A Phylogenetic Analysis of the Genus *Nocardia* with 16S rRNA Gene Sequences

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Partial sequences of the 16S rRNA genes of the type strains of nine species of the genus *Nocardia* were determined following the isolation and cloning of the amplified genes. These sequences were aligned with the sequences of representatives of the genera *Corynebacterium*, *Gordona*, *Mycobacterium*, *Rhodococcus*, and *Tsukamurella*, and phylogenetic trees were inferred by using the Fitch-Margoliash and neighbor-joining methods. The genus *Nocardia* formed a distinct clade that was most closely associated with the genus *Rhodococcus*. The average level of sequence similarity found among the type strains of the *Nocardia* species was $97.2 \pm 0.7\%$. Two sublines were recognized within the *Nocardia* clade; one encompassed *Nocardia asteroides* and related species, and the other encompassed *Nocardia otitidiscaviarum* and allied taxa. Separation of the two sublines is based on differences in helix 37-1. The results of isoprenoid quinone analyses provided evidence that nocardiae can be distinguished from all other actinomycete taxa on the basis of their characteristic menaquinone profiles. Nocardiae typically contain hexahydrogenated menaquinones with eight isoprene units in which the two end units are cyclized.

The taxonomy of actinomycetes that contain mycolic acids (high-molecular-weight, long-chain, 3-hydroxy fatty acids with an alkyl branch at position 2) has undergone sweeping changes on the basis of data derived from the use of modern taxonomic methods (18, 19, 35). The mycolic acid-containing bacteria have many phenotypic characteristics in common (6, 18), form a distinct phyletic line (48), and have been assigned to the genera *Corynebacterium*, *Gordona*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Tsukamurella* on the basis of a number of chemical and morphological markers (5, 6, 18, 19). Proposals to transfer *Nocardia amarae* (33) to the genus *Gordona* as *Gordona amarae* (20) leave the genus *Nocardia* as a relatively homogeneous taxon that contains 11 validly described species (18, 21, 58). The retention of one of these species, *Nocardia pinensis*, in the genus *Nocardia* has been debatable as this organism has many properties in common with *G. amarae* and can be readily distinguished from typical nocardiae (2). On the basis of 16S rRNA sequence data and complementary phenotypic properties, *N. pinensis* merits generic status within the family *Nocardiaceae* (11).

Members of the reconstituted genus *Nocardia* form extensively branched substrate hyphae that fragment in situ or after mechanical disruption into rod-shaped to coccoid, nonmotile elements; aerial hyphae, which at times are visible only microscopically, are almost always formed (21, 31). Nocardiae are also characterized by the presence of meso-diaminopimelic acid, arabinose, and galactose in their wall peptidoglycan (wall chemotype IV sensu Lechevalier and Lechevalier [32]) (A1 γ type), have muramic acid in the N-glycolated form, have diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylinositol mannosides as their predominant phospholipids, have major amounts of straight-chain, unsaturated, and tuberculostearic acids, have mycolic acids with 40 to 60 carbon atoms, and have DNAs that are rich in guanine plus cytosine (guanine-plus-cytosine contents, 64 to 72 mol%) (6, 18, 21). The predominant nocardial menaquinone is an unusual hexahydrogenated menaquinone with eight

isoprene units in which the end two units are cyclized (13, 25, 27).

Nocardia species have been circumscribed primarily by using chemical, numerical taxonomic, DNA relatedness, phage sensitivity, immunological, and antibiotic susceptibility data (1, 4, 6, 18, 19, 55, 56), although problems remain. It is important to distinguish between different kinds of nocardiae as some nocardiae cause a variety of suppurative diseases in humans and animals, notably actinomycete mycetoma and nocardiosis (5, 51). *Nocardia brasiliensis* and *Nocardia transvalensis* are the main causal agents of actinomycete mycetoma (35, 36), and the predominant agents of nocardiosis are *Nocardia asteroides*, *Nocardia farcinica*, and *Nocardia nova* (51, 55).

It is necessary to examine the taxonomic structure of the genus *Nocardia* in order to determine the relationships among the constituent species. Ruimy et al. (48) have shown by performing ribosomal DNA (rDNA) sequence analyses that representatives of most species belonging to the genus *Nocardia* form a distinct phyletic line, although not all of the type strains were examined by these workers nor was much light cast on the phylogenetic relationships within the genus. The study described in this paper was an extension of the previous study of Ruimy et al., although in addition to sequence analyses the menaquinone compositions of the test strains were determined.

MATERIALS AND METHODS

Organisms and culture conditions. Cultures of the test strains (Table 1) used to prepare biomass were grown in shake flasks containing modified Sauton's broth (39); these cultures were incubated for 7 to 10 days at 30°C. When maximum growth was observed, the broth cultures were checked for purity, killed with 1% (vol/vol) formaldehyde, harvested by centrifugation, washed with distilled water, and freeze-dried. The nocardiae were maintained on glucose-yeast extract agar (22) slopes and as glycerol suspensions (20%, vol/vol) at –20°C.

Menaquinone analysis. Isoprenoid quinones were extracted from freeze-dried biomass (50 mg) by using the small-scale procedure of Minnikin et al. (37). Purified menaquinones were separated by high-performance liquid chromatography, using a Pharmacia LKB instrument fitted with a Spherisorb octyldecyl silane column (5 μ m) and acetonitrile-isopropanol (75:25, vol/vol) as the mobile phase. The menaquinones were detected at 254 nm.

DNA extraction. Chromosomal DNA was isolated by using a method modified slightly from the method of Pitcher et al. (44). Small amounts of biomass from glucose-yeast extract agar plates were mixed and gently homogenized in 1.5-ml tubes containing 100 μ l of T.E. buffer (pH 8.0) supplemented with lysozyme (50 mg/ml; Sigma, Ltd., Poole, Dorset, United Kingdom). The resulting solutions

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TABLE 1. Strains used in the 16S rRNA sequence analysis

Laboratory no.	Species	Other designation ^a	Nucleotide sequence accession no.	Source of data or reference
— ^b	<i>Arthrobacter globiformis</i>	DSM 20124 ^T	M23411	RDP ^c
—	<i>Corynebacterium variabilis</i>	NCIB 9455 ^T	X53185	RDP
—	<i>Corynebacterium xerosis</i>	ATCC 373 ^T	M59058	RDP
N667	<i>Gordona amarae</i>	ATCC 27808 ^T	X75902	20
—	<i>Gordona terrae</i>	DSM 43249 ^T	X53202	RDP
—	<i>Mycobacterium bovis</i>	Strain BCG	M20940	RDP
—	<i>Mycobacterium smegmatis</i>	ATCC 14468	X52922	RDP
N317 ^T	<i>Nocardia asteroides</i>	ATCC 19247 ^T	Z36934	This study
—	<i>Nocardia asteroides</i>	DSM 43005	X57949	RDP
N318 ^T	<i>Nocardia brasiliensis</i>	ATCC 19296 ^T	Z36935	This study
N1201 ^T	<i>Nocardia brevicatena</i>	DSM 43024 ^T	Z36928	This study
N1200 ^T	<i>Nocardia carnea</i>	DSM 43397 ^T	Z36929	This study
N898 ^T	<i>Nocardia farcinica</i>	ATCC 3318 ^T	Z36936	This study
N1112 ^T	<i>Nocardia nova</i>	JCM 6044 ^T	Z36930	This study
N36 ^T	<i>Nocardia otitidiscaviarum</i>	ATCC 14629 ^T	M59056	RDP
N1116 ^T	<i>Nocardia seriolae</i>	JCM 3360 ^T	Z36925	This study
N1202 ^T	<i>Nocardia transvalensis</i>	DSM 43405 ^T	Z36926	This study
N1199 ^T	<i>Nocardia vaccinii</i>	DSM 43285 ^T	Z36927	This study
—	<i>Rhodococcus erythropolis</i>	DSM 43188 ^T	X53203	RDP
—	<i>Rhodococcus fascians</i>	DSM 20131	X53204	RDP
N663	<i>Tsukamurella paurometabola</i>	NCTC 10741	Z36933	12

^a ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; JCM, Japan Collection of Microorganisms, Wako-Shi, Saitama, Japan; NCIB, National Collection of Industrial Bacteria, Aberdeen, United Kingdom; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, Colindale, London, United Kingdom.

^b —, no laboratory number assigned.

^c RDP, Ribosomal Database Project, release 4 (30).

were incubated at 37°C overnight, and 500 µl of a guanidine-sarcosyl solution was added to each preparation. The aqueous layers were separated by centrifugation and extracted with chloroform-isoamyl alcohol (25:1, vol/vol), and the chromosomal DNA was precipitated with 0.54 volume of isopropanol, washed in 70% (vol/vol) ethanol, and dried under a vacuum. The DNA samples were redissolved in 90 µl of T.E. buffer (pH 8.0), and 10 µl of RNase A (10 mg/ml; Sigma) was added before the preparation was incubated at 37°C for 2 h. After the RNase treatment, the DNA samples were extracted with phenol and chloroform, precipitated by adding 3 volumes of ethanol in the presence of 0.8 M lithium chloride, washed with 70% ethanol, dried, and redissolved in 30 µl of water.

PCR amplification of 16S rDNA. The 16S rRNA genes (rDNA) were amplified by using universal primers p27f (5'-AGA GTT TGA TCM TGG CTC AG-3'; positions 8 to 27; *Escherichia coli* numbering of Brosius et al. [7]) and p1525r (5'-AAG GAG GTG WTC CAR CC-3'; positions 1541 to 1525) (29). Each PCR mixture (100 µl) contained primers (each at a concentration of 0.4 µM), a mixture of deoxynucleoside triphosphates (Boehringer Mannheim) (each at a concentration of 200 µM), and *Taq* polymerase buffer (Hoefer Scientific Instruments, Newcastle upon Tyne, United Kingdom). Chromosomal DNA (ca. 100 ng) was added to the solution, which was then heated at 98°C for 2 min and cooled immediately on ice. *Taq* polymerase (2.5 U) and 1 drop of mineral oil (Sigma) were then added to each of the reaction solutions. The DNA thermal cycler (Omnigene; Hybaid, Ltd., Middlesex, United Kingdom) used for thermal amplification was programmed as follows: (i) an initial extensive denaturation step consisting of 94°C for 2 min; (ii) 25 reaction cycles, with each cycle consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min; and (iii) a final extension step consisting of 72°C for 10 min.

Isolation and cloning of amplified 16S rDNA. Amplified 16S rDNA was purified from 0.8% agarose gels by using the method of Heery et al. (24). Purified rDNA was ligated into the pGEM-T vector (Promega Co., Southampton, United Kingdom) according to the manufacturer's instruction. Ligated plasmids were then transformed into *E. coli* JM109, and transformants were selected on the basis of the results of the blue-white screening procedure (50).

Sequencing of 16S rDNA. Plasmids containing 16S rDNA were isolated by using the standard purification method recommended by the manufacturer (Promega Co.). Purified plasmids were sequenced by using a *Taq* DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, Foster City, Calif.). Sequencing gel electrophoresis was performed and nucleotide sequences were automatically obtained by using an Applied Biosystems model 373A DNA sequencer and the protocol and software recommended by the manufacturer. Sequence data were recorded on an IBM PC disk. The following forward and reverse primers were used (*E. coli* numbering system [7]): universal M13 forward-reverse primer p125f (5'-GAA CGG GTG AGT AAC ACG T-3'; positions 107 to 125), p346f (5'-ACG GCC CAG ACT CCT ACG-3'; positions 329 to 346), p357f (5'-CTA CGG

GRS GCA GCA G-3'; positions 342 to 357), p692f (5'-AAT TCC TGG TGT AGC GGT-3'; positions 675 to 692), p782r (5'-ACC AGG GTA TCT AAT CCT GT-3'; positions 801 to 782), p926f (5'-AAA CTC AAA GGA ATT GAC GG-3'; positions 907 to 926), and p1208f (5'-GAC GTC AAG TCA TCA TGC C-3'; positions 1190 to 1208). The nucleotide sequences of the sequencing primers were obtained from Lane (29) or were specifically designed for actinomycetes.

Phylogenetic analysis. The 16S rRNA gene sequences which we obtained were aligned manually with sequences of representative mycolic acid-containing actinomycetes (Table 1) obtained from the Ribosomal Database Project (release 4) (30) by using the AL16S program (10) to determine information concerning secondary structures.

An evolutionary distance matrix was constructed by using the algorithm of Jukes and Cantor (26). Phylogenetic trees were constructed by using the Fitch-Margoliash (17) and neighbor-joining (49) methods. *Arthrobacter globiformis* DSM 20124^T (T = type strain) was used as the outgroup in each of these analyses. In order to determine the stability of our phylogenetic tree, the sequence data were sampled 1,000 times for bootstrap analysis (15) by using the SEQBOOT program (PHYLIP, version 3.5c [16]).

Nucleotide sequence accession numbers. The 16S rDNA sequences which we determined in this study have been deposited in the EMBL database under the accession numbers listed in Table 1. The accession numbers of the *Mycobacterium* nucleotide sequences that were compared with *Nocardia* sequences in this study are as follows: *Mycobacterium aichiense*, X55598; *M. asiaticum*, X55604; *M. aurum*, X55595; *M. avium*, M61673, M61672, M61671, M29573, M29572, X52918, X52934, M61668, M61669, M61670, and M61667; *M. bovis* BCG, M20940; *M. bovis* vallee, X55589; *M. celatum*, L08169 and L08170; *M. chelonae*, M29559 and X52921; *M. chitae*, X55603 and M61662; *M. chubuense*, X55596; *M. confluentis*, X63608; *M. cookii*, X53896; *M. diernhoferi*, X55593; *M. fallax*, M29562; *M. farcinogenes*, X55592; *M. flavescens*, X52932; *M. fortuitum*, X52933; *M. gadium*, X55594; *M. gastri*, X52919; *M. geneveuse*, X60070; *M. gilvum*, X55599; *M. gordoniae*, X52923; *M. haemophilum*, L24800; *M. hiberniae*, X67096; *M. intermedium*, X67847; *M. intracellulare*, M61685, M61686, X52927, M61683, M61684, and M61682; *M. kansasii*, M29575 and X15916; *M. komossense*, X55591; *M. leprae*, X55587, X53999, and X55022; *M. madagascariense*, X55600; *M. malmoense*, X52930; *M. marinum*, X52920; *M. neoaurum*, M29564; *M. nonchromogenicum*, X52928; *M. obuense*, X55597; *M. paratuberculosis*, M29569, M61678, M61679, M61676, M61680, M61674, M61677, and M61675; *M. phlei*, M29566; *M. scrofulaceum*, X52924; *M. senegalense*, M29567; *M. simiae*, X52931; *M. smegmatis*, X52922; *M. sphagni*, X55590; *M. szulgai*, X52926; *M. terrae*, X52925; *M. thermoresistibile*, X55602; *M. triviale*, M29571; *M. tuberculosis*, X52917; *M. ulcerans*, Z13990 and X58954; *M. vaccae*, X55601; and *M. xenopi*, X52929.

TABLE 2. Similarity values based on the 16S rDNA sequences of *Nocardia* species and related mycolic acid-containing actinomycetes

Taxon	% Similarity to:																			
	<i>Corynebacterium variabilis</i>	<i>Corynebacterium xerosis</i>	<i>Gordonia amarae</i>	<i>Gordonia terrae</i>	<i>Mycobacterium bovis</i>	<i>Mycobacterium smegmatis</i>	<i>Nocardia asteroides</i>	<i>Nocardia asteroides</i> DSM 43005	<i>Nocardia brasiliensis</i>	<i>Nocardia brevicatena</i>	<i>Nocardia carnea</i>	<i>Nocardia farcinica</i>	<i>Nocardia nova</i>	<i>Nocardia otitidiscaviarum</i>	<i>Nocardia seriolae</i>	<i>Nocardia transvalensis</i>	<i>Nocardia vaccinii</i>	<i>Rhodococcus erythropolis</i>	<i>Rhodococcus fascians</i>	<i>Tsukamurella paurometabola</i>
<i>Corynebacterium variabilis</i>	100																			
<i>Corynebacterium xerosis</i>	95.2	100																		
<i>Gordonia amarae</i>	90.8	91.3	100																	
<i>Gordonia terrae</i>	92.0	92.2	97.6	100																
<i>Mycobacterium bovis</i>	91.5	93.0	92.1	93.5	100															
<i>Mycobacterium smegmatis</i>	91.0	91.7	93.5	93.8	96.0	100														
<i>Nocardia asteroides</i>	91.0	91.1	93.5	93.9	93.2	94.0	100													
<i>Nocardia asteroides</i> DSM 43005	90.8	91.3	93.4	94.4	92.6	93.9	98.4	100												
<i>Nocardia brasiliensis</i>	90.7	90.9	94.4	94.8	92.4	93.4	97.7	97.9	100											
<i>Nocardia brevicatena</i>	90.4	90.8	93.0	93.6	93.0	94.4	97.4	97.9	96.5	100										
<i>Nocardia carnea</i>	91.2	91.2	92.9	93.6	93.2	94.2	98.0	98.3	97.0	97.8	100									
<i>Nocardia farcinica</i>	91.8	91.8	93.9	94.6	92.5	93.8	97.6	98.0	98.0	96.3	96.7	100								
<i>Nocardia nova</i>	90.6	91.4	93.5	95.0	93.0	93.6	97.5	97.8	96.7	97.1	96.7	96.3	100							
<i>Nocardia otitidiscaviarum</i>	90.6	91.1	92.7	94.4	93.2	93.5	96.9	97.7	96.3	96.8	96.6	96.0	98.4	100						
<i>Nocardia seriolae</i>	90.4	90.8	92.6	93.5	92.9	93.6	96.8	97.0	96.3	96.8	96.7	95.7	97.7	98.0	100					
<i>Nocardia transvalensis</i>	91.0	91.7	93.9	94.8	92.7	93.5	97.4	97.9	97.7	96.8	96.9	97.9	97.5	96.9	96.3	100				
<i>Nocardia vaccinii</i>	91.5	91.4	93.0	94.4	93.1	94.0	97.1	97.1	96.1	97.4	96.8	96.7	98.2	97.5	97.5	97.1	100			
<i>Rhodococcus erythropolis</i>	92.2	92.3	93.6	94.4	92.0	93.5	94.9	95.3	94.8	94.8	94.4	96.0	95.5	94.9	94.4	95.0	95.4	100		
<i>Rhodococcus fascians</i>	91.5	91.2	91.7	93.1	91.8	91.9	93.6	93.5	93.0	92.8	93.3	93.7	94.3	94.0	93.5	93.4	94.1	95.5	100	
<i>Tsukamurella paurometabola</i>	92.2	92.5	94.4	94.0	92.9	94.5	94.4	94.0	94.0	93.4	93.8	94.6	93.8	93.4	93.6	94.1	93.8	94.8	92.5	100

RESULTS AND DISCUSSION

Mycolic acid-containing organisms are classified in the families *Corynebacteriaceae* (34), *Mycobacteriaceae* (9), and *Nocardiaceae* (8); the family *Nocardiaceae* currently contains the genera *Gordona*, *Nocardia*, *Rhodococcus*, and *Tsukamurella* (18). It is evident from the phylogenetic tree derived from the sequence similarity values (Table 2) that the genus *Nocardia*, the type genus of the family *Nocardiaceae*, forms a distinct phyletic line that is most closely related to the genus *Rhodococcus* (Fig. 1). The average level of sequence similarity between representative nocardiae and rhodococci was 94.3%. The *Gordona* branch joins the *Nocardia-Rhodococcus* group, albeit at nonsignificant bootstrap values. The branching order of the *Mycobacterium* and *Tsukamurella* lines is not clear, as discordant results were found with the two methods used to generate the phylogenetic trees.

Our sequence data confirm and extend the results of Ruimy et al. (48), which were based on analyses of small-subunit rDNA sequences; however, our results also raise questions. In particular, the close relationship between *N. farcinica* and *Rhodococcus equi* found by Ruimy et al. seems to be questionable. When more nucleotides of the 16S rRNA of the type strain of *N. farcinica* are compared, this organism is clearly placed in the *Nocardia* clade.

The nocardiae were assigned to two rRNA subgroups, one corresponding to *N. asteroides* and related taxa and the other corresponding to *Nocardia otitidiscaviarum* and allied species (Fig. 1). The taxonomic significance of these subgroups is supported by the 100% bootstrap values based on 1,000 samplings. The separation of the nocardiae into two subgroups is based largely on differences found in helix 37-1 (42), as shown in Fig.

2. In this present study, almost complete 16S rRNA gene sequences (1,472 to 1,474 nucleotides) were determined. The sequences of two other nocardiae, *N. asteroides* DSM 43005 and *N. otitidiscaviarum* ATCC 14629^T, were generated by Rogall et al. (47) and Yang and Woese (57), respectively.

The six species belonging to the *N. asteroides* subgroup include *N. asteroides*, *Nocardia brevicatena*, and *Nocardia carnea*. Members of these taxa either are human pathogens or have been implicated as human pathogens (5, 18, 19, 23, 51). The close genotypic affinities among *N. asteroides*, *N. brevicatena*, and *N. carnea* are at variance given the phenotypic similarities of *N. asteroides*, *N. farcinica*, and *N. nova* strains found by Wallace et al. (55). While these latter species are readily separated by 16S rDNA sequence data, they are difficult to distinguish on the basis of simple phenotypic properties (5, 18, 19, 55). It is also interesting that the remaining members of the *N. asteroides* subgroup (namely, *N. brasiliensis*, *N. farcinica*, and *N. transvalensis*) are closely related on the basis of nucleotide sequence data. Members of all of these taxa cause actinomycete mycetoma or have been implicated as agents of actinomycete mycetoma (19, 35, 36, 38).

The second taxon, the *N. otitidiscaviarum* subgroup, consists of four species (Fig. 1). *N. otitidiscaviarum* and *N. nova* cause nocardiosis (5, 51, 55), and *Nocardia seriolae* is pathogenic for cultured fish (28). *N. nova* is an important pathogen (55) which has been separated from *N. asteroides* and *N. farcinica* strains on the basis of results of DNA relatedness analyses (28, 58) and mycolic acid analyses (58). Separation of these taxa on the basis of 16S rDNA sequence data is important as it paves the way for the development of PCR or oligonucleotide probes for diagnostic purposes (3, 14). The sharp separation of the type

FIG. 1. Phylogenetic tree showing the relationships among *Nocardia* species and between nocardiae and representatives of other mycolic acid-containing taxa. The tree was constructed by using the method of Fitch and Margoliash (17). The numbers at the nodes indicate the levels of bootstrap support based on data for 1,000 replicates; only values that are greater than 50% are shown. Bootstrap values derived from the neighbor-joining method (49) are given in parentheses. The scale bar indicates 0.01 substitution per nucleotide position. For additional details see the text.

strains of *N. asteroides* and *N. farcinica* (level of sequence similarity, 97.6%) is consistent with the results of DNA relatedness (40, 41), numerical phenetic (27, 43, 52, 54), and serological (46) studies.

The average level of sequence similarity found among the type strains of *Nocardia* species was $97.2\% \pm 0.7\%$. The closest association was found between *N. otitidiscaviarum* and *N. nova* (level of similarity, 98.4%), and the most distant association

was observed between *N. farcinica* and *N. seriolae* (level of similarity, 95.7%). These relatively modest levels of similarity are consistent with the low DNA complementary values (7 to 18%) reported between representatives of these taxa (28). The 16S rDNA nucleotide signatures that distinguish the *Nocardia* species are shown in Table 3. It is also interesting that the levels of 16S rDNA similarity found within the genus *Nocardia* are slightly greater than the values recorded for members of the genus *Mycobacterium* (45, 47, 53). There are several nucleotide signatures that distinguish *Nocardia* and *Mycobacterium* strains (Table 4).

It is evident that the genus *Nocardia* now constitutes a homogeneous taxon that can be distinguished from other mycolic acid-containing organisms on the basis of chemical, morphological, and 16S rDNA sequence data. The results of our isoprenoid quinone analyses provide evidence that nocardiae can be distinguished from all other actinomycetes, including corynebacteria, gordonae, mycobacteria, rhodococci, and tsukamurellae, on the basis of their characteristic menaquinone profiles. Our data confirm and extend the results of previous studies (13, 25, 27) and show that nocardiae typically contain hexahydrogenated menaquinones with eight isoprene units in which the two end units are cyclized [MK-8(H_4)c]. The discovery that the type strains of *N. nova*, *N. seriolae*, and *Nocardia vaccinii* contain major amounts of this compound is further evidence that these organisms are members of the genus *Nocardia*. In all 10 of the test strains the MK-8(H_4)c

FIG. 2. Signatures that distinguish the two rRNA subgroups found in the genus *Nocardia*. The P37-1 helix (42) lies between *E. coli* nucleotide positions 1006 and 1023 (7). The pair of nucleotides enclosed in a box is G-C in *N. transvalensis*. (a) *N. asteroides* rRNA subgroup. (b) *N. otitidiscaviarum* rRNA subgroup.

TABLE 3. 16S rDNA sequence signatures that differentiate *Nocardia* species

Positions ^a	<i>N. asteroides</i>	<i>N. asteroides</i> DSM 43005	<i>N. brasiliensis</i>	<i>N. brevicatena</i>	<i>N. carneae</i>	<i>N. farcinica</i>	<i>N. nova</i>	<i>N. otitidis-cavium</i>	<i>N. seriolae</i>	<i>N. trans-valensis</i>	<i>N. vaccinii</i>
137:226	U:A	U:A	U:A	U:A	U:A	C:G	U:A	U:A	U:A	U:A	U:A
199:218	A:U	A:U	A:U	A:U	A:U	A:U	A:U	G:C	A:U	A:U	A:U
258:268	G:C	G:C	G:C	G:C	G:C	G:C	G:C	G:C	A:U	G:C	G:C
293:304	G:C	G:C	A:U	G:C	G:C	G:C	A:U	G:C	G:C	G:C	G:C
407:435	A:U	A:U	A:U	A:U	A:U	A:U	A:U	A:U	A:U	C:G	A:U
444:490	A:U	A:U	A:U	A:U	U:A	A:U	A:U	A:U	A:U	A:U	A:U
445:489	G:C	G:C	G:C	G:C	C:G	G:C	G:C	G:C	G:C	G:C	G:C
446:488	G:C	G:C	G:C	G:C	C:G	G:C	G:C	G:C	G:C	G:C	G:C
591:648	C:G	U:A	U:A	U:A	U:A	U:A	C:G	U:A	U:A	U:A	C:G
603:635	C:G	C:G	C:G	C:G	C:G	C:G	U:A	C:G	C:G	C:G	C:G
614:626	G:C	G:C	G:C	A:U	G:C	G:C	G:C	G:C	A:U	G:C	A:U
615:625	G:C	G:C	G:C	C:G	G:C	G:C	C:G	C:G	C:G	G:C	C:G
616:624	G:C	G:C	G:C	A:U	G:C	G:C	A:U	A:U	A:U	G:C	A:U
824:876	U:A	U:A	U:A	U:A	U:A	C:G	U:A	U:A	U:A	U:A	C:G
825:875	A:U	A:U	A:U	A:U	A:U	G:C	A:U	A:U	A:U	A:U	G:C
1002:1038	G:C	G:C	G:C	G:C	G:C	G:C	A:U	G:C	G:C	A:U	A:U
1007:1022	C:G	C:G	C:G	C:G	C:G	C:G	G:C	G:C	G:C	G:C	G:C
1009:1020	U:A	U:A	U:A	U:A	U:A	U:A	C:G	C:G	C:G	U:A	C:G
1011:1018	C:G	C:G	C:G	C:G	C:G	C:G	U:A	U:A	U:A	C:G	U:A
1120:1153	U:A	U:A	C:G	U:A	U:A	C:G	U:A	U:A	U:A	C:G	U:A
1122:1151	A:U	A:U	G:C	A:U	A:U	G:C	A:U	A:U	A:U	G:C	A:U
1134:1140	C:G	C:G	G:C	C:G	C:G	C:G	C:G	C:G	C:G	C:G	C:G

^a *E. coli* numbering (7).

component accounted for more than 90% of the total menaquinones.

Our results highlight the power of 16S rDNA sequencing for determining relationships between organisms that have proved to be difficult to classify by more conventional methods. This approach, together with the use of appropriate chemical and morphological methods, should prove to be sufficiently powerful to determine relationships in the well-known taxonomic enigma called the "*Nocardia asteroides* complex" (18, 43).

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TABLE 4. Nucleotide signatures that distinguish *Mycobacterium* and *Nocardia* strains

Position(s) ^a	<i>Mycobacterium</i> ^b	<i>Nocardia</i>
66:103	A:U	G:C
328	U	C
580:761	C:G	U:A
694	G	A
998:1043	G:U	A:U
1001:1039	A:U	C:G
1003:1037	G:C	G:U
1189	U	C
1336	C	U

^a *E. coli* numbering (7).

^b Based on sequence data from *Mycobacterium aichiense*, *M. asiaticum*, *M. aurum*, *M. avium*, *M. bovis* BCG, *M. bovis* vallee, *M. celatum*, *M. chelonae*, *M. chitae*, *M. chubuense*, *M. confluentis*, *M. cookii*, *M. diernhoferi*, *M. fallax*, *M. farcinogenes*, *M. flavescens*, *M. fortuitum*, *M. gadium*, *M. gastris*, *M. geneveuse*, *M. gilvum*, *M. gordonae*, *M. haemophilum*, *M. hiberniae*, *M. intermedium*, *M. intracellulare*, *M. kansasii*, *M. komossense*, *M. leprae*, *M. madagascariense*, *M. malmoense*, *M. marinum*, *M. neoaurum*, *M. nonchromogenicum*, *M. obuense*, *M. paratuberculosis*, *M. phlei*, *M. scrofulaceum*, *M. senegalense*, *M. simiae*, *M. smegmatis*, *M. sphagni*, *M. szulgai*, *M. terrae*, *M. thermoresistibile*, *M. triviale*, *M. tuberculosis*, *M. ulcerans*, *M. vaccae*, and *M. xenopi*.

REFERENCES

- Berkey, P., D. Moore, and K. Rolston. 1988. In vitro susceptibilities of *Nocardia* species to newer antimicrobial agents. *Antimicrob. Agents Chemother.* **32**:1078-1079.
- Blackall, L. L., J. H. Parlett, A. C. Hayward, D. E. Minnikin, P. F. Greenfield, and A. Harbers. 1989. *Nocardia pinensis* sp. nov., an actinomycete found in activated sludge foams in Australia. *J. Gen. Microbiol.* **135**:1547-1558.
- Bödinghaus, B., T. Rogall, T. Flohr, H. Blöcker, and E. C. Böttger. 1990. Detection and identification of mycobacteria by amplification of rRNA. *J. Clin. Microbiol.* **28**:1751-1759.
- Boiron, P., and F. Provost. 1988. In vitro susceptibility testing of *Nocardia* spp. and its taxonomic implications. *J. Antimicrob. Chemother.* **22**:623-629.
- Boiron, P., F. Provost, and B. Dupont. 1993. Laboratory methods for the diagnosis of nocardiosis. Institut Pasteur, Paris.
- Bowden, G. H., and M. Goodfellow. 1990. The actinomycetes: *Actinomycetes*, *Nocardia* and related genera, p. 31-57. In M. T. Parker and B. I. Duerden (ed.), *Topley and Wilson's principles of bacteriology, virology and immunity*, 8th ed., vol. 4. Edward Arnold, London.
- Brosius, J., M. L. Palmer, P. J. Kennedy, and H. F. Noller. 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **75**:4801-4805.
- Castellani, A., and A. J. Chalmers. 1919. *Manual of tropical medicine*, 3rd ed. William, Wood and Co., New York.
- Chester, F. D. 1897. Report of the mycologist: bacteriological work. *Del. Agric. Exp. Stn. Bull.* **9**:38-145.
- Chun, J. Unpublished data.
- Chun, J., L. L. Blackall, and M. Goodfellow. Unpublished data.
- Chun, J., and M. Goodfellow. Unpublished data.
- Collins, M. D., O. W. Howarth, E. Grund, and R. M. Kroppenstedt. 1987. Isolation and structural determination of new members of the vitamin K2 series in *Nocardia brasiliensis*. *FEMS Microbiol. Lett.* **41**:35-39.
- DeLong, E. F., G. S. Wickham, and N. R. Pace. 1989. Phylogenetic strains: ribosomal RNA-based probes for the identification of single microbial cells. *Science* **243**:1360-1363.
- Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**:783-791.
- Felsenstein, J. 1993. PHYLIP (phylogenetic inference package) version 3.5c. Department of Genetics, University of Washington, Seattle.
- Fitch, W. M., and E. Margoliash. 1967. Construction of phylogenetic trees: a method based on mutation distances as estimated from cytochrome c sequences is of general applicability. *Science* **155**:279-284.
- Goodfellow, M. 1992. The family *Nocardiaceae*, p. 1188-1213. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), *The prokaryotes*, 2nd ed. Springer Verlag, New York.
- Goodfellow, M. Actinomycetes: *Actinomycetes*, *Actinomadura*, *Nocardia*, *Streptomyces* and related taxa. In J. P. Collee, J. P. Duguid, A. G. Fraser, B. P.

- Marmion, and A. Simmons (ed.), Mackie and McCartney practical medical microbiology, 14th ed., in press. Churchill Livingstone, Edinburgh.
20. Goodfellow, M., J. Chun, S. Stubbs, and A. S. Toboli. 1994. Transfer of *Nocardia amarae* Lechevalier and Lechevalier to the genus *Gordona* as *Gordona amarae* comb. nov. Lett. Appl. Microbiol. 19:401–405.
 21. Goodfellow, M., and M. P. Lechevalier. 1989. Genus *Nocardia* Trevisan 1889, 9^{AL}, p. 2350–2361. In S. T. Williams, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 4. The Williams & Wilkins Co., Baltimore.
 22. Gordon, R. E., and J. M. Milm. 1962. Identification of *Nocardia caviae* comb. nov. Ann. N. Y. Acad. Sci. 98:628–639.
 23. Gordon, R. E., S. K. Mishra, and D. A. Barnett. 1978. Some bits and pieces of the genus *Nocardia*: *N. carneae*, *N. vaccinii*, *N. transvalensis*, *N. orientalis* and *N. aerocolonigenes*. J. Gen. Microbiol. 109:69–78.
 24. Heery, D. M., F. Gannon, and R. Powell. 1990. A simple method for subcloning DNA fragments from gel slices. Trends Genet. 6:173.
 25. Howarth, O. W., E. Grund, R. M. Kroppenstedt, and M. D. Collins. 1986. Structural determination of a new naturally occurring cyclic vitamin K. Biochem. Biophys. Res. Commun. 140:916–923.
 26. Jukes, T. H., and C. R. Cantor. 1969. Evolution of protein molecules, p. 21–132. In H. N. Munro (ed.), Mammalian protein metabolism. Academic Press, New York.
 27. Kämpfer, P., W. Dott, and R. M. Kroppenstedt. 1990. Numerical classification and identification of some nocardioform bacteria. J. Gen. Appl. Microbiol. 36:309–331.
 28. Kudo, T., K. Hatai, and A. Seino. 1988. *Nocardia seriolae* sp. nov. causing nocardiosis of cultured fish. Int. J. Syst. Bacteriol. 38:173–178.
 29. Lane, D. J. 16S/23S rRNA sequencing, p. 115–175. In E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons, Chichester, United Kingdom.
 30. Larsen, N., G. J. Olsen, B. L. Madaid, M. J. McCaughey, R. Overbeek, T. J. Macke, T. L. Marsh, and C. R. Woese. 1993. The Ribosomal Database Project. Nucleic Acids Res. 21:3021–3023.
 31. Lechevalier, M. P. 1976. The taxonomy of the genus *Nocardia*, p. 1–38. In M. Goodfellow, G. H. Brownell, and J. A. Serrano (ed.), The biology of the nocardiae. Academic Press, London.
 32. Lechevalier, M. P., and H. A. Lechevalier. 1970. A critical evaluation of the genera of aerobic actinomycetes, p. 393–405. In H. Prauser (ed.), The Actinomycetales. Gustav-Fischer Verlag, Jena, Germany.
 33. Lechevalier, M. P., and H. A. Lechevalier. 1974. *Nocardia amarae* sp. nov., an actinomycete common in foaming activated sludge. Int. J. Syst. Bacteriol. 24:278–288.
 34. Lehmann, K. B., and R. Neumann. 1986. Atlas und Grundriss der Bakteriologie und Lehrbuch der speziellen bakteriologischen Diagnostik, Teil II. J. F. Lehmann, Munich.
 35. McNeil, M., and J. M. Brown. 1994. The medically important actinomycetes: epidemiology and microbiology. Clin. Microbiol. Rev. 7:357–417.
 36. McNeil, M., J. M. Brown, P. R. Georgioui, A. M. Allworth, and Z. M. Blacklock. 1992. Infections due to *Nocardia transvalensis*: clinical spectrum and antimicrobial therapy. Clin. Infect. Dis. 15:453–463.
 37. Minnikin, D. E., A. G. O'Donnell, M. Goodfellow, G. Alderson, M. Athalye, A. Schaal, and J. H. Parlett. 1984. An integrated procedure for the extraction of isoprenoid quinones and polar lipids. J. Microbiol. Methods 2:233–241.
 38. Mirza, S. H., and C. Campbell. 1994. Mycetoma caused by *Nocardia transvalensis*. J. Clin. Pathol. 47:85–86.
 39. Mordarska, H., M. Mordarski, and M. Goodfellow. 1972. Chemotaxonomic characters and classification of some nocardioform bacteria. J. Gen. Microbiol. 71:77–86.
 40. Mordarski, M., K. P. Schaal, K. Szyba, G. Pulverer, and A. Tkacz. 1977. Interrelation of *Nocardia asteroides* and related taxa as indicated by deoxyribonucleic acid reassociation. Int. J. Syst. Bacteriol. 27:66–70.
 41. Mordarski, M., K. P. Schaal, A. Tkacz, G. Pulverer, K. Szyba, and M. Goodfellow. 1978. Deoxyribonucleic acid base composition and homology studies on *Nocardia*. Zentralbl. Bakteriologie. Suppl. 6:91–97.
 42. Neefs, J.-M., Y. V. de Peer, P. De Rijk, S. Chapelle, and R. De Wachter. 1993. Compilation of small ribosomal subunit RNA structures. Nucleic Acids Res. 21:3025–3049.
 43. Orchard, V. A., and M. Goodfellow. 1980. Numerical classification of some named strains of *Nocardia asteroides* and related isolates from soil. J. Gen. Microbiol. 118:295–312.
 44. Pitcher, D. G., N. A. Saunders, and R. J. Owen. 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. 8:151–156.
 45. Pitulle, C., M. Dorsch, J. Kazda, J. Wolters, and E. Stackebrandt. 1992. Phylogeny of rapidly growing members of the genus *Mycobacterium*. Int. J. Syst. Bacteriol. 42:337–343.
 46. Ridell, M. 1975. Taxonomic study of *Nocardia farcinica* using serological and physiological characters. Int. J. Syst. Bacteriol. 25:124–132.
 47. Rogall, T., J. Wolters, T. Flohr, and E. C. Böttger. 1990. Towards a phylogeny and definition of species at the molecular level within the genus *Mycobacterium*. Int. J. Syst. Bacteriol. 40:323–330.
 48. Ruimy, R., P. Boiron, V. Boivin, and R. Christen. 1994. A phylogeny of the genus *Nocardia* deduced from the analysis of small-subunit ribosomal DNA sequences, including transfer of *Nocardia amarae* to the genus *Gordona* as *Gordona amarae* comb. nov. FEMS Microbiol. Lett. 123:261–268.
 49. Saitou, N., and M. Nei. 1987. The neighbor joining method: a new method for constructing phylogenetic trees. Mol. Biol. Evol. 4:406–425.
 50. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 51. Schaal, K. P., and H. J. Lee. 1992. Actinomycete infections in humans—a review. Gene 151:201–211.
 52. Schaal, K. P., and H. Reutersberg. 1978. Numerical taxonomy of *Nocardia asteroides*, p. 53–62. In M. Mordarski, W. Kurylowicz, and J. Jeljaszewicz (ed.), *Nocardia* and *Streptomyces*. Gustav-Fischer Verlag, Stuttgart, Germany.
 53. Stahl, D. A., and J. W. Urbance. 1990. The division between fast- and slow-growing species corresponds to natural relationships among the mycobacteria. J. Bacteriol. 172:116–124.
 54. Tsukamura, M. 1969. Numerical taxonomy of the genus *Nocardia*. J. Gen. Microbiol. 56:265–287.
 55. 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.
 56. 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.
 57. Yang, D., and C. R. Woese. Unpublished data.
 58. Yano, I., T. Imaeda, and M. Tsukamura. 1990. Characterization of *Nocardia nova*. Int. J. Syst. Bacteriol. 40:170–174.