II and VI and the *N. nova* variant) 16S rRNA sequences for the region defined by the primers described here

TABLE 3. Scheme for the identification of *Nocardia* species by RFLP pattern analysis of an amplified portion of the 16S rRNA gene

Expected sizes of fragments (bp) ^a after digestion with:				Identification ^b
<i>Hin</i> P1I ^c	<i>Dpn</i> II ^d	<i>Sph</i> I	<i>Bst</i> EII	
420, 350, 225	700, 200, 95	Uncut	Uncut	<i>N. asteroides</i> type strain
420, 350, 225	700, 200, 95	Uncut	Uncut	<i>N. asteroides</i> (I)
420, 350, 225	700, 200, 95	835, 165	730, 270	<i>N. brasiliensis</i>
420, 350, 225	700, 200, 95	Uncut	730, 270	<i>N. nova</i> variant
420, 350, 225	455, 250, 200, 95			<i>N. asteroides</i> (VI)
420, 350, 225	640, 200, 95, 60			<i>N. nova</i>
420, 350, 225	525, 200, 175, 95			<i>N. pseudobrasiliensis</i>
645, 350	705, 200, 95			<i>N. transvalensis</i> ^e
420, 225, 175, 125, 55	705, 200, 95			<i>N. farcinica</i>
420, 350, 150, 75	455, 250, 200, 95			<i>N. otitidiscaviarum</i>
640, 360	385, 250, 210, 85, 68			<i>N. asteroides</i> (II) ^f

^a Rounded to the nearest 5 bp. Fragment sizes were predicted by the sequences of reference strains as listed in GenBank.

^b Roman numerals in parentheses are drug pattern types.

^c Ranges of fragment sizes in base pairs, as determined by Molecular Analyst after digestion with *Hin*P1I, are as follows: for the *N. asteroides* type strain, *N. asteroides* drug pattern type I, *N. brasiliensis*, the *N. nova* variant, *N. asteroides* drug pattern type VI, *N. nova*, and *N. pseudobrasiliensis*, 402 to 427, 342 to 359, and 219 to 234; for *N. transvalensis*, 602 to 653 and 346 to 353; for *N. farcinica*, 403 to 427, 224 to 233, 171 to 178, and 124 to 131 (no values for smallest fragment [see text]); and for *N. otitidiscaviarum*, 402 to 419, 339 to 355, and 151 to 157 (no values for smallest fragment [see text]). For *N. asteroides* drug pattern type II, fragment sizes as determined by Molecular Analyst were 606 and 346 bp.

^d Ranges of fragment sizes in base pairs, as determined by Molecular Analyst after digestion with *Dpn*II, are as follows: for the *N. asteroides* type strain, *N. asteroides* drug pattern type I, *N. brasiliensis*, and the *N. nova* variant, 661 to 729, 186 to 204, and 85 to 102; for *N. asteroides* drug pattern type VI, 434 to 456, 250 to 253, 197 to 200, and 93 to 96; for *N. nova*, 615 to 661, 197 to 201, 94 to 98, and 65 to 68; for *N. pseudobrasiliensis*, 506 to 525, 195 to 200, 178 to 180, and 94 to 96; for *N. transvalensis*, 671 to 695, 193 to 199, and 95 to 98; for *N. farcinica*, 674 to 730, 196 to 204, and 94 to 99; and for *N. otitidiscaviarum*, 432 to 448, 240 to 252, 189 to 200, and 87 to 98. For *N. asteroides* drug pattern type II, fragment sizes as determined by Molecular Analyst were 370, 246, 195, 95, and 69 bp.

^e Results are based on the ATCC type strain.

^f Results are based on one isolate only.

were compared to determine percent similarity (Table 5). Ambiguous bases and skipped bases (maxima of 5 and 2 for all pairings, respectively) were disregarded in determining similarity.

Three isolates (A, B, and C) gave discrepant identifications by 16S rRNA RFLP pattern and biochemical testing (Table 2). Isolate A was biochemically characterized as *N. asteroides*, and the RFLP of the HSP gene showed a pattern consistent with *N. asteroides* drug pattern type VI (11). REA of the amplified area of the 16S rRNA gene gave RFLP patterns consistent with *N. farcinica*. A BLAST search of the sequenced amplified

area showed greatest similarity to *N. otitidiscaviarum* (GenBank accession no. X80611) (8), *N. farcinica*, *N. asteroides* drug pattern type I, and *N. brasiliensis*. (The sequence of this *N. otitidiscaviarum* GenBank submission is essentially identical to the sequence of the type strain of *N. farcinica* [99.9% similarity], suggesting a possible misidentification of this organism.) Alignment of the sequence of isolate A with GenBank sequences of the related organisms showed the closest relationship with *N. asteroides* drug pattern type I, with 99.5% similarity. The sequences of *N. farcinica*, *N. asteroides* drug pattern type VI, and *N. brasiliensis* showed 98.7, 97.8, and 97.7% similarity, respectively. The sequence of isolate A at the variable region was identical to that of *N. asteroides* drug pattern type I. Isolate A was also susceptible to ampicillin, consistent with an identification of *N. asteroides* drug pattern type I (16).

Isolate B was unidentifiable by conventional biochemical testing; RFLP of the HSP gene showed a pattern consistent with *N. nova* (Table 2). Although the RFLP pattern of the 16S rRNA *Hin*P1I digest resembled that of *N. farcinica* and the

FIG. 1. Bio-Rad-derived RFLP patterns of various reference strains of *Nocardia* species. Lanes 1, 6, and 16, base pair ladder, with lengths indicated at left. (A) *Hin*P1I digests. Lane 2, *N. transvalensis* and *N. asteroides* drug pattern type II; lane 3, *N. farcinica*; lane 4, *N. asteroides* type strain, *N. asteroides* drug pattern type I, *N. brasiliensis*, *N. nova* variant, *N. asteroides* drug pattern type VI, *N. nova*, and *N. pseudobrasiliensis*; lane 5, *N. otitidiscaviarum*. (B) *Dpn*II digests. Lane 7, *N. asteroides* drug pattern type II; lane 8, *N. asteroides* drug pattern type VI and *N. otitidiscaviarum*; lane 9, *N. pseudobrasiliensis*; lane 10, *N. asteroides* type strain, *N. asteroides* drug pattern type I, *N. brasiliensis*, *N. nova* variant, *N. transvalensis*, and *N. farcinica*; lane 11, *N. nova*. (C) *Sph*I and *Bst*EII digests. Lane 12, *N. brasiliensis* (*Sph*I); lane 13, *N. asteroides* type strain, *N. asteroides* drug pattern type I, and *N. nova* variant (*Sph*I); lane 14, *N. asteroides* drug pattern type I (*Bst*EII); lane 15, *N. nova* variant (*Bst*EII).

TABLE 4. Variable region

Species ^a	Base at position ^b :											
	146	147	148	149	150	151	152	153	154	155	156	
<i>N. asteroides</i> (I)	.	.	.	T	G	C	T	G	T	C	.	
<i>N. asteroides</i> (II)	.	.	.	T	T	T	G	G	T	T	.	
<i>N. asteroides</i> (VI)	.	.	.	T	T	A	C	A	T	C	.	
<i>N. brasiliensis</i>	.	.	.	T	T	T	C	A	G	T	.	
<i>N. farcinica</i>	.	.	.	T	T	A	C	A	T	C	.	
<i>N. nova</i> (type strain)	.	.	.	A	C	G	G	A	T	C	.	
<i>N. nova</i> variant	.	.	.	A	C	G	A	A	T	C	.	
<i>N. otitidiscaviarum</i>	.	.	.	A	C	G	A	A	T	C	.	
<i>N. pseudobrasiliensis</i>	.	.	.	A	T	G	G	G	A	T	.	
<i>N. transvalensis</i>	.	.	.	A	C	A	T	G	T	C	.	

^a Roman numerals in parentheses are drug pattern types.

^b As compared to *N. asteroides* ATCC 19247 (GenBank, accession no. X84850). Dots indicate conserved bases.

TABLE 5. 16S rRNA sequence similarities among *Nocardia* species^a

Species ^b	<i>N. asteroides</i> (II)	<i>N. asteroides</i> (VI)	<i>N. asteroides</i> type strain	<i>N. brasiliensis</i>	<i>N. farcinica</i>	<i>N. nova</i>	<i>N. nova</i> variant	<i>N. pseudo-</i> <i>brasiliensis</i>	<i>N. otitidis-</i> <i>caviarum</i>	<i>N. trans-</i> <i>valensis</i>
<i>N. asteroides</i> (I)	96.8	98.3	98.3	98.3	98.2	96.4	96.5	96.5	96.2	98.0
<i>N. asteroides</i> (II)		97.7	96.8	96.8	96.1	95.7	95.6	96.1	95.6	96.6
<i>N. asteroides</i> (VI)			97.8	98.2	98.0	97.2	97.4	97.1	97.1	97.8
<i>N. asteroides</i> type strain				97.7	97.2	96.6	96.8	97.0	95.9	97.1
<i>N. brasiliensis</i>					97.6	96.4	96.5	97.0	96.0	97.1
<i>N. farcinica</i>						95.7	96.0	95.5	95.5	97.3
<i>N. nova</i>							99.8	97.6	98.3	97.1
<i>N. nova</i> variant								97.6	98.4	97.2
<i>N. pseudobrasiliensis</i>									97.5	96.5
<i>N. otitidiscaviarum</i>										96.7

^a Based on GenBank and experimentally determined sequences of a 999-bp portion of the 16S rRNA gene of reference strains (see the text). Ambiguous and skipped bases (maxima of 5 and 2, respectively) are disregarded.

^b Roman numerals in parentheses are drug pattern types.

RFLP pattern of the *DpnII* digest resembled that of *N. asteroides* drug pattern type VI or *N. otitidiscaviarum*, the combination was unlike that obtained for any reference strain. A BLAST search of the sequenced amplified area showed greatest similarity to *N. vaccinii* and *N. nova*, with 98.0% similarity. *N. pseudosporangifera* and *N. otitidiscaviarum* showed 97.9 and 97.5% similarity, respectively. The sequence of isolate B at the variable region was identical to that obtained with the *N. nova* variant and *N. otitidiscaviarum*.

Isolate C was biochemically characterized as *N. otitidiscaviarum*. RFLP of the HSP gene gave a pattern consistent with *N. otitidiscaviarum* (Table 2). RFLP of the 16S rRNA gene gave a pattern consistent with *N. asteroides* drug pattern type I. A BLAST search of the sequenced amplified area showed greatest similarity to *N. seriolae*, *N. otitidiscaviarum*, *N. uniformis*, and *N. crassostreae*, with 98.2, 97.7, 96.9, and 96.7% similarity, respectively. Two attempts to sequence isolate C from different PCRs gave ambiguous sequences for the variable region each time but similar sequences for the remainder of the amplified area.

Isolate D was biochemically characterized as *N. otitidiscaviarum*, and RFLP of the 16S rRNA gene showed a pattern consistent with *N. otitidiscaviarum* (Table 2). RFLP of the HSP gene showed RFLP patterns inconsistent with those reported for this organism (11) and unlike patterns obtained for any other organism. A BLAST search of the sequenced amplified area of the 16S rRNA gene showed greatest similarity to *N. otitidiscaviarum*, *N. nova*, and *N. pseudosporangifera*. Alignment of the sequence of isolate D with GenBank sequences of these related organisms showed the closest relationship with *N. otitidiscaviarum*, with 99.8% identical bases. *N. nova* and *N. pseudosporangifera* each had 98.0% similarity to isolate D. The sequence of isolate D at the variable region was identical to that of the *N. nova* variant and *N. otitidiscaviarum*.

DISCUSSION

The 16S rRNA gene is known to be highly conserved among bacteria and is frequently used in the determination of organism relatedness (5, 6, 19); however, variable regions do exist within the gene, and some sequences within these variable regions are unique to certain species. Through amplification of a portion of the 16S rRNA gene and subsequent REA with the enzymes *HinP1I* and *DpnII*, we have taken advantage of this variability and propose an alternative identification method for the rapid identification of *Nocardia* species of clinical significance which is perhaps even more sensitive than REA of the HSP gene.

The use of the 16S rRNA gene as the target for PCR and REA-based identifications of *Nocardia* takes advantage of the information already accumulated about 16S gene sequences. Most of the sequences available in sequence data banks for type strains of *Nocardia* species include at least portions of the 16S rRNA gene. Thus, a comparison of the 16S rRNA sequences of unusual patient isolates with the well-characterized genomes included in the databases is possible.

All patient isolates that we examined which morphologically resembled *Nocardia* species gave good amplification with our 16S-specific primers. Only one ATCC strain of *N. asteroides* drug pattern type IV, which reportedly accounts for only 5% of patient isolates (16), was not consistently amplified.

Steingrube et al. report an identification scheme for non-*Nocardia* actinomycetes using the same HSP gene amplification and REA methods used for the identification of *Nocardia* species (11). Similarly, the amplification of the 16S rRNA gene described here was not specific for the genus *Nocardia*. However, RFLP patterns obtained for non-*Nocardia* species were easily distinguished from those obtained for the *Nocardia* species (data not shown).

REA of the 16S rRNA gene resulted in unique RFLP patterns for all common clinically isolated *Nocardia* species except *N. nova*, which showed two distinct patterns (Table 3; Fig. 1). Organisms giving unusual RFLP patterns, or patterns which were inconsistent with the biochemical identification, were clinical isolates with 16S sequences which differed significantly from any of the type strains.

The *N. nova* variant isolates we describe most likely belong to the species *N. nova* and illustrate the difficulties potentially encountered in relying solely on RFLP to make definitive organism identifications. Minor base substitutions can result in changes in restriction endonuclease recognition sites and consequently in variant RFLP patterns for members of the same species.

As with the analysis of RFLP patterns obtained from the HSP gene, RFLP of the 16S rRNA gene shows unique patterns for the various drug pattern types of *N. asteroides* (10, 11, 16) (Table 3; Fig. 1), further illustrating the heterogeneous nature of this species (10, 16). The *N. asteroides* drug pattern types are distinguishable in the routine diagnostic laboratory only by differences in susceptibility test results and not by biochemical reactions. Sequence analysis shows that the various drug pattern types have 96.8 to 98.3% similarity to the *N. asteroides* type strain and one another (Table 5).

RFLP fragment sizes reported here are based on restriction endonuclease recognition sites determined from published or experimental sequences (Table 3). The range of fragment sizes

for each organism (Table 3) was determined experimentally and reflects values reported by the Molecular Analyst Software. The small differences from predicted band sizes occasionally found for measured band sizes were presumably due to variations in gels, buffers, ethidium bromide concentration, and electrophoresis conditions. In addition, because agarose concentration and electrophoresis conditions were selected to allow distinction of as many different sizes of bands as possible, some bands smaller than 80 bases were not easily distinguished and therefore were not recognized by the Molecular Analyst Software.

Consensus does not yet exist regarding how similar 16S rRNA sequences of two organisms must be in order for them to be considered to belong to the same species. In a study of the phylogenetic relatedness of *Nocardia* species, Chun and Goodfellow (2) report 98.4% similarity in a 1472- to 1474-base sequence of the 16S rRNA gene between the most closely related species of *Nocardia* (*N. otitidiscaviarum* and *N. nova*). Likewise, Ruimy et al. (9) report 98.5% similarity between the same two species in a 1,500-base sequence of the same gene. In a similar study of *Bacillus* species, Fox et al. (3) state that for sequences of approximately 1,000 bases, 16S rRNA sequences from most species that are recognized to be separate differ in at least 1.5% of their bases (98.5% similarity).

Ruimy et al. (9) also report 99.4 to 100% similarity among the 1,500-base sequences of the 16S rRNA gene of five isolates of *N. pseudobrasiliensis*. For the sequences of the region amplified by the primers used in our study for these five *N. pseudobrasiliensis* isolates, a similarity of 99.2 to 100% was calculated. Likewise, for three patient isolates of the *N. nova* variant for which sequencing was performed, a comparison of the portion of the 16S rRNA gene defined by our primers showed 99.6 to 100% similarity (data not shown).

Thus, it seems reasonable to consider *Nocardia* isolates to belong to different species if they have $\leq 98.5\%$ similarity. Isolates belonging to the same species generally have a similarity of at least 99.2%. By these criteria, isolates B and C may each represent new *Nocardia* species.

Analysis of the results of all three of the methods that we used for the identification of isolates A, B, and C (16S rRNA REA, HSP gene REA, and biochemical tests) illustrates the particular usefulness of REA analysis of the 16S rRNA region. For all three isolates, the discrepancy between the 16S rRNA REA and biochemical identifications suggested the unusual nature of these organisms. Comparison of HSP gene RFLP with biochemical results would have resulted in incorrect identifications of the isolates, as the HSP gene and biochemical identifications agreed with each other. Interestingly, the different identifications obtained with 16S RFLP and HSP gene RFLP together would also have suggested the unusual nature of these isolates.

Isolate D gave ambiguous results by HSP gene RFLP; 16S RFLP, biochemical characterization, and sequence analysis all indicated that this organism belongs to the species *N. otitidiscaviarum* (Table 2).

We also noted the presence of a variable site within the 16S rRNA region being amplified (Table 4). This site holds potential for the development of nucleic acid probes which, together with 16S rRNA amplification, may serve as another means of rapid species identification for certain *Nocardia* isolates.

From the results of 16S REA, HSP gene REA, and conventional biochemical identification, it is apparent that the use of only one of these methods will result in inaccurate identifications of occasional unusual isolates. Conventional methods appear to be the most unreliable, due to the small number of discriminatory tests available and the expertise needed to in-

terpret these tests. However, both RFLP methods also have drawbacks. The HSP gene PCR method is unable to amplify some isolates of *N. otitidiscaviarum*, and REA for those isolates of *N. otitidiscaviarum* which do amplify result in a variety of RFLP patterns which may be difficult to interpret. In addition, the lack of sequence data for the HSP gene area of the genome precludes comparison of the region with entries in sequence databases. Compared to biochemical methods, HSP gene REA appears to correlate well, but it may also fail to suggest the unusual nature of some isolates. In comparison, the 16S rRNA method described here did not consistently amplify *N. asteroides* drug pattern type IV; however, this organism is less commonly isolated than *N. otitidiscaviarum*. When combined with biochemical identification, 16S rRNA REA seems to be more sensitive for the recognition of unusual isolates. Sequence information on this 16S region is also readily available in GenBank and other sequence databases. Notably, when both REA procedures are used together, discrepancies in identification are very useful for suggesting that an isolate may be unusual.

RFLP methodologies are a means for the rapid and accurate identification of clinical isolates of *Nocardia* species and far surpass the conventional biochemical methods in their discriminatory capabilities. While few organisms will be misidentified by either HSP gene or 16S rRNA analysis alone, it is now apparent that if REA of only one area of the genome is used to identify these organisms, unusual or undescribed species might remain undetected. To maximize both the speed and the accuracy of identification, RFLP analysis of two different loci may be the most useful method for identification of *Nocardia* species. Inconsistencies between RFLP identifications should prompt sequence analysis of the relevant portion of the genome and comparison with published sequences; for this purpose, analysis of the 16S rRNA gene region may be most useful.

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REFERENCES

1. Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. Wertheim-van Dillen, and J. van Der Noordaa. 1990. Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* **28**:495-503.
2. Chun, J., and M. Goodfellow. 1995. A phylogenetic analysis of the genus *Nocardia* with 16S rRNA gene sequences. *Int. J. Syst. Bacteriol.* **45**:240-245.
3. Fox, G. E., J. D. Wisotzkey, and P. Jurtshuk, Jr. 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int. J. Syst. Bacteriol.* **42**:166-170.
4. Gordon, R. E., D. A. Barnett, J. E. Handerman, and C. Hor-Nay Pang. 1974. *Nocardia coeliaca*, *Nocardia autotrophica*, and the nocardin strain. *Int. J. Syst. Bacteriol.* **24**:54-63.
5. Gray, M. W., D. Sankoff, and R. J. Cedergren. 1984. On the evolutionary descent of organisms and organelles: a global phylogeny based on a highly conserved structural core in small subunit ribosomal RNA. *Nucleic Acids Res.* **12**:5837-5852.
6. Ludwig, W., and K. H. Schleifer. 1994. Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. *FEMS Microbiol. Rev.* **15**:155-173.
7. McNeil, M. M., and J. M. Brown. 1994. The medically important aerobic actinomycetes: epidemiology and microbiology. *Clin. Microbiol. Rev.* **7**:357-417.
8. Rainey, F. A., J. Burghardt, R. M. Kroppenstedt, S. Klatte, and E. Stackebrandt. 1995. Phylogenetic analysis of the genera *Rhodococcus* and *Nocardia* and evidence for the evolutionary origin of the genus *Nocardia* from within the radiation of *Rhodococcus* species. *Microbiology* **141**:523-528.
9. Ruimy, R., P. Riegel, A. Carlotti, P. Boiron, G. Bernardin, H. Monteil, R. J. Wallace, Jr., and R. Christen. 1996. *Nocardia pseudobrasiliensis* sp. nov., a

- new species of *Nocardia* which groups bacterial strains previously identified as *Nocardia brasiliensis* and associated with invasive diseases. *Int. J. Syst. Bacteriol.* **46**:259–264.
10. Steingrube, V. A., B. A. Brown, J. L. Gibson, R. W. Wilson, J. Brown, Z. Blacklock, K. Jost, S. Locke, R. F. Ulrich, and R. J. Wallace, Jr. 1995. DNA amplification and restriction endonuclease analysis for differentiation of 12 species and taxa of *Nocardia*, including recognition of four new taxa within the *Nocardia asteroides* complex. *J. Clin. Microbiol.* **33**:3096–3101.
 11. Steingrube, V. A., R. W. Wilson, B. A. Brown, K. C. Jost, Jr., Z. Blacklock, J. L. Gibson, and R. J. Wallace, Jr. 1997. Rapid identification of clinically significant species and taxa of aerobic actinomycetes, including *Actinomyces*, *Gordonia*, *Nocardia*, *Rhodococcus*, *Streptomyces*, and *Tsukamurella* isolates, by DNA amplification and restriction endonuclease analysis. *J. Clin. Microbiol.* **35**:817–822.
 12. Telenti, A., F. Marchesi, M. Balz, F. Bally, E. C. Böttger, and T. Bodmer. 1993. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J. Clin. Microbiol.* **31**:175–178.
 13. Wallace, R. J., Jr., B. A. Brown, Z. Blacklock, R. Ulrich, K. Jost, J. M. Brown, M. M. McNeil, G. Onyi, V. A. Steingrube, and J. Gibson. 1995. New *Nocardia* taxon among isolates of *Nocardia brasiliensis* associated with invasive disease. *J. Clin. Microbiol.* **33**:1528–1533.
 14. Wallace, R. J., Jr., B. A. Brown, M. Tsukamura, J. M. Brown, and G. O. Onyi. 1991. Clinical and laboratory features of *Nocardia nova*. *J. Clin. Microbiol.* **29**:2407–2411.
 15. Wallace, R. J., Jr., and L. C. Steele. 1988. Susceptibility testing of *Nocardia* species for the clinical laboratory. *Diagn. Microbiol. Infect. Dis.* **9**:155–166.
 16. Wallace, R. J., Jr., L. C. Steele, G. Sumter, and J. M. Smith. 1988. Antimicrobial susceptibility patterns of *Nocardia asteroides*. *Antimicrob. Agents Chemother.* **32**:1776–1779.
 17. Wallace, R. J., Jr., M. Tsukamura, B. A. Brown, J. Brown, V. A. Steingrube, Y. Zhang, and D. R. Nash. 1990. Cefotaxime-resistant *Nocardia asteroides* strains are isolates of the controversial species *Nocardia farcinica*. *J. Clin. Microbiol.* **28**:2726–2732.
 18. Wilson, R. W., V. A. Steingrube, B. A. Brown, Z. Blacklock, K. C. Jost, Jr., A. McNabb, W. D. Colby, J. R. Biehle, J. L. Gibson, and R. J. Wallace, Jr. 1997. Recognition of a *Nocardia transvalensis* complex by resistance to aminoglycosides, including amikacin, and PCR-restriction fragment length polymorphism analysis. *J. Clin. Microbiol.* **35**:2235–2242.
 19. Woese, C. R. 1987. Bacterial evolution. *Microbiol. Rev.* **51**:221–271.