

***Mycobacterium shottsii* sp. nov., a slowly growing species isolated from  
Chesapeake Bay striped bass (*Morone saxatilis*)**

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The GenBank accession number for the 16S rRNA sequence of *Mycobacterium shottsii* ATCC 700981<sup>T</sup> (strain M175<sup>T</sup>) is AY005147.

**Summary.** Slowly growing nonpigmented mycobacteria were isolated from striped bass (*Morone saxatilis*) during an epizootic of mycobacteriosis in the Chesapeake Bay. Growth characteristics, acid-fastness, and sequencing results of the 16S rRNA gene were consistent with those of the genus *Mycobacterium*. A unique profile of biochemical reactions was observed among the 21 isolates. A single cluster of eight peaks identified by analysis of mycolic acids (HPLC) resembled those of reference patterns but differed in peak elution times to profiles of reference species of the *M. tuberculosis* complex. One isolate (M175<sup>T</sup>) was placed within the slowly growing mycobacteria by analysis of aligned 16S rRNA gene sequences and was proximate in phylogeny to *M. ulcerans* and *M. marinum*. However, distinct nucleotide differences in the 16S rRNA gene sequence among M175<sup>T</sup>, *M. ulcerans* and *M. marinum* (99.2% similarity) were detected. M175<sup>T</sup> could be differentiated from other slow growing nonpigmented mycobacteria by its inability to grow at 37°C, production of niacin and urease, absence of nitrate reductase, and resistance to isoniazid (1 µg/ml), thiacetazone and thiophene-2-carboxylic hydrazide. Based upon these genetic and phenotypic differences, we propose isolate M175<sup>T</sup> as representing a new species, *Mycobacterium shottsii*. The type strain M175<sup>T</sup> has been deposited in the American Type Culture Collection as ATCC 700981<sup>T</sup> and the National Collection of Type Cultures (UK) as NCTC 13215<sup>T</sup>.

Mycobacteria are widely distributed in both fresh and marine waters, and include species pathogenic to marine animals and humans (Collins *et al.*, 1984; Falkinham, 1996; Dailloux *et al.*, 1999). An increased awareness of the diversity and complexity within the genus has resulted from the application of molecular techniques to the analysis of isolates from environmental sources and clinical specimens (Springer *et al.*, 1993). During a recent epizootic of mycobacteriosis in striped bass, *Morone saxatilis*, from the Chesapeake Bay, various mycobacteria were isolated including a homogenous group which on the basis of traditional biochemical tests, mycolic acid analyses, and a distinct 16S rRNA gene sequence, could not be assigned to any recognized species (Rhodes *et al.*, 2001). More extensive characterization using additional biochemical tests, antimicrobial

susceptibility, and HPLC mycolic acid pattern analysis of additional isolates indicated that these isolates belong to a new taxon. In this report we describe the results of a taxonomic study of these isolates and propose that they are representative of a new species, *Mycobacterium shottsii*.

Isolate M175<sup>T</sup>, and 20 similar isolates were recovered from granulomatous lesions in striped bass (Rhodes *et al.*, 2001). One isolate each (M23 and M216) was recovered from kidney and skin lesions, respectively, and all others from the spleen. Additional isolates included M115, M120, M121, M148, M177, M179, M182, M200, M202, M203, M205, M208, M210, M211, M217, M218, M219, and M220. Growth and biochemical testing included reference *M. avium* (M1), *M. chelonae* (M3), *M. flavescens* (M4), *M. fortuitum* (M6), *M. gordonae* (M8), *M. kansasii* (M10), *M. marinum* (M11, M12, M13), *M. nonchromogenicum* (M14), *M. phlei* (M15), *M. scrofulaceum* (M17), *M. simiae* (M19, M20), and *M. terrae* (M21) obtained from the Environmental Protection Agency, Cincinnati, OH, USA, and Consolidated Laboratory Services, Commonwealth of Virginia, Richmond, VA, USA.

Colony morphology and the ability to grow at temperatures ranging from 23 to 42°C were determined after 1 and 2 months incubation on Middlebrook 7H10 agar with albumin-dextrose-catalase (ADC) enrichment. The following tests were performed at 23°C using accepted methods (Kent & Kubica, 1985; Lévy-Frébault & Portaels, 1992): production of acid phosphatase, arylsulfatase, catalase,  $\beta$ -galactosidase, nitrate reductase, niacin, pyrazinamidase, Tween 80 hydrolysis, urease and growth on media containing hydroxylamine (500  $\mu$ g/ml), isoniazid (1 and 10  $\mu$ g/ml),  $\beta$ -nitrobenzoic acid (500  $\mu$ g/ml), NaCl (50 mg/ml), thiactone (10  $\mu$ g/ml) and thiophene-2-carboxylic hydrazide (2  $\mu$ g/ml). Drug susceptibility tests were performed using the disc method (Kent & Kubica, 1985).

High Performance Liquid Chromatography (HPLC) was used to analyze mycolic acids from 4 isolates (M115, M121, M148 and M175<sup>T</sup>) as p-bromophenacyl esters as described by Butler *et al.*,

(1986). Specimens were processed as described previously using the standard method for sample preparation and ultraviolet analysis (Butler et.al., 1996). Differentiation of specific *Mycobacterium* species was with a visual decision method using an internal size standard, an identification process recently reviewed by Butler & Guthertz (2001).

Sequencing of 140 bp for the signature region “A” of the 16S rRNA gene from 4 isolates (M115, M121, M148 and M175<sup>T</sup>) was completed using standard protocols (Rhodes *et al.*, 2001).

A phylogenetic tree was constructed using the Neighbor-Joining method with Jukes-Cantor distances (data not shown), aligning the entire 16S rRNA gene sequence of strain M175<sup>T</sup> with 23 selected *Mycobacterium* species. *Nocardia asteroides* and *N. farcinica* were used as outgroups in the reconstruction of the evolutionary relationships among M175<sup>T</sup> and other species within the genus *Mycobacterium*.

The sequences were aligned using the PILEUP program in the Wisconsin package of the Genetics Computer Group (Madison, Wis.). Aligned sequences were analyzed using the Molecular Evolutionary Genetics Analysis (MEGA) package version 1.01 (Kumar *et al.*, 1993), which also was used to generate bootstrap confidence values using 500 permutations of the data sets.

Cells grown on Middlebrook 7H10 agar were acid-fast coccobacilli (0.4 to 0.6  $\mu\text{m}$  by 0.8 to 1  $\mu\text{m}$ ), which tended to occur in cell aggregates. Cell branching or spores were not observed. On Middlebrook 7H10 agar isolates grew as dysgonic, rough and nonpigmented colonies of 0.5 to 1 mm after 4 to 6 weeks at 23°C. The dominant colony morphology was initially flat with a slightly irregular margin and becoming umbonate upon continued incubation. A second colony type was smooth, slightly raised and with a more entire margin. Little or no growth occurred at 30°C and none at 37°C or above. Colonies did not produce pigment following exposure to light for several

hours or upon prolonged exposure for several days. Phenotypic characteristics are presented in Table 1.

Representative mycolic acid chromatograms from M175<sup>T</sup> and *M. marinum* and *M. tuberculosis* are shown Fig. 1. Although M175<sup>T</sup> exhibited a single cluster of eight peaks that resembled reference patterns for species of the *M. tuberculosis* complex, peak elution times differed. Shorter elution times indicated that M175<sup>T</sup> contained more polar, shorter, carbon chain-length mycolic acids than species of the *M. tuberculosis* complex. A profile comparable to that obtained with M175<sup>T</sup> was not available in the *Mycobacterium* HPLC mycolic acid database at the Centers for Disease Control and Prevention.

The 140 bp signature region “A” of the 16S rRNA genes of isolates M115, M121, M148 and M175<sup>T</sup> was identical. This variable region of the 16S rRNA gene is sequence-specific for slowly-growing mycobacteria (Kirschner *et al.*, 1993) and between our isolates and *M. ulcerans* or *M. marinum* differed only by a single base pair. A phylogenetic tree reconstructed from the aligned 16S rRNA gene sequences of M175<sup>T</sup> and 23 other *Mycobacterium* spp, (Fig. 2) also indicated a close relationship with *M. ulcerans* and *M. marinum* within the slow growing *Mycobacterium* spp. The 16S rRNA genes of M175<sup>T</sup> differed from *M. ulcerans* by 3 insertions and 8 substitutions (one base of the *M. ulcerans* sequence in GenBank is N) and from *M. marinum* by 4 insertions and 7 substitutions (one base of the *M. marinum* sequence in Gen Bank is N). Most of these differences were located at the 3' end of the gene. We previously reported (Rhodes *et al.*, 2001) this close relationship based on the high sequence similarity in 16S rRNA gene sequences between M175<sup>T</sup> and *M. marinum* (99.2%), *M. ulcerans* (99.2%), *M. bovis* (98.7%) and *M. tuberculosis* (98.7%). Similar differences of a few nucleotides in the 16S rRNA gene sequence between other *Mycobacterium* spp. also have been reported (Tønjum *et al.*, 1998).

**Description of *Mycobacterium shottsii* sp. nov.** (shott'si.i.M. L.gen.n. *shottsii* of Shotts; named after Dr. Emmett Shotts, American fish bacteriologist) is an acid-fast coccobacillus (0.4 to 0.6  $\mu\text{m}$  by 0.8 to 1  $\mu\text{m}$ ) which may form cell aggregates in culture. The type strain was isolated from granulomatous lesions in splenic tissue from a striped bass (*Morone saxatilis*). Spores and cell branching are not present. Colonies on Middlebrook 7H10 agar are dysgonic, rough, nonpigmented, and typically flat with an irregular margin and becoming umbonate upon aging. Smooth colonies with an entire margin are less frequently seen. Visible colonies from a dilute inoculum are observed after 4-6 weeks incubation at 23°C. Little or no growth occurs at 30°C and none at 37°C or above. Isolates do not grow on MacConkey agar and Löwenstein-Jensen with 5% NaCl, are negative for arylsulfatase,  $\beta$ -galactosidase, nitrate reductase, pyrazinamidase (7d), semiquantitative catalase, Tween 80 hydrolysis, Tween opacity, and have variable reactions for acid phosphatase and catalase at 68°C. Positive pyrazinamidase reactions occur when incubation is extended to 14-21 days. Isolates are positive for urease and niacin production. Colonies do not produce pigment following exposure to light for several hours or upon prolonged exposure for several days. These mycobacteria tolerate isoniazid at 1  $\mu\text{g}/\text{ml}$  (but not at 10  $\mu\text{g}/\text{ml}$ ), thiacetazone, and thiophene-2-carboxylic hydrazide. Growth is inhibited in media containing hydroxylamine. Isolates are resistant to  $\alpha$ -aminosalicylic acid and isoniazid but susceptible to ethambutol, ethionamide, kanamycin, rifampin, and streptomycin in disc susceptibility tests. The mycolic acid HPLC pattern consists of a single cluster of eight peaks resembling reference patterns for species of the *M. tuberculosis* complex but that elute more rapidly. The 16S rRNA gene sequence is unique among species of *Mycobacterium* and is most similar to those of *M. ulcerans* and *M. marinum*. Strain M175<sup>T</sup> had been deposited in the American Type Culture Collection as ATCC 700981<sup>T</sup> and the National Collection of Type Cultures (UK) as NCTC 13215<sup>T</sup>.

Slowly growing mycobacteria which either grow poorly or not at all at 37°C or produce niacin are compared with *M. shottsii* in Table 2. Accumulation of niacin in culture media easily distinguishes *M. shottsii* from other slowly growing mycobacteria which grow optimally at

temperatures  $\leq 30^{\circ}\text{C}$ . In addition, *M. marinum* and *M. cookii* differ from *M. shottsii* by producing pigment. Urease activity by *M. shottsii* distinguishes it from *M. ulcerans* and *M. haemophilum*. Other slowly growing mycobacteria which may accumulate niacin are differentiated from *M. shottsii* by growth at  $37^{\circ}\text{C}$  and resistance profiles to inhibitory agents. *M. shottsii* differs from *M. chesapeaki*, a proposed species also isolated from striped bass (Heckert *et al.*, 2001), by tests for growth at  $37^{\circ}\text{C}$ , niacin production, pyrazinamidase activity (7d) and 16S rRNA gene sequence.

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Table 1. Characteristics of strain M175<sup>T</sup> and 20 similar isolates recovered from striped bass.

Characteristic	% of strains giving positive reaction*
Pigment production in the dark .....	0
Photochromogenicity .....	0
Growth at:	
23°C .....	100
30°C .....	38 (+)
37°C .....	0
42°C .....	0
Enzymatic activities	
Acid phosphatase .....	67 (+)
Arylsulfatase (14d).....	0
Catalase at 68°C .....	33 (-)
β-Galactosidase .....	0
Nitrate reductase .....	0
Production of niacin.....	100
Pyrazinamidase (7d).....	0
Semiquantitative catalase .....	0
Tween hydrolysis (10 d).....	0
Urease .....	95 (+)
Resistance to:	
Hydroxylamine (500 μg/ml) .....	0
Isoniazid (1 μg/ml) .....	100
Isoniazid (10 μg/ml) .....	5 (-)

p-Nitrobenzoic acid (500 µg/ml) .....	10 (-)
Oleic acid (0.25 µg/ml) .....	0
Sodium chloride (50 mg/ml) .....	0
Thiacetazone (10 µg/ml) .....	100
Thiophene-2-carboxylic hydrazide (2 µg/ml) .....	100
Resistance (disc method) to :	
p-Aminosalicylic acid (2 µg/ml) .....	80 (+)
p-Aminosalicylic acid (10 µg/ml) .....	10 (-)
Ethambutol (5 µg/ml) .....	0
Ethambutol (10 µg/ml) .....	0
Ethionamide (5 µg/ml) .....	0
Isoniazid (0.2 µg/ml) .....	100
Isoniazid (1 µg/ml) .....	100
Kanamycin (6 µg/ml) .....	0
Rifampin (1 µg/ml) .....	10 (-)
Rifampin (5 µg/ml) .....	0
Streptomycin (2 µg/ml) .....	0
Streptomycin (10 µg/ml) .....	0

\*For characteristics that vary among strains the reaction of the type strain is indicated in parentheses; +, positive; -, negative.

Table 2. Distinguishing characteristics of selected *Mycobacterium* species

Data for species other than *M. shottsii* sp. nov. cited from Levy-Frebault & Portaels 1992.

Abbreviations: N = nonpigmented; P = photochromogenic; S = scotochromogenic, + = at least 85% strains positive; - = less than 15% positive; M = 50-80% positive; F = 15 to 49 % positive; ND = not determined.

Characteristic	<i>M. shottsii</i> sp. nov.	<i>M. marinum</i>	<i>M. ulcerans</i>	<i>M. cookii</i>	<i>M. haemophilum</i>	<i>M. africanum</i>	<i>M. microtii</i>	<i>M. simiae</i>	<i>M. tuberculosis</i>
Growth at:									
23-25°C	+	+	+	+	+	-	ND	+	ND
30°C	F	+	+	+	+	M	ND	+	+
37°C	-	F	F	-	-	+	+	+	+
42°C	-	-	-	-	-	F	-	+	-
Pigmentation	N	P	N	S	N	N	N	P (M)	N
Resistance to:									
Isoniazid 1µg/ml	+	M	+	-	ND	-	ND	+	-
Isoniazid 10µg/ml	-	M	M	-	-	-	ND	M	-
TCH	+	+	+	ND	+	-	-	+	+
Hydroxylamine	-	+	F	ND	+	-	ND	+	-
□-Nitrobenzoic	-	M	-	ND	-	-	-	+	-

acid									
NaCl	-	-	-	ND	-	-	-	-	-
Thiacetazone	+	M	+	ND	ND	-	-	+	M
Oleate	-	F	ND	+	ND	ND	ND	+	M
Catalase									
activity:									
>45 mm	-	F	-	ND	-	-	-	+	-
heat stable	F	+	-	-	-	-	-	+	-
Tween 80	-	+	-	-	-	F	-	-	M
hydrolysis									
Urease	+	+	F	-	-	+	+	+	+
Niacin	+	-	-	-	-	F	+	F	+
production									
Nitrate reduction	-	-	-	+	-	M	-	-	+
Acid	M	+	-	+	+	ND	ND	-	M
phosphatase									
Arylsulfatase	-	F	ND	ND	ND	-	-	-	-
(3d)									
Pyrazinamidase	-	M	ND	ND	ND	ND	ND	+	+
(7d)									

## Text for Figures

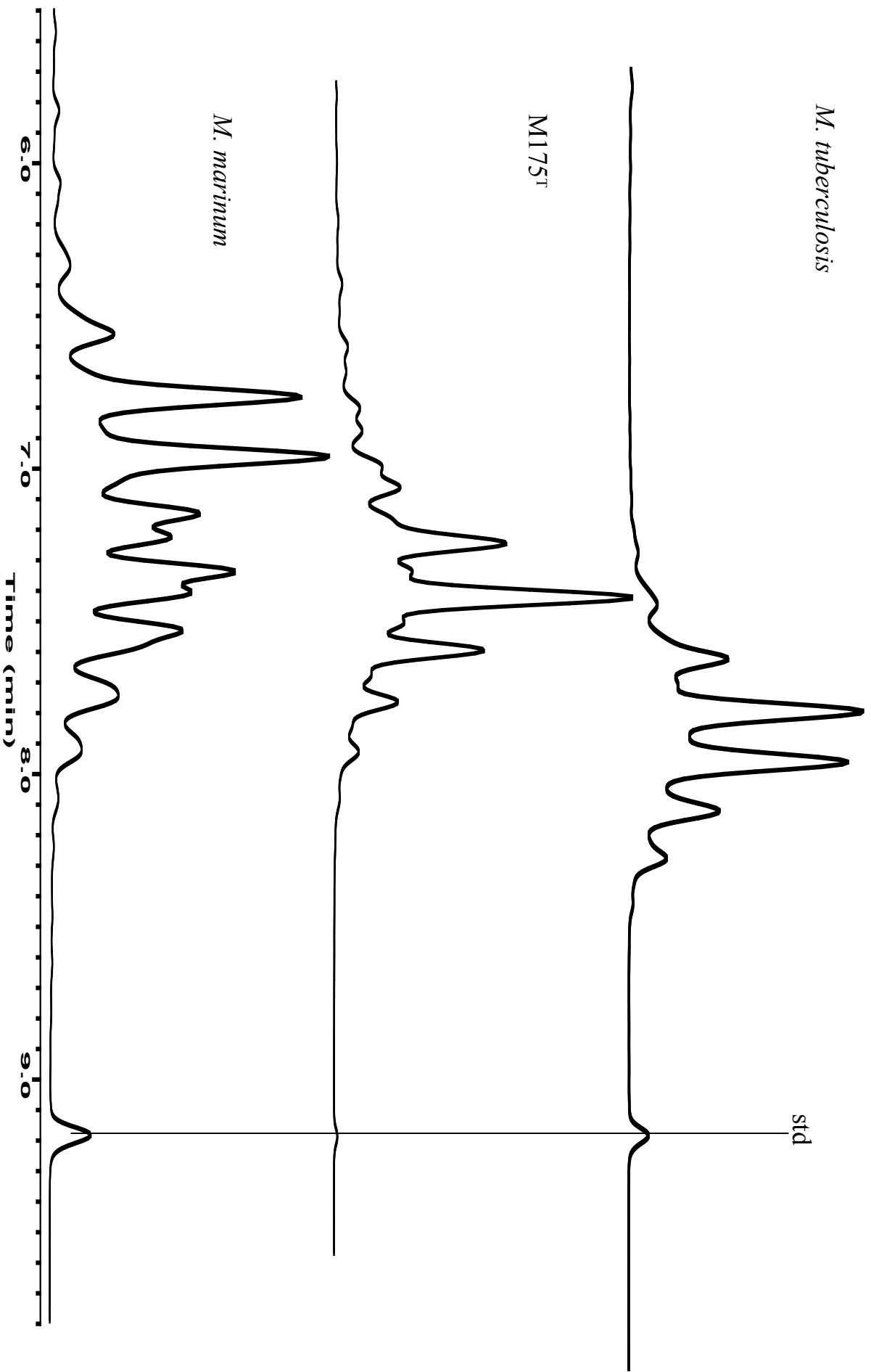
Figure 1. Comparison of mycolic acid HPLC profiles for M175<sup>T</sup>, *M. marinum* and *M. tuberculosis*.

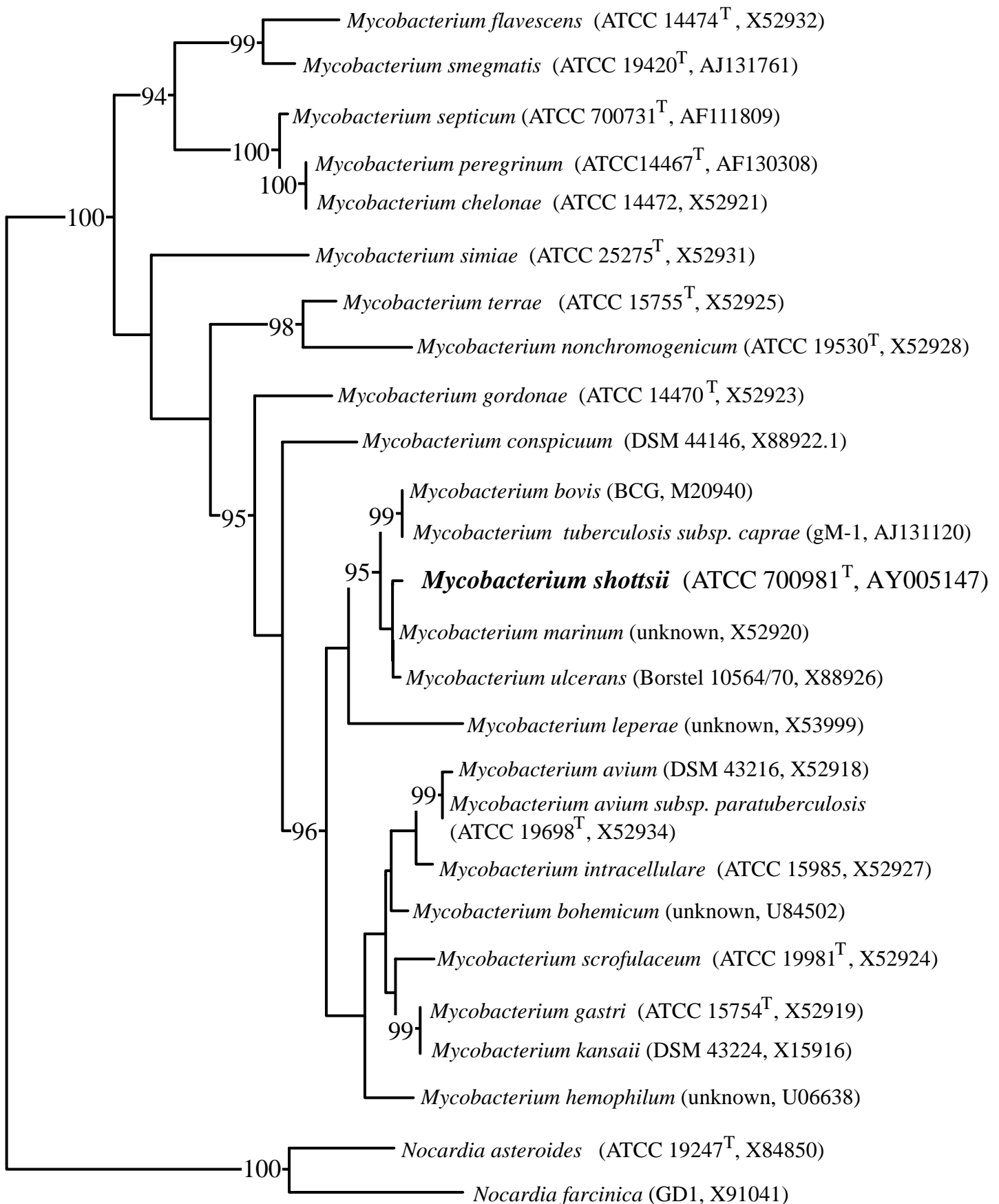
Figure 2. Phylogenetic relationship of, *M. shottsii* M175<sup>T</sup> among other *Mycobacterium* spp. based upon sequences of the 16S rRNA gene. Jukes-Cantor distances were derived from the aligned sequences to construct an optimal tree using the Neighbor-Joining (NJ) method. Five hundred replicate trees were generated in a bootstrap analysis to derive a majority consensus tree. The levels of support for the presence of nodes are indicated in the NJ tree. Genbank accession numbers and strain numbers for sequences used to construct the tree are shown in parentheses following each species.

*M. tuberculosis*

M175<sup>T</sup>

*M. marinum*





0 .01