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Recognition of a *Nocardia transvalensis* Complex by Resistance to Aminoglycosides, Including Amikacin, and PCR-Restriction Fragment Length Polymorphism Analysis

REBECCA W. WILSON,[1] VINCENT A. STEINGRUBE,[2]* BARBARA A. BROWN,[2] ZETA BLACKLOCK,[3] KENNETH C. JOST, JR.,[4] ALAN MCNABB,[5] W. DAVID COLBY,[5] JON R. BIEHLE,[6] JEREMY L. GIBSON,[2] AND RICHARD J. WALLACE, JR.[1,2]

Center for Pulmonary and Infectious Disease Control[1] and the Department of Microbiology,[2] The University of Texas Health Center at Tyler, Tyler, Texas; State Health Laboratory, Tuberculosis Section, Brisbane, Queensland, Australia[3]; the Texas Department of Health, Austin, Texas[4]; Department of Microbiology, University Campus, London Health Sciences Center, London, Ontario, Canada[5]; and Department of Pathology, Creighton University Medical Center, Omaha, Nebraska[6]

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Amikacin resistance, rare among nocardiae, was observed in 58 clinical isolates of nocardiae. All of these isolates hydrolyzed hypoxanthine, and 75 to 100% utilized citrate, D-galactose, and D-trehalose as sole carbon sources. Based on utilization of I-erythritol, D-glucitol, i-*myo*-inositol, D-mannitol, and ribitol and susceptibility to amoxicillin-clavulanic acid, the 58 isolates were separable into four groups. One group was negative for I-erythritol and ribitol and included all the isolates belonging to *Nocardia asteroides* complex antibiogram type IV. The remaining three groups were positive for I-erythritol and ribitol and were grouped within *Nocardia transvalensis*. The group that included the type strain was designated *N. transvalensis* sensu stricto, and the other two groups were designated new taxons 1 and 2. PCR-restriction fragment length polymorphism (RFLP) analysis of a 439-bp segment of the 65-kDa heat shock protein gene with *Xho*I and *Hin*fI produced identical patterns for 53 (91%) and 58 (100%) isolates, respectively, and differentiated them from all other *Nocardia* taxa. *Nar*I- and *Hae*III-derived RFLP patterns clearly differentiated each of the four biochemically defined taxa. These four groups were also distinguishable by using the chromogenic substrates in Dade MicroScan test panels. By high-performance liquid chromatography, these isolates exhibited the same unique mycolic acid-ester elution patterns that differed from those of all other clinically significant nocardiae. Gas-liquid chromatographic analysis of fatty acids also produced similar patterns for all isolates that distinguished them from all other *Nocardia* taxa, but did not differentiate the four taxa within the complex. We propose the designation *N. transvalensis* complex for these four groups of nocardiae, pending further genetic evaluation.

The taxonomy of nocardiae, after years of confusion and a paucity of satisfactory phenotypic tests, is undergoing an evolution. *Nocardia nova*, *N. farcinica*, and, most recently, *N. pseudobrasiliensis* are now established species, although they are still not readily identifiable in most clinical laboratories (6, 17, 19). Interest in the nocardiae and their taxonomy has risen as a result of the apparent increase in the occurrence of nocardioses. This increase has been due, in part, to the increase in the proportion of immune-suppressed individuals in the population, particularly those with advanced human immunodeficiency virus disease, who are at greater risk for such infections (2, 6, 12, 13). *N. transvalensis* is a relatively rare species clinically, and it has not been thoroughly evaluated by newer taxonomic techniques that include antimicrobial susceptibility patterns and PCR-restriction fragment length polymorphism (RFLP) analysis (7, 10, 12, 13, 17, 18, 23, 24). In earlier studies, nocardial isolates of antibiogram type IV within the *N. asteroides* complex and most isolates of *N. transvalensis* have demonstrated amikacin resistance, a rare phenotype among isolates of *Nocardia* (12, 17, 24, 25). Previous studies of nocardiae by PCR-RFLP analysis protocols with either *Msp*I and *Bsa*HI (17) or *Msp*I, *Hin*fI, and *Bsa*HI (19) have not resulted in a clear-cut differentiation of *N. asteroides* complex antibiogram type IV isolates and those of *N. transvalensis*. This study was therefore undertaken to apply these newer identification methodologies to an expanded population of amikacin-resistant nocardial isolates in an effort to better define the species *N. transvalensis.*

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MATERIALS AND METHODS

Organisms. This study included clinical isolates and strains of nocardiae from the American Type Culture Collection (ATCC; Rockville, Md) (Table 1). The clinical isolates were selected from those submitted for susceptibility testing to (i) one of the authors (R.J.W.) while in Houston, Texas, between 1976 and 1982; (ii) the Mycobacteria/Nocardia Laboratory at the University of Texas Health Center at Tyler (UTHCT) between 1982 and the present; and (iii) the State Health Laboratory, Tuberculosis Section, Brisbane, Queensland, Australia, between 1985 and the present.

Biochemical, growth, and aminoglycoside resistance characteristics. The clinical isolates of nocardiae chosen for this study were initially selected on the basis of resistance to amikacin and other aminoglycosides including gentamicin, kanamycin, and tobramycin by methods detailed in the section below (24). Isolates identified biochemically as *N. transvalensis* were also included for study, irrespective of their aminoglycoside susceptibilities. Isolates were then tested for the following phenotypic characteristics: (i) resistance to lysozyme; (ii) the ability to hydrolyze hypoxanthine but not xanthine or casein by the methods of Mishra et al. (15); and (iii) utilization of citrate, D-galactose, and D-trehalose, but not L-rhamnose, by the methods of Tsukamura (21).

Isolates were identified as *N. asteroides* complex antibiogram type IV if they met the four criteria presented above together with the inability to utilize I-erythritol or ribitol (adonitol) as sole carbon sources. Isolates were identified as

* Corresponding author. Mailing address: Department of Microbiology, The University of Texas Health Center at Tyler, P.O. Box 2003, Tyler, TX 75710-2003. Phone: (903) 877-7685. Fax: (903) 877-7652.

TABLE 1. Clinical and reference isolates of the *N. transvalensis* complex used in this study

Species or taxa	Total no. of isolates	No. of clinical isolates[a]		ATCC reference isolates	
		United States[b]	Australia[b]	United States[b]	Australia[b]
N. asteroides complex type IV[c]	31	29	0	49872	
				49873	
N. transvalensis					
Sensu stricto	10	4	4	6865[T]	
				29982	
New taxon 1	8	1	5	700035	700034
New taxon 2	9	9	0		
Total	58	43	9	5	1

[a] The total number of clinical nocardial isolates screened was 1,840, including 1,481 from the United States and 359 from Australia. Only 10 of 15 Australian isolates of the *N. transvalensis* complex were available for this study.
[b] Designates country of origin of clinical isolates and reference strains.
[c] Described by Wallace et al. (24).

N. transvalensis by the same four initial characteristics, together with their ability to utilize I-erythritol and ribitol.

In an effort to further differentiate these isolates, additional tests were performed. These included tests for the utilization of D-glucitol (D-sorbitol), i-*myo*-inositol, and D-mannitol as sole carbon sources; hydrolysis of tyrosine; and hydrolysis of the chromogenic nitrophenyl- and β-naphthylamide-labeled substrates in the Rapid Anaerobe Identification (ANA) and HNID (*Haemophilus-Neisseria*) Dade MicroScan panels (Dade MicroScan, Inc., West Sacramento, Calif.) (3).

Susceptibility testing. Susceptibility testing by the broth microdilution method was performed in Mueller-Hinton broth with amoxicillin-clavulanic acid and amikacin as described previously (23, 24). An MIC of ≥16 μg/ml was defined as the breakpoint indicating resistance of these isolates to amoxicillin-clavulanic acid and amikacin. Disk diffusion susceptibility testing was performed with amikacin, gentamicin, kanamycin, and tobramycin as described previously (23). A zone size of ≤10 mm was defined as the breakpoint indicating resistance to gentamicin and tobramycin, while that for amikacin and kanamycin was defined as ≤20 mm after 72 h of incubation at 35°C.

HPLC. Nocardial isolates were cultured on brain heart infusion agar slants at 37°C until mature. A loopful of growth was harvested, processed for mycolic acid-*p*-bromophenacyl-esters, and analyzed by high-performance liquid chromatography (HPLC) (4, 5), as modified by Jost and Dunbar (9). In steps involving evaporation, a Rapid-Vap model 79000-02 (Labconco, Kansas City, Mo.) evaporation system was used at 85°C to dry the samples. A synthetic high-molecular-weight mycolic acid surrogate (Ribi ImmunoChem Research, Hamilton, Mont.) was added to each sample prior to analysis as an internal standard.

Fatty acid analysis. Cells of nocardial isolates were harvested from tryptic soy agar plates after 96 h of incubation at 28°C, and extracts were prepared by standard methods (11, 16). Gas-liquid chromatographic analyses were carried out on a 5890 series II (Hewlett-Packard, Avondale, Pa.) gas chromatograph fitted with a Hewlett-Packard Ultra 2 capillary column and a flame ionization detector by the methods of Smid and Salfinger (16). The Microbial Identification System (MIS; Microbial ID, Newark, Del.) was used for the calibration of standards and the development of MIS identification libraries and similarity values as described previously (11, 16).

PCR amplification. Nocardial DNA was prepared from cells harvested from tryptic soy agar cultures, and a 439-bp segment of the 65-kDa heat shock protein (HSP) gene was amplified from ground cell supernatants by PCR as described previously (17) by using the appropriate positive and negative controls, according to a modification of the method of Telenti et al. (20).

RFLP analysis. Commercially available restriction endonucleases (New England Biolabs, Beverly, Mass., and Promega, Madison, Wis.) were screened for those that produced optimal species- and subgroup-specific RFLP band patterns by incubation at the appropriate temperatures and with the buffers recommended by the manufacturers, with the exception of the temperatures and buffers for *Bsa*HI. To achieve complete digestion with *Bsa*HI, acetylated bovine serum albumin was substituted for bovine serum albumin, and the digestion mixture was incubated at 60°C for 1 h.

Restriction fragments were electrophoresed on 3% Metaphor agarose (4-bp resolution; FMC Bioproducts, Rockland, Maine) containing ethidium bromide (0.625 μg/ml) in a Mini-Sub-Cell electrophoresis system (Bio-Rad, Richmond, Calif.) at 95 V for 1.5 to 2.0 h. Fragment sizes (in base pairs) were estimated on a computerized Bio Image system (Millipore, Bedford, Mass.). Fragments of ≤60 bp were disregarded as PCR primer artifacts, as discussed by Telenti et al. (20).

RESULTS

Organisms. Fifty-six clinical nocardial isolates that met the selected biochemical and antibiogram criteria were identified (15, 17, 24). Thirty-one isolates of *N. asteroides* complex antibiogram type IV were identified from clinical samples originating in the United States. These isolates included 31 of the 1,482 (2.1%) clinical nocardial isolates submitted to the two Texas laboratories over the past 20-year period. No isolates belonging to this group were recovered in the Queensland, Australia, laboratory. Twenty-four isolates of *N. transvalensis* were identified; 14 of these were from the United States and represented 0.9% of the 1,482 clinical isolates evaluated. The remaining 10 isolates came from a total of 15 isolates that represented 4.2% of 359 clinical nocardial isolates identified in the Queensland, Australia, laboratory over the past 11-year period.

Four of the six ATCC reference strains studied had been selected from the clinical isolates described above and submitted to ATCC as examples of two of the four taxa identified in this study. These were ATCC 49872, ATCC 49873, ATCC 700034, and ATCC 700035 (Table 1). The remaining two strains were isolates of *N. transvalensis* (including ATCC 29982 and the type strain, ATCC 6865), kindly provided by P. Pienta of ATCC.

Biochemical, growth, and drug susceptibility characteristics. Of the 52 clinical isolates and 6 ATCC reference strains, 52 (89.7%) were resistant to amikacin according to the MIC (Table 2), a unique characteristic among nocardiae (12, 17, 24). A similar number of isolates were also resistant to the other aminoglycosides. Resistance rates were 9 of 9 by MIC determinations and 28 of 29 (97%) by the disk diffusion method for gentamicin, 9 of 9 by MIC determinations and 30 of 30 (100%) by the disk diffusion method for kanamycin, and 5 of 5 by MIC determinations and 28 of 28 (100%) by the disk diffusion method for tobramycin. These isolates utilized citrate, D-galactose, and D-trehalose, did not utilize L-rhamnose, and hydrolyzed hypoxanthine.

Thirty-one isolates were identified as belonging to *N. asteroides* complex type IV and exhibited the following phenotypic characteristics: (i) no (0%) isolates hydrolyzed tyrosine or utilized I-erythritol, i-*myo*-inositol, or ribitol; (ii) 1 of 31 (3%) isolates utilized D-glucitol and D-mannitol; (iii) for 4 of 31 (13%) isolates amoxicillin-clavulanic acid MICs were >8 μg/ml (resistant) (Table 2); (iv) for 30 of 31 (97%) isolates MICs were ≥16 μg/ml (resistant) to amikacin and 27 of 28 (96%) isolates were resistant to amikacin by the disk diffusion method. All type IV isolates were resistant to amikacin by at least one of the two methods.

With the exception of one isolate negative for I-erythritol utilization, all *N. transvalensis* isolates grew and produced acid from I-erythritol and ribitol, characteristics that differentiated them from isolates of *N. asteroides* complex type IV. On the basis of the utilization of D-glucitol, i-*myo*-inositol, and D-mannitol and resistance to amoxicillin-clavulanic acid, *N. transvalensis* isolates could be divided into three subgroups. Ten isolates, including the ATCC 6865 type strain, that utilized i-*myo*-inositol, that were variable in their utilization of D-glucitol and hydrolysis of tyrosine, and that were all resistant to amoxicillin-clavulanic acid were designated *N. transvalensis* sensu stricto. These 10 isolates were all resistant to amikacin by

TABLE 2. Biochemical and antibiogram characteristics of the *N. transvalensis* complex subgroups

Test	Test result for the following isolate groups[a]:									
	N. asteroides complex type IV[b]			*N. transvalensis*						
				Sensu stricto			New taxon 1			
	% of 31 isolates	ATCC 49872	ATCC 49873	% of 10 isolates	ATCC 6865^T	ATCC 29982	% of 8 isolates	ATCC 700034	ATCC 700035	% of 9 new taxon 2 isolates
Utilization of the following:										
Citrate	100	+	+	100	+	+	75	+	+	78
D-Galactose	94	+	+	100	+	+	88	+	−	89
D-Trehalose	100	+	+	90	+	−	100	+	+	100
L-Rhamnose	0	−	−	0	−	−	0	−	−	0
I-Erythritol	0	−	−	100	+	+	100	+	+	89
Ribitol (adonitol)	0	−	−	100	+	+	100	+	+	100
D-Mannitol	3	−	−	80	+	+	100	+	+	11
D-Glucitol (D-sorbitol)	3	−	−	90	+	+	100	+	+	0
i-*myo*-Inositol	0	−	−	100	+	+	0	−	−	0
Hydrolysis of the following:										
Hypoxanthine	100	+	+	90	+	+	100	+	+	100
Tyrosine	0	−	−	40	−	−	38	−	−	0
Resistance to the following:										
Aminoglycosides										
Amikacin										
MICs	97	+	+	100	+	+	88	+	+	67
30-μg disk	96	+	+	100	+	+	75	−	+	67
Gentamicin (10-μg disk)	96	+	+	80	+	+	88	+	+	100
Kanamycin (30-μg disk)	100	+	+	100	+	+	100	+	+	100
Tobramycin (10-μg disk)	100	+	+	100	+	+	88	+	+	100
Amox/clav acid (MICs)[c]	13	−	+	100	+	+	13	−	−	0

[a] Numbers, percentage of isolates positive for each test: +, positive reaction or drug resistant; −, negative reaction or absence of drug resistance.
[b] Described by Wallace et al. (24).
[c] Amox/clav acid, amoxicillin-clavulanic acid.

MIC determinations, and 75% were resistant by the disk diffusion method.

Isolates of the other two subgroups were considered new taxa and were designated new taxon 1 ($n = 8$) and new taxon 2 ($n = 9$). They were susceptible, with the exception of one of eight (13%) new taxon 1 isolates, to amoxicillin-clavulanic acid, did not utilize i-*myo*-inositol, and were separated from each other on the basis of differences in utilization of D-glucitol and D-mannitol and variable hydrolysis of tyrosine, as indicated in Table 2. Seven of eight (88%) of the new taxon 1 isolates were resistant to amikacin by MIC determinations and three of four (75%) were resistant to amikacin by the disk diffusion method. Six of nine (67%) new taxon 2 isolates were resistant to amikacin by MIC determinations and six of nine (67%) were resistant to amikacin by the disk diffusion method.

Isolates of *N. transvalensis* sensu stricto and the new taxon 1 were clearly differentiated from one another and from those of *N. asteroides* complex type IV and the new taxon 2 by reactions with ρ-nitrophenyl-α-D-mannopyranoside (MNP), glycylglycine-β-naphthylamide (GGLY), and *o*-nitrophenyl-β-D-galactoside (GAL) (Table 3). These three chromogenic substrates together with 3-indoxyl phosphate (IDX) and L-pyrrolidonyl-

TABLE 3. MicroScan panel chromogenic substrate hydrolysis characteristics of the *N. transvalensis* complex subgroups

Chromogenic substrate	Test result for the following isolate groups[a]:									
	N. asteroides complex type IV[b]			*N. transvalensis*						
				Sensu stricto			New taxon 1			
	% of 31 isolates	ATCC 49872	ATCC 49873	% of 10 isolates	ATCC 6865^T	ATCC 29982	% of 8 isolates	ATCC 700034	ATCC 700035	% of 9 new taxon 2 isolates
MNP	0	−	−	100	+	+	0	−	−	0
GGLY	46	−	+	0	−	−	100	+	+	44
PYR	0	−	−	0	−	−	0	−	−	0
IDX	0	−	−	0	−	−	0	−	−	0
GAL	18	−	−	0	−	−	0	−	−	56

[a] Numbers, percentage of isolates positive for each test; +, positive reaction; −, negative reaction.
[b] Described by Wallace et al. (24).

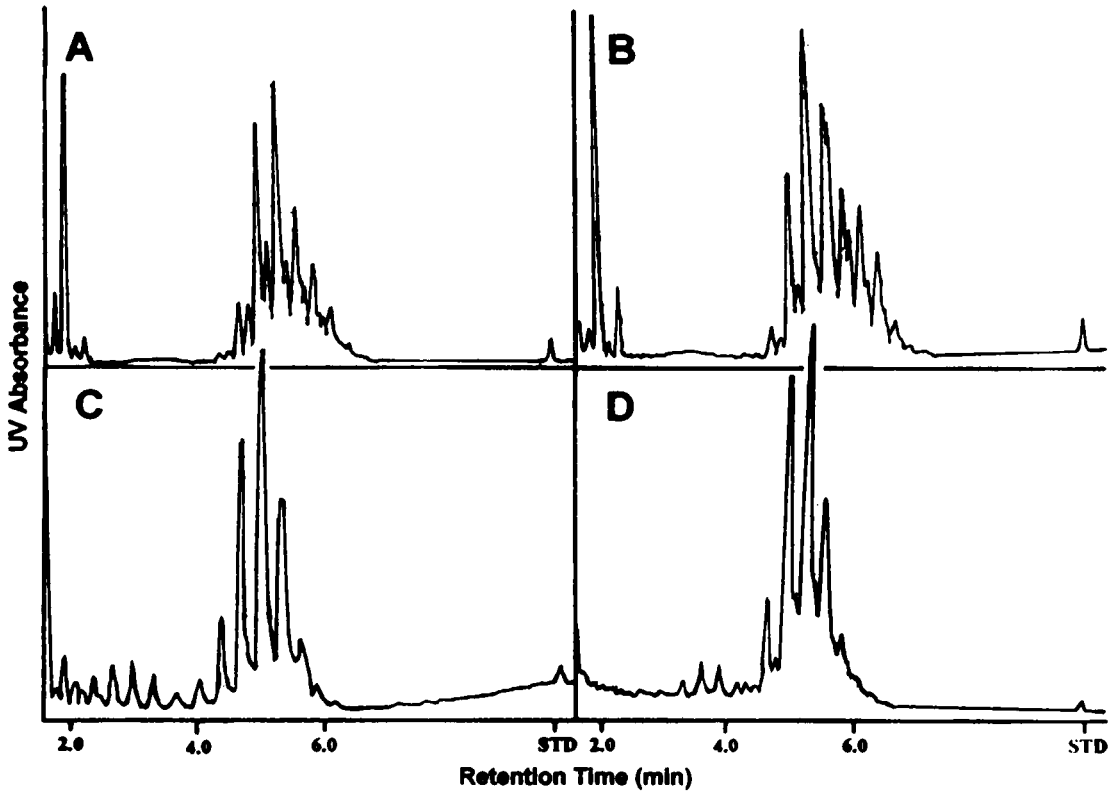

FIG. 1. Comparison of HPLC-generated mycolic acid-*p*-bromophenacyl-ester elution patterns from isolates of the *N. transvalensis* complex with those of other *Nocardia* species. (A) *N. transvalensis* sensu stricto ATCC 6865. (B) *N. asteroides* complex antibiogram type IV isolate ATCC 49872. (C) *N. farcinica* ATCC 3318. (D) *N. nova* ATCC 33727. STD represents the internal standard peak that eluted at approximately 9.55 min.

β-naphthylamide (PYR) also differentiated strains of the *N. transvalensis* complex from other previously studied *Nocardia* species except *N. asteroides* sensu stricto (ATCC 19247 type strain) (3).

HPLC analysis. All 58 isolates belonging to the *N. transvalensis* complex produced mycolic acids. By HPLC analysis, these isolates demonstrated mycolic acid patterns that were characterized by two sets of major peaks. The first and generally largest peak eluted between 1.85 and 1.95 min. Typically, no peaks or only minor peaks eluted between 2.5 and 4.5 min. The second set of major peaks, consisting of four to six individual peaks, eluted between 5.0 and 7.0 min. These mycolic acid-ester HPLC elution patterns were similar for all isolates of the *N. transvalensis* complex and clearly distinguished them from isolates of all other commonly occurring *Nocardia* taxa including *N. brasiliensis*, *N. farcinica*, and *N. nova*. A comparison of HPLC-generated mycolic acid-*p*-bromphenacyl-ester elution patterns from isolates of the *N. transvalensis* complex with those from isolates of *N. farcinica* and *N. nova* is presented in Fig. 1.

Fatty acid analysis. As reported previously (11), considerable heterogeneity was observed for the fatty acid profiles of the *N. transvalensis* isolates. *N. transvalensis* ATCC 6865, ATCC 29982, ATCC 700034, and ATCC 700035, as well as the new taxon 2 clinical isolate N 897 and the *N. asteroides* complex type IV strain ATCC 49873, could be differentiated on the basis of their fatty acid compositions and were used to produce library entries. Differentiation of some of the clinical isolates was possible by using these library entries, but incorrect identifications occurred and it was not possible to reliably distinguish the four taxa within the complex. The fatty acid profiles for isolates of this group are very similar and clearly separate them from all other clinically significant *Nocardia* taxa, as indicated in the dendrogram in Fig. 2.

PCR amplification and RFLP analysis. Satisfactory amplicons were readily obtained from all test isolates. Screening of 30 restriction endonucleases in previous studies (17, 19) resulted in the selection of seven enzymes, *Bsa*HI, *Hae*III, *Hin*fI, *Msp*I, *Nae*I, *Nar*I, and *Xho*I, for use in further characterization of these isolates. As observed previously (17–19), restriction fragment sizes were consistent within each taxonomic group, exhibiting standard deviations of ≤2 bp on the same gel and ≤4 bp on multiple gels. Since agarose with a 4-bp resolution (3% Metaphor) was used, the restriction fragment sizes given in Fig. 3 were rounded to the nearest 5 bp, as recommended by Telenti et al. (20).

*Xho*I and *Hin*fI digests of PCR amplicons produced two very similar RFLP patterns from 53 (91.4%) and 58 (100%) isolates, respectively, (Fig. 3 and 4A and B). The remaining five isolates, including three isolates of *N. transvalensis* sensu stricto and two isolates of the new taxon 2 that failed to exhibit the common *Xho*I-derived pattern, produced amplicons devoid of *Xho*I recognition sites (Fig. 4A, lane 3). The *Hin*fI band pattern by RFLP analysis was the same as that observed in a previous study with isolates of the *N. asteroides* complex type IV and *N. transvalensis* (19). Isolates of the two new taxa and *N. asteroides* complex type IV (lanes 4 to 6, 9 to 11, and 12 to 14, respectively, in Fig. 4C and 5A and B) also produced common band patterns by RFLP analysis with three other enzymes: *Msp*I, *Bsa*HI, and *Nar*I (Fig. 4C, and 5A and B, respectively). The *Msp*I- and *Bsa*HI-derived patterns were the same as those reported previously for isolates of *N. asteroides*

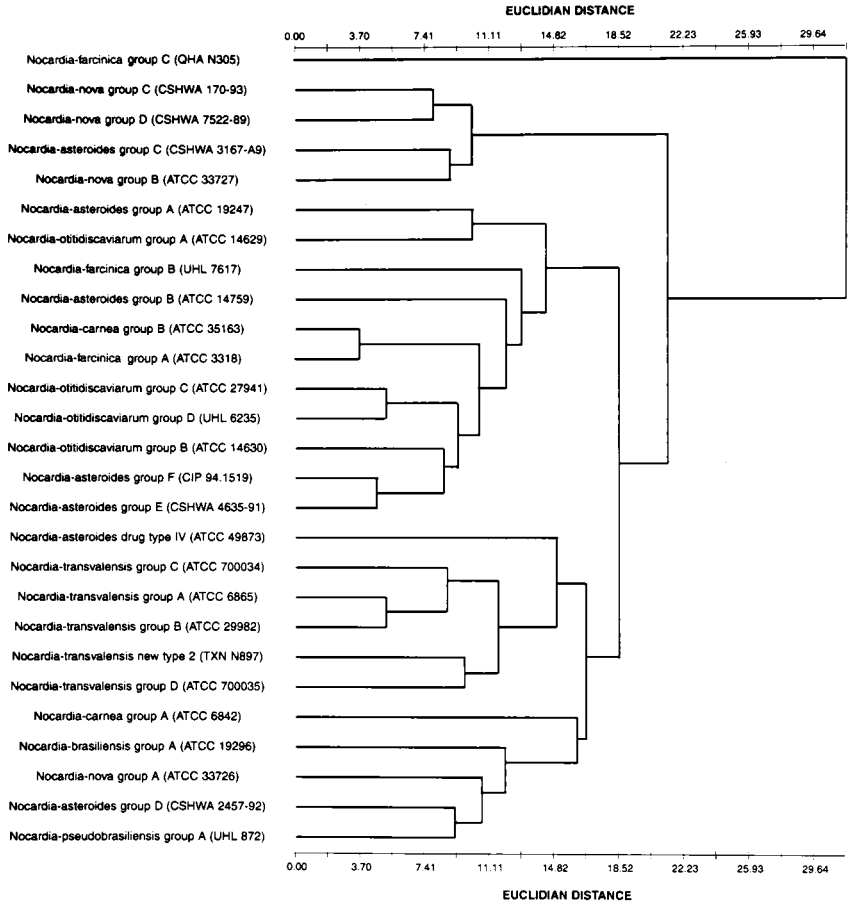

FIG. 2. Dendrogram representing phylogenetic clustering of isolates of the *N. transvalensis* complex based on fatty acid analysis by gas-liquid chromatography and evaluation by the MIS. The dendrogram is based on the cellular fatty acid compositions of *Nocardia* species grown on Trypticase soy agar for 4 days at 28°C.

complex type IV (17, 19). *N. transvalensis* sensu stricto isolates exhibited multiple band patterns by RFLP analysis with *Msp*I and *Bsa*HI, including one pattern with *Msp*I that was common to that of the other members of the complex (Fig. 4C and 5A, lanes 1 to 3). Likewise, *Nae*I produced band patterns by RFLP analysis that included a common pattern of 270- and 180-bp bands for isolates of the former three subgroups and multiple patterns for isolates of *N. transvalensis* sensu stricto (data not shown).

The four biochemically definable subgroups of nocardiae

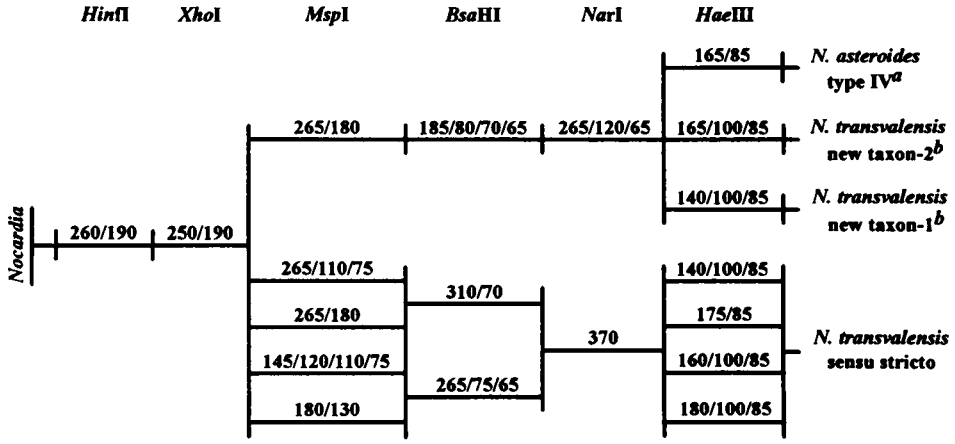

FIG. 3. Practical schematic illustrating the similarities and differences between each of the biochemically and antibiogram-defined subgroups of the *N. transvalensis* complex by restriction endonuclease analysis of a PCR-amplified 65-kDa HSP gene sequence. RFLP band values are expressed as the number of nucleotide base pairs rounded to the nearest 5 bp, as described in the text. *a*, *N. asteroides* complex antibiogram type IV described by Wallace et al. (24); *b*, new taxon within *N. transvalensis* described in the text.

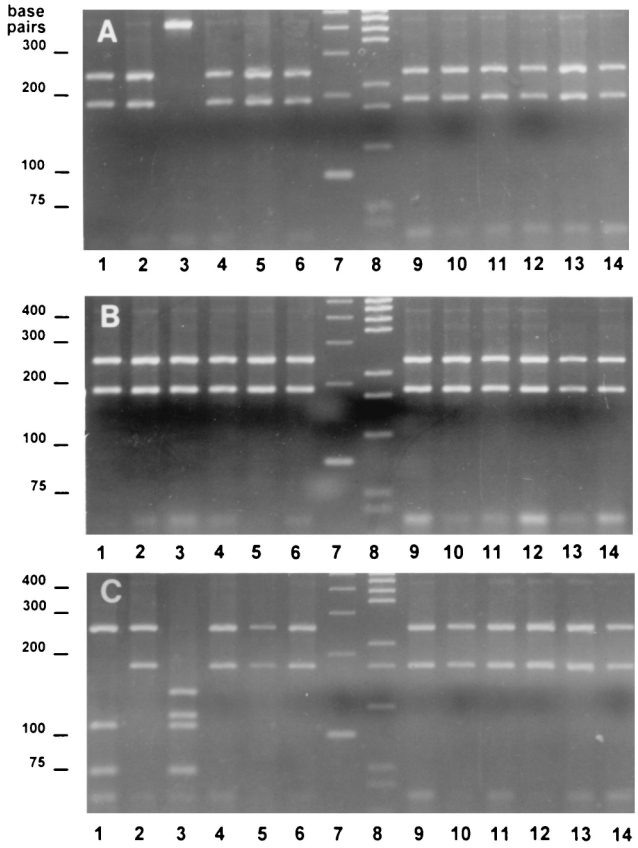

FIG. 4. RFLP band patterns from *Xho*I, *Hin*fI, and *Msp*I digests of PCR-amplified sequences of the 65-kDa HSP gene. (A) *Xho*I digests. Lanes 1 to 3, *N. transvalensis* sensu stricto isolates ATCC 6865, N 1429, and N 1085, respectively; lanes 4 to 6, new taxon 1 isolates ATCC 700034, ATCC 700035, and N 1114, respectively; lanes 7 and 8, size markers (100-bp and pGEM base pair ladders, respectively); lanes 9 to 11, new taxon 2 isolates N 330, N 814, and N 1173, respectively; lanes 12 to 14, *N. asteroides* complex antibiogram type IV isolates ATCC 49872, ATCC 49873, and N 1435, respectively. (B) *Hin*fI digests. Lane designations are the same as those for panel A. (C) *Msp*I digests. Lane designations are the same as those for panel A.

were clearly distinguishable with *Nar*I and *Hae*III. *Nar*I produced a single, unique pattern for all 10 isolates of *N. transvalensis* sensu stricto (Fig. 5B, lanes 1 to 3) and a common pattern for the other three subgroups (Fig. 5B, lanes 4 to 6, 9 to 11, and 12 to 14, respectively). Although *Hae*III gave multiple patterns for isolates of *N. transvalensis* sensu stricto (Fig. 3 and 5C, lanes 1 to 3), it produced unique patterns for each of the other three subgroups (Fig. 3 and 5C, lanes 4 to 6, 9 to 11, and 12 to 14, respectively). The *Hae*III-derived common band patterns by RFLP analysis were distinctive for all eight isolates of the new taxon 1, 8 of 9 (89%) isolates of the new taxon 2, and all 31 isolates of *N. asteroides* complex type IV. One of nine (11%) new taxon 2 isolates (N 875) produced an RFLP pattern like that of the *N. asteroides* complex type IV isolates.

DISCUSSION

Isolates of *N. transvalensis* and *N. asteroides* complex antibiogram type IV (24) are unique among clinically significant nocardiae in their resistance to amikacin and aminoglycosides in general (1, 12, 14, 17, 24, 25). These two taxa exhibited several common biochemical characteristics, including utilization of citrate, D-galactose, and D-trehalose; hydrolysis of hypoxanthine; and similar mycolic acid-ester HPLC elution patterns. They therefore appeared to represent a family of *Nocardia* taxa that are herein referred to as the *N. transvalensis* complex. Detailed testing of a large population of these isolates (the largest taxonomic study of these isolates to date [12]) led to the observation that four biochemically distinct subgroups appeared to exist within the *N. transvalensis* complex. For the purposes of this study these four subgroups have been designated *N. asteroides* complex antibiogram type IV, *N. transvalensis* sensu stricto, new taxon 1, and new taxon 2.

Few clinical isolates of *N. transvalensis* have been reported (7, 12–14), in part because of the requirement for hypoxanthine hydrolysis and carbohydrate utilization testing (i.e., I-erythritol, i-*myo*-inositol, and ribitol) to clearly separate them from isolates of the *N. asteroides* complex. Clinical laboratories do not routinely run such specialized tests, thereby making accurate diagnosis of these infrequent clinical isolates unlikely (6, 7, 12, 13) unless they are submitted to a regional or national reference laboratory such as the Centers for Disease Control and Prevention. An apparent increase in the frequency of nocardial infections has been reported (2), particularly in association with recent increases in the population of immunocompromised patients (12, 13). The occurrence of clinical iso-

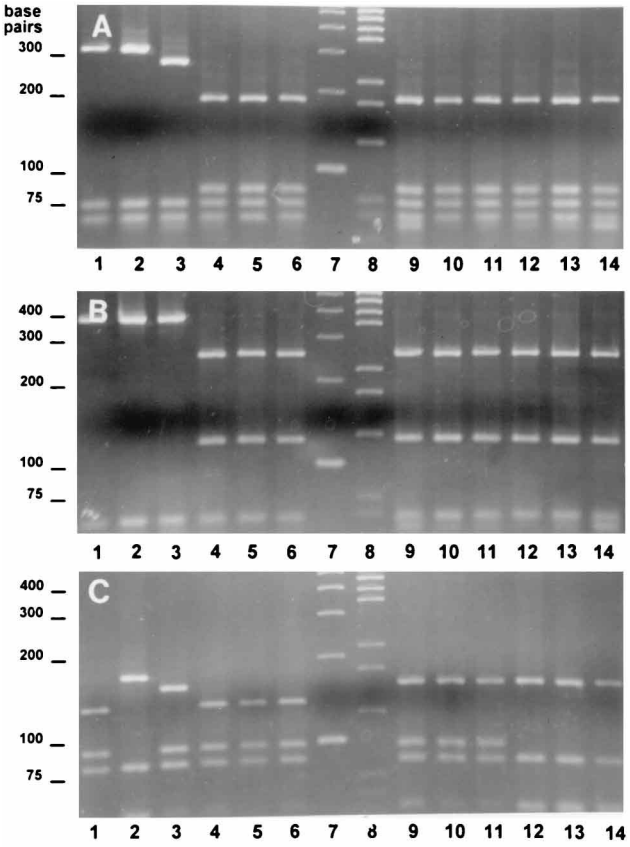

FIG. 5. RFLP band patterns from *Bsa*HI, *Nar*I, and *Hae*III digests of PCR-amplified sequences of the 65-kDa HSP gene. (A) *Bsa*HI digests. Lanes 1 to 3, *N. transvalensis* sensu stricto ATCC 6865, N 1429, and N 1085, respectively; lanes 4 to 6, new taxon 1 isolates ATCC 700034, ATCC 700035, and N 1114, respectively; lanes 7 and 8, size markers (100-bp and pGEM base pair ladders, respectively); lanes 9 to 11, new taxon 2 isolates N 330, N 814, and N 1173, respectively; and lanes 12 to 14, *N. asteroides* complex antibiogram type IV isolates ATCC 49872, ATCC 49873, and N 1435, respectively. (B) *Nar*I digests. Lane designations are the same as those for panel A. (C) *Hae*III digests. Lane designations are the same as those for panel A.

lates of the *N. transvalensis* complex that are inherently resistant to aminoglycosides increases the significance of accurate species (complex) recognition (12). With the increased use and availability of susceptibility testing and attention to drug resistance patterns (17, 22, 24), an increase in the rate of recognition of clinical isolates of *N. transvalensis* and the *N. transvalensis* complex should be possible.

Biehle et al. (3) were able to differentiate five species of *Nocardia* on the basis of the hydrolysis of chromogenic substrates commercially available in Dade MicroScan panels. The three most useful substrates included IDX, MNP, and PYR. *N. transvalensis* sensu stricto was differentiated from *N. asteroides* sensu stricto, *N. brasiliensis*, *N. farcinica*, *N. otitidiscaviarum*, and *N. nova* by reactions with GAL, IDX, and MNP (3). However, the chromogenic substrate hydrolysis patterns for the remaining three taxa of the *N. transvalensis* complex essentially resembled that of *N. asteroides* sensu stricto. Differentiation of the four taxa within the *N. transvalensis* complex was possible with GGLY, MNP, and GAL, although the patterns obtained with *N. asteroides* complex type IV and the new taxon 2 were variable and did not provide an unequivocal differentiation of these two groups.

Minimal phenotypic criteria for the identification of nocardial isolates as *N. transvalensis* have not been established. On the basis of the studies of Gordon et al. (8) and Mishra et al. (15), together with data in the current study, we propose that utilization of both I-erythritol and ribitol (adonitol) as sole carbon sources, hydrolysis of hypoxanthine, and resistance to amikacin be adopted as primary criteria for the identification of *N. transvalensis*. Testing with the chromogenic substrates GAL, IDX, and MNP also provided useful criteria for the identification of *N. transvalensis* (3). All other biochemical tests evaluated produced results that either were variable or were shared with other species or taxa of clinically significant nocardiae. These tests should be conducted by using positive and negative reference strains as controls. Previous studies in which such minimal identification criteria were not adopted may have resulted in the inclusion of nocardial isolates of species or taxa other than *N. transvalensis.*

Several investigators have noted high MICs of amikacin or resistance to amikacin for most (1, 19, 24) but not all (12, 14) isolates identified as *N. transvalensis*. In disk diffusion susceptibility studies, the development of a small zone of inhibition tends to occur with mature growth of nocardial isolates and may be overlooked when incubation periods of less than 72 h are used (23). The results of MIC studies may also be similarly affected. We recommend that amikacin disk susceptibility test results be read at 72 h and that only clear zones around the disk be recorded. This 72-h incubation period for both disk diffusion and MIC studies has been recommended previously (23) because this longer growth period was found to yield results that were clinically significant.

HPLC analysis has been used to detect unique mycolic acid-ester patterns from species of *Mycobacterium* and other aerobic actinomycetes (19). These patterns offer another aid to the differentiation of isolates of the *N. transvalensis* complex from those of other *Nocardia* taxa studied previously (19, 22). Although the identification of *Nocardia* to the species level by HPLC has not been completely developed, differences in HPLC mycolic acid-ester elution patterns have been useful in recognizing a new species or taxon within a previously established taxon (e.g., *N. pseudobrasiliensis* within *N. brasiliensis*) (22).

The dendrogram based on fatty acid analysis by gas-liquid chromatography demonstrated close relatedness between *N. asteroides* complex type IV and *N. transvalensis*, although as reported previously (11), *N. transvalensis* isolates exhibited considerable heterogeneity in their fatty acid profiles. The *N. transvalensis* complex was also found to be separate from other clinically significant taxa of *Nocardia.*

In previously reported studies, species and taxa of clinically significant *Nocardia* as well as those of other aerobic actinomycetes have been rapidly and accurately differentiated by PCR-based protocols involving restriction endonuclease analysis (10, 17–20, 22). Isolates belonging to the *N. transvalensis* complex were clearly separable from all other *Nocardia* and aerobic actinomycete species and taxa, although those of *N. transvalensis* and *N. asteroides* complex type IV were not completely differentiated from one another (17, 19). In this study, PCR-RFLP analysis with *Xho*I and *Hin*fI appeared to further substantiate the existence of a close relationship between these two taxa, thereby justifying their being regarded as an *N. transvalensis* complex. Analysis with *Nar*I clearly differentiated isolates comprising *N. transvalensis* sensu stricto from those of the other three subgroups comprising this complex, namely, *N. asteroides* complex type IV, new taxon 1, and new taxon 2. Further analysis with *Hae*III resulted in the complete separation of *N. asteroides* complex type IV and both new taxa within *N. transvalensis*. Interestingly, *Hae*III digests of *N. transvalensis* sensu stricto amplicons exhibited considerable intraspecies polymorphism, as observed previously with other *Nocardia* species (17, 19). These data correlate with the observed heterogeneity in fatty acid profiles observed for *N. transvalensis* (11).

Striking differences in the geographical distribution of the subgroups within the *N. transvalensis* complex were evident. The 10 Australian isolates included in this study represented only two of the three *N. transvalensis* complex subgroups, including 4 of 10 isolates of *N. transvalensis* sensu stricto and 6 of 8 isolates of the new taxon 1. *N. transvalensis* isolates comprised 15 of 359 (4.2%) clinical nocardial isolates recovered in Queensland, Australia, while all isolates of this taxon received from sources within the United States by the two Texas laboratories represented only 17 of 1,482 (1.1%) of all clinical nocardial isolates submitted for susceptibility testing. These observations correlated very closely with those of similar reports in the literature (7, 12). While no isolates of the *N. asteroides* complex type IV have been identified among the 359 nocardial isolates identified in the Queensland, Australia, laboratory, they represented 4 of 78 (5.1%) clinical *N. asteroides* complex isolates reported by the UTHCT laboratory in a previous study (24) and 31 of 1,482 (2.1%) of all clinical nocardial isolates submitted for susceptibility testing to the two Texas laboratories from sources within the United States.

PCR-based methodologies have been demonstrated to offer clear-cut advantages over traditional methods for the identification of clinical isolates of *Nocardia* and other aerobic actinomycetes (17–20). These methods are particularly valuable for the identification of clinical isolates of the *N. transvalensis* complex that are difficult to recognize by traditional methods and pose potential chemotherapeutic complications arising from their inherent resistance to aminoglycosides and amikacin in particular (12, 17, 24, 25). In the course of this study, isolates of the *N. transvalensis* complex were found to cluster into four biochemically distinct subgroups that were distinguishable by PCR-RFLP analysis. The designation of these subgroups, comprising at least two taxa, as the *N. transvalensis* complex has been recommended, pending more definitive genetic analyses such as 16S rRNA sequencing or DNA-DNA homology studies. In order to facilitate further genetic studies, two isolates, N 444 from the United States and N 1408 from Australia, representing the new taxon 1 have been deposited

with ATCC and are listed as ATCC 700035 and ATCC 700034, respectively. Two clinical isolates of *N. asteroides* complex antibiogram type IV used in this study had been deposited previously with ATCC as ATCC 49872 and ATCC 49873, respectively.

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