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Identification and Subspecific Differentiation of *Mycobacterium scrofulaceum* by Automated Sequencing of a Region of the Gene (*hsp65*) Encoding a 65-Kilodalton Heat Shock Protein

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***Mycobacterium scrofulaceum* is most commonly recovered from children with cervical lymphadenitis, although it also accounts for approximately 2% of the mycobacterial infections in AIDS patients. Species assignment of *M. scrofulaceum* isolates by conventional techniques can be difficult and time-consuming. To develop a strategy for rapid species assignment of these organisms, a 360-bp region of the gene (*hsp65*) encoding a 65-kDa heat shock protein in 37 isolates from diverse sources was sequenced. Eight *hsp65* alleles were identified, and these sequences formed phylogenetic clusters and lineages largely distinct from other *Mycobacterium* species. There was incomplete correlation between serovar designation and *hsp65* allele assignment. The *hsp65* data correlated strongly with the results of sequence analysis of the gene coding for 16S rRNA. Automated DNA sequencing of a 360-bp region of the *hsp65* gene provides a rapid and unambiguous method for species assignment of these acid-fast organisms for diagnostic purposes.**

Mycobacterium scrofulaceum is an opportunistic pathogen of humans that can be isolated from environmental sources such as house dust, soil, water, and sewage (6, 17, 23, 24, 60) and foods such as pooled oysters, raw milk, and other dairy products (8, 10, 21, 25, 31). The species name for *M. scrofulaceum* is derived from the word scrofula, the historic term used to describe mycobacterial infections of cervical lymph nodes. The organism is most commonly recovered from children of preschool age with unilateral cervical lymphadenitis (36, 39, 40, 60, 61). Less common manifestations of *M. scrofulaceum* infection include pulmonary disease (18), disseminated disease (22, 32), conjunctivitis, osteomyelitis (30), meningitis (63), and granulomatous hepatitis (37). *M. scrofulaceum* accounts for 2% of mycobacterial isolates recovered from clinical specimens in the United States (14, 16, 29) and is responsible for approximately 2 and 15% of the nontuberculous mycobacterial infections in AIDS patients from the United States (15) and Sweden (20), respectively.

Differentiation of *M. scrofulaceum* from other slowly growing mycobacteria with conventional methods is complex and time-consuming (58). Several methods have been developed for more rapid mycobacterial species assignment. DNA probes are commercially available for identification of the *Mycobacterium tuberculosis* complex, *Mycobacterium avium* complex, *Mycobacterium kansasii*, and *Mycobacterium gordonae*, but not for other species of mycobacteria. Seroagglutination testing has been used for subspecific differentiation and typing of strains of several mycobacterial species, including *M. scrofulaceum*. Mycobacterial serovars 27 and 41 through 43 are generally recognized as being *M. scrofulaceum*; however, serovar designations do not always correspond to the species assignment (15, 59). Additional strategies developed for species designation of mycobacteria include analysis of cell wall lipid composition by thin-layer chromatography (5, 55), gas-liquid chromatography (11, 45, 53), and high-performance liquid chromatography (7, 52); restriction fragment length polymorphism profiling of a 360-bp fragment of the *hsp65* gene (51); and nucleotide sequencing of several gene targets (12, 13, 46, 47, 49), primarily the gene encoding 16S rRNA (16S rDNA) (1, 28, 41, 42, 48).

Recently, automated DNA sequence analysis of a 360-bp segment of the gene (*hsp65*) encoding a 65-kDa heat shock protein has been used for species assignment of mycobacteria (27). The gene is present in all known *Mycobacterium* species, and oligonucleotide primers have been described that amplify a segment of the gene from all *Mycobacterium* species tested, but not from 19 non-*Mycobacterium* species (51). In mycobacteria, nucleotide polymorphism in *hsp65* is, on average, greater than in the 16S rDNA (27). This observation suggests that in addition to species identification, sequence analysis of *hsp65* may provide important subspecific information. To assess the utility of species assignment and subspecific differentiation of *M. scrofulaceum* organisms by characterization of *hsp65* polymorphism, we studied sequence variation among 37 *M. scrofulaceum* isolates, including reference strains and isolates recovered over four decades from children with cervical lymphadenitis.

MATERIALS AND METHODS

Bacterial isolates. The year of isolation, serovar designation, and *hsp65* allele assignments of the 37 *M. scrofulaceum* isolates studied are listed in Table 1. Thirty-five strains were obtained from a collection of cervical lymph node isolates held by Emanuel Wolinsky (MetroHealth Medical Center, Cleveland, Ohio), and most of these strains were typed by seroagglutination in his laboratory. The two other strains studied are reference isolates obtained from Richard Frothingham (Duke University Medical Center, Durham, N.C.). Bacteria were identified to the species level by conventional methods, including growth rate, colony morphology, pigmentation, and biochemical profiles.

Isolation of DNA. Strains were cultured on slants of Lowenstein-Jensen solid media for 2 to 4 weeks. All of the following procedures were performed in a biological safety cabinet. A small sample of the culture was collected on the end of a sterile wooden applicator stick and emulsified in an Eppendorf tube containing 500 μl of STET buffer (10 mM Tris-HCl–1 mM EDTA, pH 8.0, with 1% Triton X-100); the bacteria were harvested by centrifugation in a microcentrifuge

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TABLE 1. Characteristics of 37 *M. scrofulaceum* strains studied

Strain[a]	Yr of isolation	Serovar[b]	*hsp65* allele
NTB 126	1961	43	*hsp65.27*[c]
NTB 136	1966	43	*hsp65.27*[c]
NTB 138	1966	43	*hsp65.27*[c]
NTB 159	1983	ND[d]	*hsp65.27*[c]
Lane 3081	NA[e]	ND	*hsp65.27*[c]
NTB 154	1979	41	*hsp65.28*[c]
ATCC 19981	1955	ND	*hsp65.35*[c]
NTB 122	1958	42	*hsp65.35*
NTB 123	1958	42	*hsp65.35*
NTB 125	1960	42	*hsp65.35*
NTB 127	1962	42	*hsp65.35*
NTB 128	1962	41	*hsp65.35*
NTB 129	1962	41	*hsp65.35*
NTB 132	1962	42	*hsp65.35*
NTB 133	1962	42	*hsp65.35*
NTB 134	1963	42	*hsp65.35*[c]
NTB 135	1964	42	*hsp65.35*
NTB 139	1967	42	*hsp65.35*
NTB 141	1967	41	*hsp65.35*
NTB 143	1967	42	*hsp65.35*
NTB 145	1968	41	*hsp65.35*
NTB 146	1969	42	*hsp65.35*
NTB 147	1969	42	*hsp65.35*
NTB 148	1970	42	*hsp65.35*
NTB 149	1970	42	*hsp65.35*
NTB 150	1970	42	*hsp65.35*
NTB 151	1971	42	*hsp65.35*
NTB 152	1971	41	*hsp65.35*[c]
NTB 153	1975	42	*hsp65.35*
NTB 131	1962	16	*hsp65.36*[c]
NTB 142	1967	4	*hsp65.36*[c]
NTB 155	1979	43	*hsp65.36*[c]
NTB 15	1981	43	*hsp65.36*[c]
NTB 124	1958	42	*hsp65.37*[c]
NTB 144	1967	NT[f]	*hsp65.38*[c]
NTB 156	1980	8/21	*hsp65.39*[c]
NTB 130	1962	41	*hsp65.40*[c]

[a] Strain designations beginning with NTB are from a collection of cervical lymph node isolates held by E. Wolinsky. ATCC 19981 (the *M. scrofulaceum* type strain from the American Type Culture Collection) and Lane 3081 are reference strains and were obtained from R. Frothingham.

[b] Serovar designations were assigned in the laboratory of E. Wolinsky.

[c] A partial 16S rDNA sequence was obtained for this strain. In addition, the partial 16S rDNA sequence for *M. scrofulaceum* 1374 (*hsp65.35*) (Fig. 2) was determined.

[d] ND, not done.

[e] NA, not available.

[f] NT, nontypeable.

designed to minimize the risk of aerosol generation (model VS-13; Shelton Scientific, Shelton, Conn.) at 20,000 × *g* for 10 min. The supernatant was discarded, and the cell pellet was resuspended in 100 μl of 95% ethanol. After overnight incubation at 4°C, the suspension was centrifuged at 20,000 × *g* for 10 min and the supernatant was discarded. Following the addition of 0.1 ml of STET buffer and 50 μg of 0.1-mm-diameter zirconium oxide beads, the bacteria were lysed by agitation at 5,000 rpm for 100 s with a Mini-BeadBeater (Biospec Products, Bartlesville, Okla.). The supernatant, containing the genomic DNA, was used for analysis.

Automated DNA sequencing of *hsp65*. A 441-bp segment (nucleotides 396 to 836) (44) of *hsp65* was amplified in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) using the following oligonucleotide primers as described previously: forward (Tb11), 5′-ACCAACGATGGTGTGTCCAT-3′; and reverse (Tb12), 5′-CTTGTCGAACCGCATACCCT-3′ (27). Unincorporated nucleotides and primers were removed by filtration with Microcon 100 microconcentrators (Amicon Inc., Beverly, Mass.). Sequencing reactions were performed with the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, Calif.). An automated DNA sequencer (model 373A; Applied Biosystems) was used to characterize both strands of the target *hsp65* segment. Unambiguous sequence data were obtained for a 360-bp region of the 441-bp fragment in all strains. The data were assembled and edited with the EDITSEQ, ALIGN, and MEGALIGN programs (DNASTAR, Madison, Wis.).

Automated DNA sequencing of 16S rDNA. A 1,036-bp region of the 16S rDNA was amplified by PCR with the forward primer 285 (5′-GAGAGTTTGATCCT GGCTCAG-3′, corresponding to *Escherichia coli* 16S rDNA from positions 9 to 29) and reverse primer 264 (5′-TGCACACAGGCCACAAGGGA-3′, corresponding to *E. coli* 16S rDNA from positions 1046 to 1027) as described previously (48). The thermal-cycler parameters used were 39 cycles of denaturation at 94°C for 1 min, annealing at 68°C for 1 min, and extension at 72°C for 2 min. Unincorporated nucleotides and primers were removed by filtration with Microcon 100 microconcentrators. Sequencing reactions were performed with the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit using primer 285 as well as primer 259 (5′-TTCACGAACAACGCGACAA-3′, corresponding to *E. coli* 16S rDNA from positions 609 to 590). An automated DNA sequencer (model 373A or 377; Applied Biosystems) was used to characterize both strands of the target 16S rDNA segment. For all strains evaluated, unambiguous sequence data were obtained for a segment of the 16S rDNA fragment containing both hypervariable regions A and B. The size of this segment ranged from 387 to 398 bp because of the presence of insertions or deletions in some of the sequences. The data were assembled and edited with the EDITSEQ, ALIGN, and MEGALIGN programs.

Phylogenetic trees were constructed by maximum-parsimony analysis with the computer program PAUP 3.1.1 using the branch swapping and nearest-neighbor interchange options (50).

RESULTS

Nucleotide polymorphism. Characterization of the 360-bp *hsp65* fragment from 37 *M. scrofulaceum* strains revealed that 8 of the 37 sequences were unique. All of the nucleotide variation was due to point mutations. Each distinctive sequence was given an arbitrary allele designation, such as *hsp65.35*, *hsp65.36*, etc. A total of 50 polymorphic nucleotide sites was identified (Fig. 1), which would result in 12 amino acid substitutions. Pairs of alleles differed, on average, at 19 (5.3%) of the nucleotide sites and 4 (3.3%) of the inferred amino acid positions. Phylogenetic analysis revealed that the eight alleles grouped into one lineage (A) and two clusters (B and C) when compared with 66 unique *hsp65* sequences representing 23 mycobacterial species (Fig. 2), including a previously reported sequence from one *M. scrofulaceum* strain (27).

Lineage A. Twenty-three of the 37 isolates evaluated had allele *hsp65.35*, which formed lineage A in the phylogenetic tree (Fig. 2). This lineage represented the majority (62%) of the isolates studied, including the *M. scrofulaceum* type strain (ATCC 19981) and a previously studied *M. scrofulaceum* strain (1374) (27). The *hsp65.35* allele differed from the other seven *hsp65* alleles by 19 nucleotides, on average (range, 16 to 22) (Fig. 1).

Cluster B. Twelve of the 37 isolates evaluated had either allele *hsp65.27* or alleles *hsp65.36* through *hsp65.39*, which formed cluster B in the phylogenetic tree (Fig. 2). *M. scrofulaceum* reference strain Lane 3081 (*hsp65.27*) grouped within this cluster. No *hsp65* alleles from other mycobacterial species occurred in cluster B. Pairs of sequences within this cluster differed, on average, at 17 (4.7%) nucleotide sites and 4 (3.3%) of the inferred amino acid positions.

Cluster C. Two strains had one of two alleles (*hsp65.28* or *hsp65.40*) which were allied with cluster C (Fig. 2). These two alleles differed from the other six *hsp65* alleles by 22 nucleotides, on average (range, 17 to 26). Cluster C also contained three sequences from previously studied *M. avium* complex strains. Only nine polymorphic nucleotide sites were identified among the *hsp65* alleles within this cluster, and none of the variants would result in amino acid substitutions. Pairs of sequences in cluster C differed, on average, at 4 (1.1%) nucleotide sites.

Comparison of *hsp65* allele assignment and serovar assignment. Seroagglutination testing separated 34 of the 37 isolates into six different serovars (Table 1). The most commonly assigned serovars were 42 (50%), 41 (20%), and 43 (15%). The

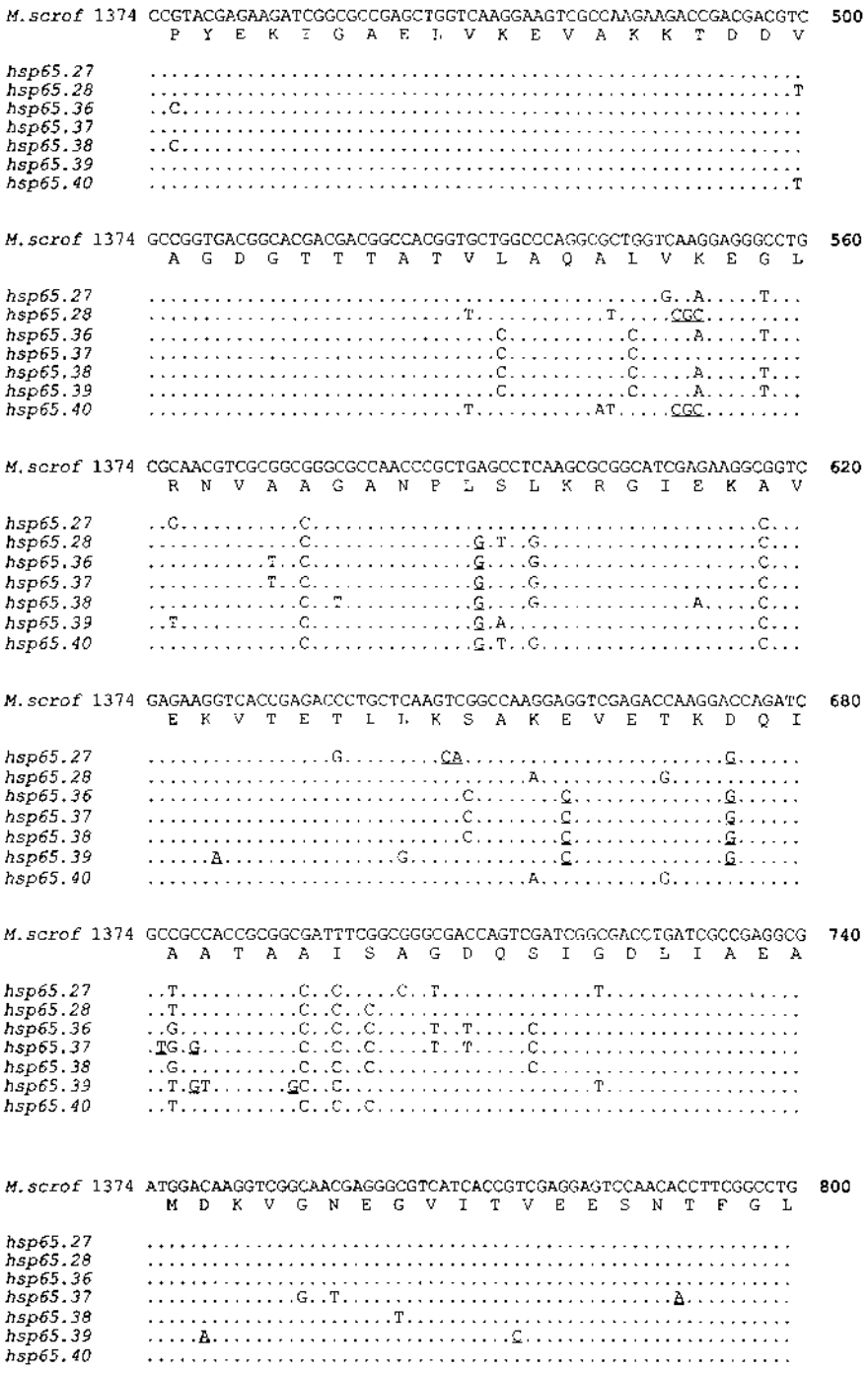

FIG. 1. Nucleotide sequence from positions 441 to 800 in a 360-bp fragment of the *hsp65* gene from 37 strains (eight alleles) of *M. scrofulaceum*, compared with the published nucleotide and amino acid sequences of the *hsp65* fragment for *M. scrofulaceum* (*M. scrof*) 1374 (GenBank accession number U17955) (27). The sequence of allele *hsp65.35* is identical to the published sequence from *M. scrofulaceum* 1374 and therefore is not shown. Nucleotide numbering is based on a published sequence of *hsp65* from *M. tuberculosis* (44) and is displayed at the top of the figure. Invariant sites are shown as dots. Nonsynonymous nucleotide changes are underlined.

distribution pattern for *hsp65* allele assignments was similar. The most commonly assigned alleles resulting from sequencing analysis were *hsp65.35* (62%), *hsp65.27* (14%), and *hsp65.36* (11%). Three isolates found in cluster B (NTB 131, NTB 142, and NTB 156) had serovars traditionally assigned to *M. avium* complex organisms. One isolate (NTB 144) was nontypeable, and another isolate (NTB 156) reacted with antisera to two different serovars. In general, there was no simple correlation between serovar designation and *hsp65* allele assignment. For example, 23 strains with the *hsp65.35* allele had either serovar 41 or 42, and 4 strains with *hsp65.36* had one of three serovars (4, 16, or 43). Conversely, strains with serovar 41 had one of three *hsp65* alleles (*hsp65.28*, *hsp65.35*, or *hsp65.40*).

16S rDNA sequences. 16S rDNA sequences have frequently been used to define taxonomic relationships among organisms and to assign a species status to new or difficult-to-classify

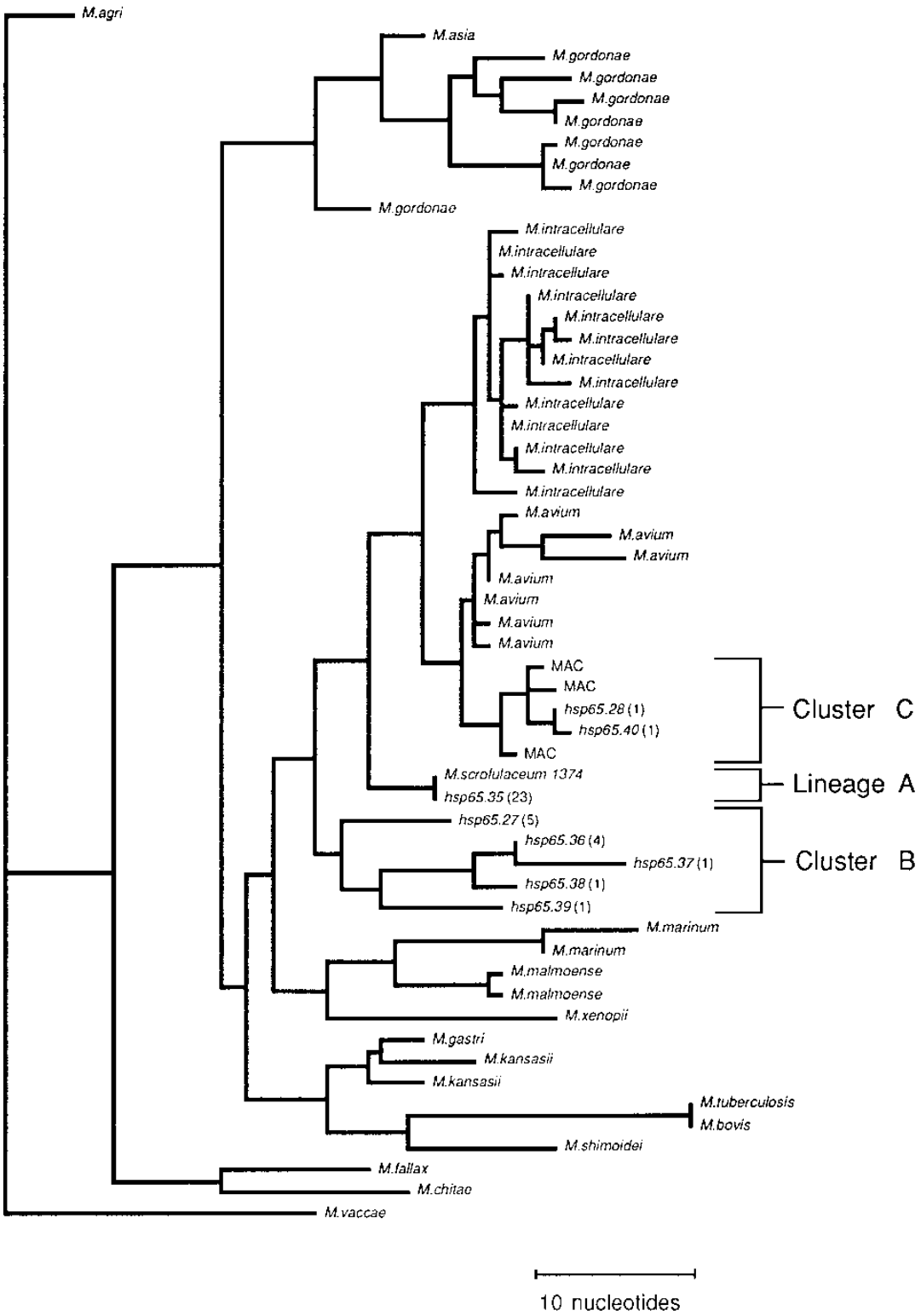

FIG. 2. Phylogenetic tree of eight *hsp65* alleles obtained from 37 *M. scrofulaceum* isolates, compared with 66 *hsp65* alleles representing 23 mycobacterial species, including one previously reported allele from a *M. scrofulaceum* isolate (*M. scrofulaceum* 1374) (27). The phylogenetic tree was constructed with the program PAUP 3.1.1 (50), using the branch swapping and nearest-neighbor interchanges. The number of strains with each allele is indicated in parentheses. Horizontal lengths represent genetic distances.

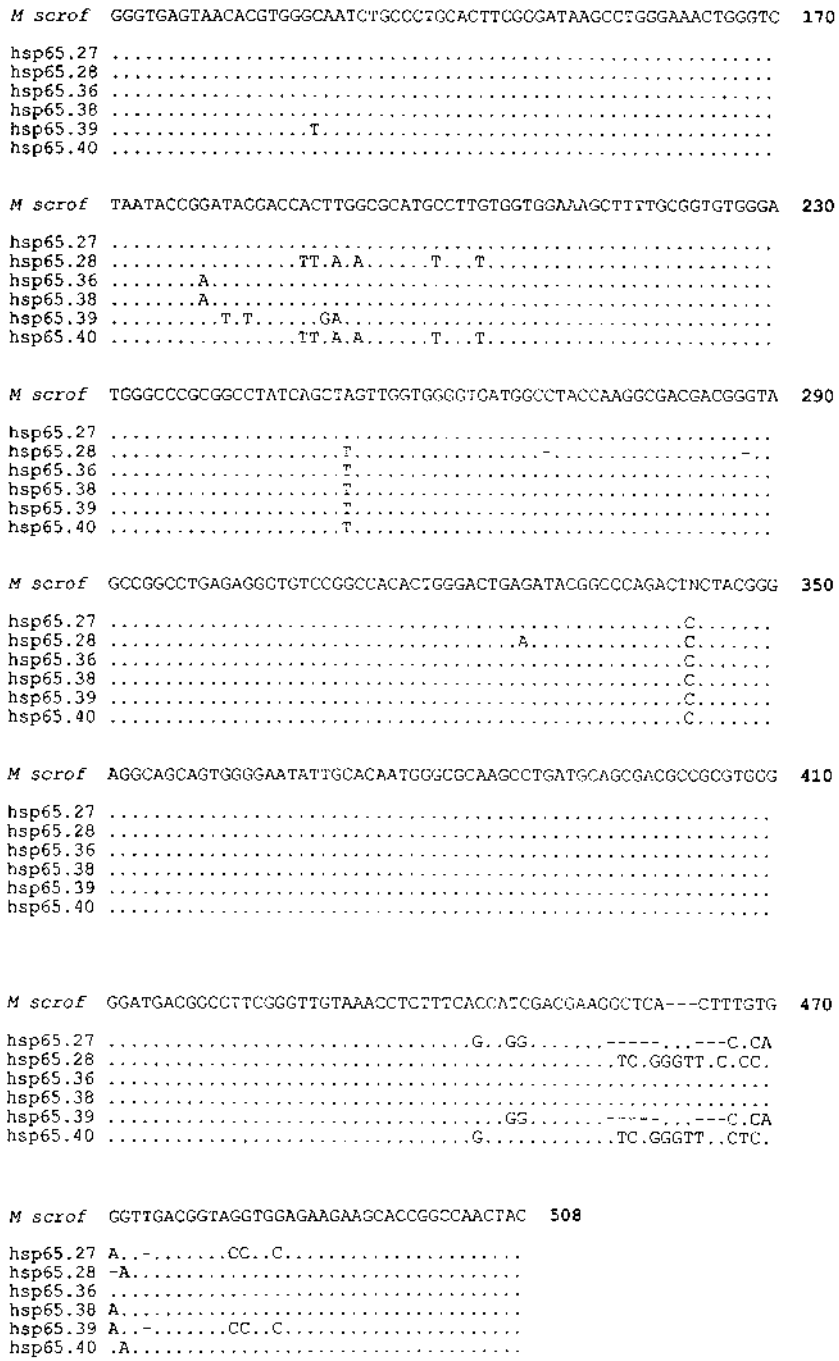

FIG. 3. Nucleotide sequence of a segment of the 16S rDNA fragment, containing both the hypervariable A and B regions, from 19 strains (six alleles) of *M. scrofulaceum* representing each of the different *hsp65* alleles, compared with the nucleotide sequence of the 16S rDNA fragment for *M. scrofulaceum* (*M. scrof*) (GenBank accession number X52924). The sequences of isolates with alleles *hsp65.35* and *hsp65.37* are identical to the reference sequence and therefore are not shown. The first nucleotide corresponds to *E. coli* 16S rDNA position 129, and numbering is displayed at the top of the figure. Invariant sites are shown as dots, and deletions are indicated by dashes.

organisms (1–3, 9, 28, 41, 42, 48, 56, 57). In an attempt to clarify the species identity of some of the isolates, we sequenced a segment of the 16S rDNA containing hypervariable regions A and B from 19 isolates, which represented each of the different *hsp65* alleles (Table 1 and Fig. 3), and compared these results with published mycobacterial 16S rDNA sequences (Fig. 4). 16S rDNA sequences from 11 isolates with alleles *hsp65.35* through *hsp65.38* were closely allied to one another and clustered with the sequence from the *M. scrofulaceum* type strain (ATCC 19981). Two 16S rDNA sequences from isolates with alleles *hsp65.28* and *hsp65.40* were closely related to sequences from members of the *M. avium* complex. The remaining six sequences were not closely related to other strains in our sample. Five of these six sequences had allele *hsp65.27* and had an identical 16S rDNA sequence that was similar to a sequence (X93030) identified recently in a myco-

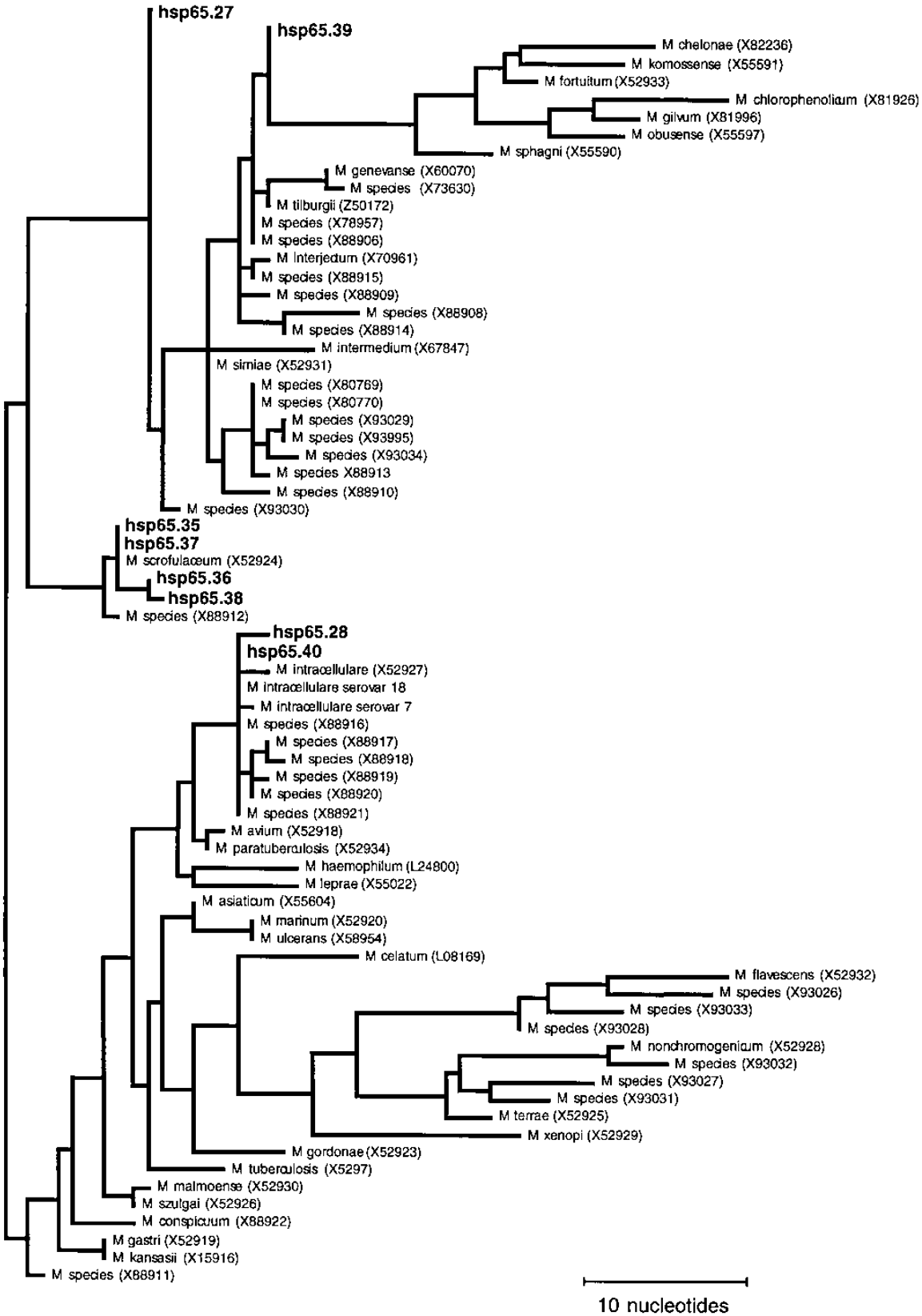

FIG. 4. Phylogenetic tree of 16S rDNA sequences obtained from 19 *M. scrofulaceum* isolates, representing each of the different *hsp65* alleles, compared with 16S rDNA sequences from numerous mycobacterial strains. In parentheses are the GenBank sequence identification numbers. The sequences for *M. intracellulare* serovars 18 and 7 are from reference 2. The phylogenetic tree was constructed with the program PAUP 3.1.1 (50), using the branch swapping and nearest-neighbor interchanges. Horizontal lengths represent genetic distances.

bacterial strain of uncertain taxonomic position (48). Biochemical test results identified the strain as *M. avium-M. intracellulare*, whereas 16S rDNA sequence results identified it as a unique isolate related to *Mycobacterium simiae* (48). The sixth sequence was related to a sequence reported for *Mycobacterium tilburgii*, an organism recovered from an immunocompetent patient with disseminated infection (56).

DISCUSSION

Sequence-specific differentiation of *M. scrofulaceum*. The conventional method for identifying mycobacteria to the species level is based on growth characteristics and biochemical test reactivity. These methods are time-consuming, and it is not uncommon for species identification to require an additional 2 to 4 weeks following the initial isolation (58). Furthermore, variation in phenotypic characteristics of atypical mycobacterial species makes the unambiguous interpretation of biochemical test results difficult. Several strategies formulated to improve the conventional methods of mycobacterial strain identification include the use of commercial nucleic acid probe kits (34), analysis of cell wall lipid composition by various chromatographic methods (5, 7, 11, 52, 53, 55), restriction fragment length polymorphism profiling of a 360-bp fragment of the *hsp65* gene (51), and nucleotide sequencing of the gene encoding 16S rRNA (1, 28, 41, 42, 48). Each of these techniques has several advantages and disadvantages. For example, DNA probes for rapid species identification are not commercially available for most atypical mycobacteria, including *M. scrofulaceum*. Species assignment of mycobacteria by analysis of cell wall lipid composition by thin-layer chromatography, gas-liquid chromatography, or high-performance liquid chromatography requires substantial bacterial growth under standardized conditions. Finally, subspecific differentiation of mycobacteria by restriction fragment length polymorphism profiling of a 360-bp fragment of the *hsp65* gene or nucleotide sequencing of the gene encoding 16S rRNA is not as discriminatory as nucleotide sequencing of a segment of the *hsp65* gene (27).

We have demonstrated that *M. scrofulaceum* strains can be readily assigned a species designation by sequence analysis of a segment of the *hsp65* gene. This sequencing approach has been used effectively to differentiate *Mycobacterium* species (27) and offers distinct advantages over the current method of identifying *M. scrofulaceum* strains by phenotypic and biochemical characteristics. Before performing conventional species assignment, it is necessary to subculture the isolates for 2 to 4 weeks following initial isolation. In contrast, the sequencing technique is rapid and can be used to identify the species of *Mycobacterium* growing in early positive (growth index of approximately 50 to 100) BACTEC cultures or in some smear-positive sputum specimens (27). Phenotypic and biochemical profiles for strains can vary, and in many instances it is necessary to identify the organisms on the basis of a best-fit analysis (35). In contrast, nucleic acid sequencing provides direct and unambiguous results, thereby avoiding the frustration and the considerable commitment of technologist time that often accompany biochemical testing.

Subspecific differentiation of *M. scrofulaceum*. Seroagglutination testing has been the method commonly used for subspecific differentiation and typing of strains of *M. scrofulaceum*. However, standardized antisera are not generally available, and serovar designations are not always cognate with species assignment. For example, strains of *M. simiae* and *M. scrofulaceum* have been found to type as *M. avium* complex serovars rather than as serovars traditionally assigned to their own species (15, 59). Furthermore, strains may cross-react with antisera to several serovars or may fail to react with any antiserum. Isolates NTB 156 and NTB 144 are examples of organisms with these problems. Finally, discrepancies in the characterization of strains occur between laboratories (15, 59). Unlike seroagglutination testing, *hsp65* analysis provides direct and unambiguous differentiation of these strains and may have applications for molecular epidemiology studies and identification of medically important subspecific strain groups.

***hsp65* alleles and 16S rDNA sequences.** Based on 16S rDNA and *hsp65* sequence analyses of a sample of 37 strains, it is likely that 29 isolates that we studied represent true *M. scrofulaceum*. However, our analysis identified one phylogenetic group (cluster C) containing strains of both the *M. avium* complex and *M. scrofulaceum*, an observation suggesting that the organisms within this cluster may represent an intermediate group between the *M. avium* complex and *M. scrofulaceum*. Several reports have described difficulties in distinguishing some strains of the *M. avium* complex from *M. scrofulaceum* organisms. Hawkins and Portaels reported strains with biochemical characteristics intermediate between *M. avium* and *M. scrofulaceum* (19, 38). *M. scrofulaceum* isolates that belong to established serovars of *M. avium* complex strains (59, 62) and isolates that carry plasmids present in *M. avium* and *M. intracellulare* strains (26, 33) have been identified. Furthermore, strains identified as *M. scrofulaceum* based on α-antigen analysis and biochemical properties have been reported to react with DNA probes specific for the *M. avium* complex (43, 54). Additional studies support the notion that an intermediate species exists (12, 13, 48).

The specific status of the six isolates with alleles *hsp65.27* and *hsp65.39* is unknown and clearly warrants additional study. In particular, the five organisms with the *hsp65.27* allele and identical 16S rDNA sequences that are not closely related to other mycobacterial species probably represent a taxonomic entity that may justify a new species designation. In this regard, it is noteworthy that isolates with *hsp65.27* have a 16S rDNA sequence that is identical to a novel species which is phylogenetically related to *M. simiae*. These isolates have been recovered from a variety of geographic regions.

Summary. The principal goal of our study was to assess the level of *hsp65* polymorphism in *M. scrofulaceum* and thereby determine the potential utility of this strategy for species assignment and subspecific strain differentiation. The results suggest that automated sequence analysis of a 360-bp region of *hsp65* has several advantages over the current methods used to identify and subtype *M. scrofulaceum* strains. It produces rapid and unambiguous data. The strategy is PCR based and therefore may be applied to some primary samples or early positive cultures (27). In addition, it may contribute new insight into mycobacterial strains which appear to represent an intermediate species between *M. scrofulaceum* and the *M. avium* complex.

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REFERENCES

1. **Böddinghaus, 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.
2. **Böddinghaus, B., J. Wolters, W. Hiekens, and E. C. Böttger.** 1990. Phylogenetic analysis and identification of different serovars of *Mycobacterium intracellulare* at the molecular level. FEMS Microbiol. Lett. **70:**197–204.
3. **Bosquée, L., E. C. Böttger, H. De Beenhouwer, P. A. Fonteyne, B. Hirschel, L. Larsson, W. M. Meyers, J. C. Palomino, L. Realini, L. Rigouts, M. T. Silva, A. Teske, P. Van der Auwera, and F. Portaels.** 1995. Cervical lymphadenitis caused by a fastidious mycobacterium closely related to *Mycobacterium genavense* in an apparently immunocompetent woman: diagnosis by culture-free microbiological methods. J. Clin. Microbiol. **33:**2670–2674.
4. **Böttger, E. C.** Personal communication.
5. **Brennan, P. J., M. Heifets, and B. P. Ullom.** 1982. Thin-layer chromatography of lipid antigens as a means of identifying nontuberculous mycobacteria. J. Clin. Microbiol. **15:**447–455.
6. **Brooks, R. W., B. C. Parker, H. Gruft, and J. O. Falkinham III.** 1984. Epidemiology of infection by nontuberculous mycobacteria. V. Numbers in eastern United States soils and correlation with soil characteristics. Am. Rev. Respir. Dis. **130:**630–633.
7. **Butler, W. R., K. C. Jost, Jr., and J. O. Kilburn.** 1991. Identification of mycobacteria by high-performance liquid chromatography. J. Clin. Microbiol. **29:**2468–2472.
8. **Chapman, J. S., and M. Speight.** 1968. Isolation of atypical mycobacteria from pasteurized milk. Am. Rev. Respir. Dis. **98:**1052–1054.
9. **Coyle, M. B., L. C. Carlson, C. K. Wallis, R. B. Leonard, V. A. Raisys, J. O. Kilburn, M. Samadpour, and E. C. Böttger.** 1992. Laboratory aspects of "*Mycobacterium genavense*," a proposed species isolated from AIDS patients. J. Clin. Microbiol. **30:**3206–3212.
10. **Dunn, B. L., and D. J. Hodgson.** 1982. 'Atypical' mycobacteria in milk. J. Appl. Bacteriol. **52:**373–376.
11. **Fourche, J., M. Capdepuy, J. Maugein, and F. Le Moigne.** 1990. Analysis of cellular fatty acids and proteins by capillary gas chromatography and sodium dodecyl sulphate polyacrylamide gel electrophoresis to differentiate *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum* (MAIS) complex species. J. Chromatogr. **532:**209–216.
12. **Frothingham, R., and K. H. Wilson.** 1993. Sequence-based differentiation of strains in the *Mycobacterium avium* complex. J. Bacteriol. **175:**2818–2825.
13. **Frothingham, R., and K. H. Wilson.** 1994. Molecular phylogeny of the *Mycobacterium avium* complex demonstrates clinically meaningful divisions. J. Infect. Dis. **169:**305–312.
14. **Good, R. C.** 1980. From the Center for Disease Control. Isolation of nontuberculous mycobacteria in the United States, 1979. J. Infect. Dis. **142:**779–783.
15. **Good, R. C.** 1985. Opportunistic pathogens in the genus *Mycobacterium*. Annu. Rev. Microbiol. **39:**347–369.
16. **Good, R. C., and D. E. Snider, Jr.** 1982. Isolation of nontuberculous mycobacteria in the United States, 1980. J. Infect. Dis. **146:**829–833.
17. **Goslee, S., and E. Wolinsky.** 1976. Water as a source of potentially pathogenic mycobacteria. Am. Rev. Respir. Dis. **113:**287–292.
18. **Gracey, D. R., and R. B. Byrd.** 1970. Scotochromogens and pulmonary disease. Five years' experience at a pulmonary disease center with report of a case. Am. Rev. Respir. Dis. **101:**959–963.
19. **Hawkins, J. E.** 1977. Scotochromogenic mycobacteria which appear intermediate between *Mycobacterium avium-intracellulare* and *Mycobacterium scrofulaceum*. Am. Rev. Respir. Dis. **116:**963–964. (Letter.)
20. **Hoffner, S. E., B. Petrini, P. J. Brennan, A. Y. Tsang, and G. Källenius.** 1989. AIDS and *Mycobacterium avium* serovars in Sweden. Lancet **2:**336–337. (Letter.)
21. **Hosty, T. S., and C. I. McDurmont.** 1975. Isolation of acid-fast organisms from milk and oysters. Health Lab. Sci. **12:**16–19.
22. **Hsueh, P.-R., T.-R. Hsiue, J.-J. Jarn, S.-W. Ho, and W.-C. Hsieh.** 1996. Disseminated infection due to *Mycobacterium scrofulaceum* in an immunocompetent host. Clin. Infect. Dis. **22:**159–161.
23. **Ichiyama, S., K. Shimokata, and M. Tsukamura.** 1988. The isolation of *Mycobacterium avium* complex from soil, water, and dusts. Microbiol. Immunol. **32:**733–739.
24. **Jin, B. W., H. Saito, and Z. Yoshii.** 1984. Environmental mycobacteria in Korea. I. Distribution of the organisms. Microbiol. Immunol. **28:**667–677.
25. **Jones, R. J., D. E. Jenkins, and K. H. Hsu.** 1966. Raw milk as a source of mycobacteria. Can. J. Microbiol. **12:**979–984.
26. **Jucker, M. T., and J. O. Falkinham III.** 1990. Epidemiology of infection by nontuberculous mycobacteria. IX. Evidence for two DNA homology groups among small plasmids in *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum*. Am. Rev. Respir. Dis. **142:**858–862.
27. **Kapur, V., L.-L. Li, M. R. Hamrick, B. B. Plikaytis, T. M. Shinnick, A. Telenti, W. R. Jacobs, Jr., A. Banerjee, S. Cole, K. Y. Yuen, J. E. Clarridge III, B. N. Kreiswirth, and J. M. Musser.** 1995. Rapid *Mycobacterium* species assignment and unambiguous identification of mutations associated with antimicrobial resistance in *Mycobacterium tuberculosis* by automated DNA sequencing. Arch. Pathol. Lab. Med. **119:**66–73.
28. **Kirschner, P., B. Springer, U. Vogel, A. Meier, A. Wrede, M. Kiekenbeck, F.-C. Bange, and E. C. Böttger.** 1993. Genotypic identification of mycobacteria by nucleic acid sequence determination: report of a 2-year experience in a clinical laboratory. J. Clin. Microbiol. **31:**2882–2889.
29. **Krajnack, M. A., and H. Dowda.** 1981. Non-tuberculous mycobacteria in South Carolina, 1971 to 1980. J. S. C. Med. Assoc. **77:**551–555.
30. **Lincoln, E. M., and L. A. Gilbert.** 1972. Disease in children due to mycobacteria other than *Mycobacterium tuberculosis*. Am. Rev. Respir. Dis. **105:**683–714.
31. **Matthews, P. R., P. Collins, and P. W. Jones.** 1976. Isolation of mycobacteria from dairy creamery effluent sludge. J. Hyg. **76:**407–413.
32. **McNutt, D. R., and H. H. Fudenberg.** 1971. Disseminated scotochromogen infection and unusual myeloproliferative disorder. Report of a case and review of the literature. Ann. Intern. Med. **75:**737–744.
33. **Meissner, P. S., and J. O. Falkinham III.** 1986. Plasmid DNA profiles as epidemiological markers for clinical and environmental isolates of *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum*. J. Infect. Dis. **153:**325–331.
34. **Musial, C. E., L. S. Tice, L. Stockman, and G. D. Roberts.** 1988. Identification of mycobacteria from culture by using the Gen-Probe Rapid Diagnostic System for *Mycobacterium avium* complex and *Mycobacterium tuberculosis* complex. J. Clin. Microbiol. **26:**2120–2123.
35. **Nolte, F. S., and B. Metchock.** 1995. *Mycobacterium*, p. 400–437. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 6th ed. ASM Press, Washington, D.C.
36. **Olson, N. R.** 1967. Atypical mycobacterial infections of the neck. Laryngoscope **77:**1376–1389.
37. **Patel, K. M.** 1981. Granulomatous hepatitis due to *Mycobacterium scrofulaceum*: report of a case. Gastroenterology **81:**156–158.
38. **Portaels, F.** 1978. Difficulties encountered in identification of *M. avium-M. intracellulare*, *M. scrofulaceum*, and related strains. Am. Rev. Respir. Dis. **118:**969. (Letter.)
39. **Prissick, F. H., and A. M. Masson.** 1956. Cervical lymphadenitis in children caused by chromogenic mycobacteria. Can. Med. Assoc. J. **75:**798–803.
40. **Prissick, F. H., and A. M. Masson.** 1957. Yellow-pigmented pathogenic mycobacteria from cervical lymphadenitis. Can. J. Microbiol. **3:**91–99.
41. **Rogall, T., T. Flohr, and E. C. Böttger.** 1990. Differentiation of *Mycobacterium* species by direct sequencing of amplified DNA. J. Gen. Microbiol. **136:**1915–1920.
42. **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.
43. **Saito, H., H. Tomioka, K. Sato, H. Tasaka, and D. J. Dawson.** 1990. Identification of various serovar strains of *Mycobacterium avium* complex by using DNA probes specific for *Mycobacterium avium* and *Mycobacterium intracellulare*. J. Clin. Microbiol. **28:**1694–1697.
44. **Shinnick, T. M.** 1987. The 65-kilodalton antigen of *Mycobacterium tuberculosis*. J. Bacteriol. **169:**1080–1088.
45. **Smid, I., and M. Salfinger.** 1994. Mycobacterial identification by computer-aided gas-liquid chromatography. Diagn. Microbiol. Infect. Dis. **19:**81–88.
46. **Soini, H., E. C. Böttger, and M. K. Viljanen.** 1994. Identification of mycobacteria by PCR-based sequence determination of the 32-kilodalton protein gene. J. Clin. Microbiol. **32:**2944–2947.
47. **Soini, H., E. Eerola, and M. K. Viljanen.** 1996. Gentic diversity among *Mycobacterium avium* complex Accuprobe-positive isolates. J. Clin. Microbiol. **34:**55–57.
48. **Springer, B., L. Stockman, K. Teschner, G. D. Roberts, and E. C. Böttger.** 1996. Two-laboratory collaborative study on identification of mycobacteria: molecular versus phenotypic methods. J. Clin. Microbiol. **34:**296–303.
49. **Stone, B. B., R. M. Nietupski, G. L. Breton, and W. G. Weisburg.** 1995. Comparison of *Mycobacterium* 23S rRNA sequences by high-temperature reverse transcription and PCR. Int. J. Syst. Bacteriol. **45:**811–819.
50. **Swofford, D. L.** 1991. PAUP, Phylogenetic Analysis Using Parsimony, version 3.1.1. Illinois Natural History Survey, University of Illinois, Champaign.
51. **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.
52. **Thibert, L., and S. Lapierre.** 1993. Routine application of high-performance liquid chromatography for identification of mycobacteria. J. Clin. Microbiol. **31:**1759–1763.
53. **Tisdall, P. A., G. D. Roberts, and J. P. Anhalt.** 1979. Identification of clinical isolates of mycobacteria with gas-liquid chromatography alone. J. Clin. Microbiol. **10:**506–514.
54. **Tomioka, H., H. Saito, K. Sato, H. Tasaka, and D. J. Dawson.** 1993. Identification of *Mycobacterium avium* complex strains belonging to serovars 21–28 by three commercial DNA probe tests. Tubercle Lung Dis. **74:**91–95.
55. **Tsukamura, M., and S. Mizuno.** 1981. Studies on thin-layer chromatography after incubation with [^{35}S]methionine as an aid to identification of mycobacteria. Microbiol. Immunol. **25:**215–227.
56. **Vos, M. C., A. M. Bergmans, A. G. Buiting, H. Teepen, J. R. Juttman, and L. M. Schouls.** Disseminated infection with a newly identified mycobacterial

species in an immunocompetent patient. GenBank entry Z50172.

57. **Wayne, L. G., R. C. Good, E. C. Böttger, R. Butler, M. Dorsch, T. Ezaki, W. Gross, V. Jonas, J. Kilburn, P. Kirschner, M. I. Krichevsky, M. Ridell, T. M. Shinnick, B. Springer, E. Stackebrandt, I. Tarnok, Z. Tarnok, H. Tasaka, V. Vincent, N. G. Warren, C. A. Knott, and R. Johnson.** 1996. Semantide- and chemotaxonomy-based analyses of some problematic phenotypic clusters of slowly growing mycobacteria, a cooperative study of the International Working Group on Mycobacterial Taxonomy. Int. J. Syst. Bacteriol. **46:**280–297.
58. **Wayne, L. G., R. C. Good, M. I. Krichevsky, Z. Blacklock, H. L. David, D. Dawson, W. Gross, J. Hawkins, V. V. Levy-Frebault, C. McManus, F. Portaels, S. Rüsch-Gerdes, K. H. Schröder, V. A. Silcox, M. Tsukamura, L. Van Den Breen, and M. A. Yakrus.** 1991. Fourth report of the cooperative, open-ended study of slowly growing mycobacteria by the International Working Group on Mycobacterial Taxonomy. Int. J. Syst. Bacteriol. **41:**463–472.
59. **Wayne, L. G., R. C. Good, A. Tsang, R. Butler, D. Dawson, D. Groothuis, W. Gross, J. Hawkins, J. Kilburn, M. Kubin, K. H. Schröder, V. A. Silcox, C. Smith, M.-F. Thorel, C. Woodley, and M. A. Yakrus.** 1993. Serovar determination and molecular taxonomic correlation in *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum*: a cooperative study of the International Working Group on Mycobacterial Taxonomy. Int. J. Syst. Bacteriol. **43:**482–489.
60. **Wolinsky, E.** 1979. Nontuberculous mycobacteria and associated diseases. Am. Rev. Respir. Dis. **119:**107–159.
61. **Wolinsky, E.** 1995. Mycobacterial lymphadenitis in children: a prospective study of 105 nontuberculous cases with long-term follow-up. Clin. Infect. Dis. **20:**954–963.
62. **Wolinsky, E., and W. B. Schaefer.** 1973. Proposed numbering scheme for mycobacterial serotypes by agglutination. Int. J. Syst. Bacteriol. **23:**182–183.
63. **Yamamoto, M., K. Sudo, M. Taga, and S. Hibino.** 1967. A study of diseases caused by atypical mycobacteria in Japan. Am. Rev. Respir. Dis. **96:**779–787.