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

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Note

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

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Slowly growing, non-pigmented mycobacteria were isolated from striped bass (*Morone saxatilis*) during an epizootic of mycobacteriosis in the Chesapeake Bay. Growth characteristics, acid-fastness and results of 16S rRNA gene sequencing 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 from profiles of reference species of the *Mycobacterium tuberculosis* complex. One isolate (M175^T) was placed within the slowly growing mycobacteria by analysis of aligned 16S rRNA gene sequences and was proximate in phylogeny to *Mycobacterium ulcerans* and *Mycobacterium marinum*. However, distinct nucleotide differences were detected in the 16S rRNA gene sequence among M175^T, *M. ulcerans* and *M. marinum* (99·2 % similarity). Isolate M175^T could be differentiated from other slowly growing, non-pigmented 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^{-1}), thiacetazone and thiophene-2-carboxylic hydrazide. Based upon these genetic and phenotypic differences, isolate M175^T (=ATCC 700981^T =NCTC 13215^T) is proposed as the type strain of a novel species, *Mycobacterium shottsii* sp. nov.

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 homogeneous group that, 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 novel taxon. In this report, we describe the results of a taxonomic study of these isolates and propose that they are representative of a novel species, *Mycobacterium shottsii* sp. nov.

Isolate M175^T 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 and all others were from the spleen. Additional isolates included M115, M120, M121, M148, M177, M179, M182, M200, M202, M203, M205, M208, M210, M211 and M217–M220. Growth and biochemical testing included reference strains of *Mycobacterium avium*

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The GenBank accession number for the 16S rRNA sequence of *Mycobacterium shottsii* M175^T is AY005147.

(M1), *Mycobacterium chelonae* (M3), *Mycobacterium flavescens* (M4), *Mycobacterium fortuitum* (M6), *Mycobacterium gordonae* (M8), *Mycobacterium kansasii* (M10), *Mycobacterium marinum* (M11–M13), *Mycobacterium nonchromogenicum* (M14), *Mycobacterium phlei* (M15), *Mycobacterium scrofulaceum* (M17), *Mycobacterium simiae* (M19, M20) and *Mycobacterium 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; Vincent Lévy-Frébault & Portaels, 1992): production of acid phosphatase, arylsulfatase, catalase, β-galactosidase, nitrate reductase, niacin, pyrazinamidase, Tween 80 hydrolysis, urease and growth on media containing hydroxylamine (500 μg ml^{-1}), isoniazid (1 and 10 μg ml^{-1}), *p*-nitrobenzoic acid (500 μg ml^{-1}), NaCl (50 mg ml^{-1}), thiacetone (10 μg ml^{-1}) and thiophene-2-carboxylic hydrazide (TCH; 2 μg ml^{-1}). Drug susceptibility tests were performed using the disc method (Kent & Kubica, 1985).

HPLC was used to analyse mycolic acids from four isolates (M115, M121, M148 and M175^T) 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 UV analysis (Butler *et al.*, 1996). Differentiation of specific *Mycobacterium* species was by a visual decision method using an internal size standard, an identification process reviewed recently by Butler & Guthertz (2001).

Sequencing of 140 bp for the signature region 'A' of the 16S rRNA gene from four isolates (M115, M121, M148 and M175^T) was completed using standard protocols (Rhodes *et al.*, 2001).

A phylogenetic tree was constructed using the neighbour-joining method with Jukes–Cantor distances (data not shown), aligning the entire 16S rRNA gene sequence of strain M175^T with 23 selected *Mycobacterium* species. *Nocardia asteroides* and *Nocardia farcinica* were used as outgroups in the reconstruction of the evolutionary relationships among M175^T and other species within the genus *Mycobacterium*. The sequences were aligned using the program PILEUP in the Wisconsin package of the Genetics Computer Group (Madison, WI, USA). Aligned sequences were analysed using the Molecular Evolutionary Genetics Analysis (MEGA) package version 1.01 (Kumar *et al.*, 1993), which was also used to generate bootstrap confidence values using 500 permutations of the datasets.

Cells grown on Middlebrook 7H10 agar were acid-fast coccobacilli (0·4–0·6 × 0·8–1 μm) that tended to occur in cell aggregates. Cell branching and spores were not observed. On Middlebrook 7H10 agar, isolates grew as dysgonic, rough and non-pigmented colonies of 0·5–1 mm after 4–6 weeks at 23 °C. The dominant colony morphology was initially flat with a slightly irregular margin, 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 that gave varying results for the novel isolates are presented in Table 1. Other characteristics are given in the species description below.

Representative mycolic acid chromatograms from isolate M175^T and *M. marinum* and *Mycobacterium tuberculosis* are shown Fig. 1. Although isolate M175^T 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 isolate M175^T contained more polar, shorter carbon chain-length mycolic acids than species of the *M. tuberculosis* complex. A profile comparable to that obtained with isolate M175^T 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^T 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 *Mycobacterium ulcerans* or *M. marinum*, differed by only a single base pair. A

Table 1. Characteristics that gave variable results for strain M175^T and 20 similar isolates recovered from striped bass

The reaction of the type strain is indicated in parentheses.

Characteristic	Positive strains (%)
Growth at:	
23 °C	100
30 °C	38 (+)
37 °C	0
Enzyme activities:	
Acid phosphatase	67 (+)
Catalase at 68 °C	33 (−)
Urease	95 (+)
Resistance to:	
Isoniazid (1 μg ml^{-1})	100
Isoniazid (10 μg ml^{-1})	5 (−)
p-Nitrobenzoic acid (500 μg ml^{-1})	10 (−)
Resistance (disc method) to:	
p-Aminosalicylic acid (2 μg ml^{-1})	80 (+)
p-Aminosalicylic acid (10 μg ml^{-1})	10 (−)
Rifampicin (1 μg ml^{-1})	10 (−)
Rifampicin (5 μg ml^{-1})	0

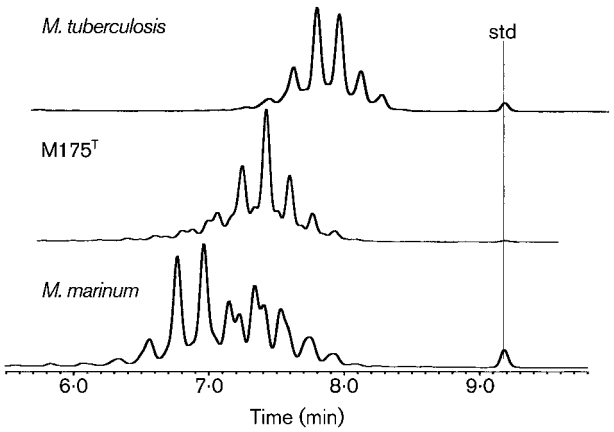

Fig. 1. Comparison of mycolic acid HPLC profiles for isolate M175^T, *M. marinum* and *M. tuberculosis*. std, Internal standard.

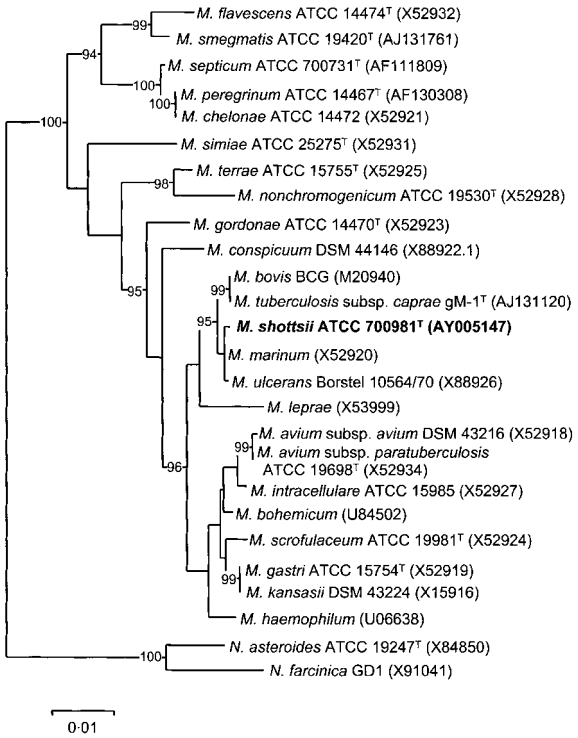

Fig. 2. Phylogenetic relationships of isolate M175^T among other *Mycobacterium* species based on sequences of the 16S rRNA gene. Jukes–Cantor distances were derived from the aligned sequences to construct an optimal tree using the neighbour-joining method. Five hundred replicate trees were generated in a bootstrap analysis to derive a majority consensus tree. Levels of support for the presence of nodes are indicated in the tree. GenBank accession numbers for sequences used to construct the tree are shown in parentheses. Bar, 0·01 substitutions per nucleotide position.

phylogenetic tree reconstructed from the aligned 16S rRNA gene sequences of isolate M175^T and 23 other *Mycobacterium* species (Fig. 2) also indicated a close relationship with *M. ulcerans* and *M. marinum* within the slow-growing *Mycobacterium* species. The 16S rRNA gene of isolate M175^T differed from *M. ulcerans* by three insertions and eight substitutions (one base of the *M. ulcerans* sequence in GenBank is N) and from *M. marinum* by four insertions and seven substitutions (one base of the *M. marinum* sequence in GenBank is N). Most of these differences were located at the 3′ end of the gene. We reported this close relationship previously (Rhodes *et al.*, 2001) based on the high sequence similarity in 16S rRNA gene sequences between isolate M175^T and *M. marinum* (99·2 %), *M. ulcerans* (99·2 %), *Mycobacterium bovis* (98·7 %) and *M. tuberculosis* (98·7 %). Similar differences of a few nucleotides in 16S rRNA gene sequences between other *Mycobacterium* species have been reported previously (Tønjum *et al.*, 1998).

Slowly growing mycobacteria that grow either poorly or not at all at 37 °C or produce niacin are compared with the novel isolates in Table 2. Accumulation of niacin in culture media easily distinguishes the novel isolates from other slowly growing mycobacteria that grow optimally at temperatures $\leqslant$30 °C. In addition, *M. marinum* and *Mycobacterium cookii* differ from the novel isolates by producing pigment. The presence of urease activity in the novel isolates distinguishes them from *M. ulcerans* and *Mycobacterium haemophilum*. Other slowly growing mycobacteria that may accumulate niacin are differentiated from the novel isolates by growth at 37 °C and resistance profiles to inhibitory agents. The novel isolates differ from '*Mycobacterium chesapeaki*', a proposed species also isolated from striped bass (Heckert *et al.*, 2001), by tests for growth at 37 °C, niacin production, pyrazinamidase activity (7 days) and 16S rRNA gene sequence. These results indicate that isolate M175^T represents a novel member of the genus *Mycobacterium*, for which the name *Mycobacterium shottsii* sp. nov. is proposed.

Description of *Mycobacterium shottsii* sp. nov.

Mycobacterium shottsii (shott′si.i. N.L. gen. n. *shottsii* of Shotts, named after Emmett Shotts, an American fish bacteriologist).

Acid-fast coccobacilli (0·4–0·6 × 0·8–1 μm) that may form cell aggregates in culture. Spores and cell branching are not present. Colonies on Middlebrook 7H10 agar are dysgonic, rough, non-pigmented and typically flat with an irregular margin, becoming umbonate upon ageing. Smooth colonies with an entire margin are seen less frequently. 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 or Löwenstein–Jensen medium with 5 % NaCl, are negative for arylsulfatase (14 days), β-galactosidase, nitrate reductase, pyrazinamidase

Table 2. Distinguishing characteristics of selected *Mycobacterium* species

Species: 1, *M. shottsii* sp. nov.; 2, *M. marinum*; 3, *M. ulcerans*; 4, *M. cookii*; 5, *M. haemophilum*; 6, *M. africanum*; 7, *M. microtii*; 8, *M. simiae*; 9, *M. tuberculosis*. Data for species other than *M. shottsii* sp. nov. were taken from Vincent Lévy-Frébault & Portaels (1992). Characteristics are scored as: +, ⩾85 % strains positive; M, 50–80 % positive; F, 15–49 % positive; −, <15 % positive; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9
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 (1 μg ml^{-1})	+	M	+	−	ND	−	ND	+	−
Isoniazid (10 μg ml^{-1})	−	M	M	−	−	−	ND	M	−
TCH	+	+	+	ND	+	−	−	+	+
Hydroxylamine	−	+	F	ND	+	−	ND	+	−
p-Nitrobenzoic acid	−	M	−	ND	−	−	−	+	−
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 hydrolysis	−	+	−	−	−	F	−	−	M
Urease	+	+	F	−	−	+	+	+	+
Niacin production	+	−	−	−	−	F	+	F	+
Nitrate reduction	−	−	−	+	−	M	−	−	+
Acid phosphatase	M	+	−	+	+	ND	ND	−	M
Arylsulfatase (3 days)	−	F	ND	ND	ND	−	−	−	−
Pyrazinamidase (7 days)	−	M	ND	ND	ND	ND	ND	+	+

*N, Non-pigmented; P, photochromogenic; S, scotochromogenic.

(7 days), semiquantitative catalase, Tween 80 hydrolysis and 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. 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. Tolerates isoniazid at 1 μg ml^{-1} (but not at 10 μg ml^{-1}), thiacetazone and TCH. Growth is inhibited in media containing hydroxylamine. Resistant to *p*-aminosalicylic acid and isoniazid but susceptible to ethambutol, ethionamide, kanamycin, rifampicin 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 the peaks 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*. The type strain, isolate M175^T (=ATCC 700981^T =NCTC 13215^T), was isolated from granulomatous lesions in splenic tissue from a striped bass (*Morone saxatilis*).

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