5-2005

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

MW Rhodes
Virginia Institute of Marine Science

H Kator
Virginia Institute of Marine Science

et al

I Kaattari
Virginia Institute of Marine Science

Kimberly S. Reece
Virginia Institute of Marine Science

See next page for additional authors

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

Martha W. Rhodes,[1] Howard Kator,[1] Alan McNabb,[2] Caroline Deshayes,[3] Jean-Marc Reyrat,[3] Barbara A. Brown-Elliott,[4] Richard Wallace Jr,[4] Kristin A. Trott,[5] John M. Parker,[6] Barry Lifland,[7] Gerard Osterhout,[8] Ilsa Kaattari,[1] Kimberly Reece,[1] Wolfgang Vogelbein[1] and Christopher A. Ottinger[9]

Correspondence
Martha W. Rhodes
martha@vims.edu

[1]Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062, USA

[2]Laboratory Services, British Columbia Centre for Disease Control, Vancouver BC, Canada V5Z 4R4

[3]Avenir Group, Inserm U570, Avenir Group, Faculté de Médecine Necker-Enfants Malades, 75730 Paris Cedex 15, France

[4]Mycobacteria/Nocardia Laboratory, University of Texas Health Center, Tyler, TX 75708, USA

[5]Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA

[6]Office of Laboratory Animal Care, University of California, Berkeley, CA 94720, USA

[7]Department of Comparative Medicine, Stanford University, Stanford, CA 94305, USA

[8]MIDI, Inc. Research and Development, Newark, DE 19713, USA

[9]US Geological Survey, Leetown Science Center, National Fish Health Research Laboratory, Kearneysville, WV 25430, USA

A group of slowly growing photochromogenic mycobacteria was isolated from Chesapeake Bay striped bass (*Morone saxatilis*) during an epizootic of mycobacteriosis. Growth characteristics, acid-fastness and 16S rRNA gene sequencing results were consistent with those of the genus *Mycobacterium*. Biochemical reactions, growth characteristics and mycolic acid profiles (HPLC) resembled those of *Mycobacterium shottsii*, a non-pigmented mycobacterium also isolated during the same epizootic. Sequencing of the 16S rRNA genes, the gene encoding the exported repeated protein (*erp*) and the gene encoding the 65 kDa heat-shock protein (*hsp65*) and restriction enzyme analysis of the *hsp65* gene demonstrated that this group of isolates is unique. Insertion sequences associated with *Mycobacterium ulcerans*, IS*2404* and IS*2606*, were detected by PCR. These isolates could be differentiated from other slowly growing pigmented mycobacteria by their inability to grow at 37 °C, production of niacin and urease, absence of nitrate reductase, negative Tween 80 hydrolysis and resistance to isoniazid (1 μg ml^{-1}), *p*-nitrobenzoic acid, thiacetazone and thiophene-2-carboxylic hydrazide. On the basis of this polyphasic study, it is proposed that these isolates represent a novel species, *Mycobacterium pseudoshottsii* sp. nov. The type strain, L15^T, has been deposited in the American Type Culture Collection as ATCC BAA-883^T and the National Collection of Type Cultures (UK) as NCTC 13318^T.

Published online ahead of print on 23 December 2004 as DOI 10.1099/ijs.0.63343-0.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *hsp65* gene sequences of *Mycobacterium pseudoshottsii* ATCC BAA-883^T are AY570988 and AY571788, respectively.

Aligned *erp* gene sequences of *Mycobacterium pseudoshottsii* sp. nov., *Mycobacterium marinum*, *Mycobacterium shottsii*, *Mycobacterium ulcerans* and *Mycobacterium tuberculosis* are available as a supplementary figure in IJSEM Online.

Since the early 1990s, the availability of a variety of molecular techniques for characterizing bacteria has had a major impact on mycobacterial taxonomy. The greater discriminatory power of molecular methods compared with traditional phenotypic techniques has resulted in a dramatic increase in the identification of previously unrecognized species (Tortoli, 2003). These new taxa comprise isolates of both clinical and environmental origin. DNA-based methods are particularly valuable for characterizing the latter, which typically have lower growth-temperature preferences and biochemical characteristics that often do not allow conclusive species identification using diagnostic techniques developed almost exclusively for clinical isolates.

The primary pathogens of mycobacteriosis in fish are reported to be *Mycobacterium abscessus* (formerly *Mycobacterium chelonae* subsp. *abscessus*), *Mycobacterium chelonae*, *Mycobacterium fortuitum* and *Mycobacterium marinum* (Belas *et al.*, 1995). Recently, three previously unrecognized species were isolated: '*Mycobacterium chesapeaki*' (Heckert *et al.*, 2001) and *Mycobacterium shottsii* from an epizootic in striped bass (*Morone saxatilis*) (Rhodes *et al.*, 2003), and *Mycobacterium montefiorense* from captive moray eels (*Gymnothorax funebris* and *Gymnothorax moringa*) (Levi *et al.*, 2003). Characterization using molecular methods allowed discrimination of *Mycobacterium shottsii* and *Mycobacterium montefiorense* from the clinically significant related species *Mycobacterium marinum* (Dobos *et al.*, 1999) and *Mycobacterium triplex*, respectively (Floyd *et al.*, 1996; Hazra *et al.*, 2001).

During an epizootic of mycobacteriosis in Chesapeake Bay striped bass, a variety of mycobacteria were isolated and then characterized using traditional biochemical and growth features (Rhodes *et al.*, 2004). In this study, we report the isolation, from striped bass, of a group of mycobacteria phenotypically resembling *Mycobacterium shottsii*, which shares with *Mycobacterium marinum* a cladic relationship with *Mycobacterium tuberculosis*, *Mycobacterium bovis* and several other species (Goodfellow & Magee, 1998). Polyphasic studies involving biochemical testing, mycolic acid analysis, sequencing of the 16S rRNA gene, the 65 kDa heat-shock-protein gene (*hsp65*) and the exported repetitive protein (*erp*) gene, and gene amplification of *Mycobacterium ulcerans*-specific insertion sequences (IS*2404* and IS*2606*) indicate that these organisms belong to a novel species.

All of the mycobacterial isolates (L1, L6, L9, L15^T, L23, L25, L28, L31, L32, L37, L39, L43, L50, L58, L62, R97, R129, R174, R177 and R179) examined in this study were isolated from *Morone saxatilis* collected from Chesapeake Bay (the largest estuary in the US and bordered by the states of Maryland and Virginia) or one of its tributaries (i.e. York and Rappahannock rivers). Splenic tissue was dissected aseptically, homogenized in phosphate buffer, spread-plated on Middlebrook 7H10 agar (Difco/Becton Dickinson Diagnostic Systems) with oleic acid/albumin/dextrose/catalase (OADC) enrichment, then incubated at 23 °C for 3 months. Growth and biochemical testing included reference strains of *Mycobacterium avium* (M1), *Mycobacterium fortuitum* (M6), *Mycobacterium gordonae* (M8), *Mycobacterium kansasii* (M10) and *Mycobacterium marinum* (M11) (all of which were obtained from the Environmental Protection Agency, Cincinnati, OH, USA, and Consolidated Laboratory Services, Commonwealth of Virginia, Richmond, VA, USA), *Mycobacterium ulcerans* (ATCC 19423^T) and *Mycobacterium shottsii* M175^T (previously deposited, from our laboratory, in the American Type Culture Collection, Manassas, VA, USA as ATCC 700981^T).

Colony morphology and the ability to grow at temperatures ranging from 23 to 37 °C were determined after 1 and 2 months incubation on Middlebrook 7H10 agar with OADC enrichment. The following characters were assessed at 23 °C: production of acid phosphatase, arylsulfatase after 3 and 14 days, catalase after heating at 68 °C, β-galactosidase, nitrate reductase, niacin, pyrazinamidase, semi-quantitative catalase, Tween 80 hydrolysis, urease and growth on Löwenstein–Jensen medium containing NaCl (50 mg ml^{-1}) (Kent & Kubica, 1985; Lutz, 1995). The proportion method (Kent & Kubica, 1985; Lévy-Frébault & Portaels, 1992) was used to determine drug susceptibility. Testing media were prepared by adding hydroxylamine, isoniazid, *p*-nitrobenzoic acid, thiacetone and thiophene-2-carboxylic hydrazide to Middlebrook 7H10 agar with OADC; alternatively, the desired drug concentration was achieved by elution from submerged discs (BBL/Becton Dickinson Diagnostic Systems). Oleate resistance was determined in the same manner using Dubos oleic acid agar (control) (BBL/Becton Dickinson Diagnostic Systems) and the same base medium supplemented with oleic acid.

The results of HPLC analysis of mycolic acids from L15^T and *Mycobacterium shottsii* M175^T were compared using the Sherlock Mycobacteria Identification System (SMIS; MIDI). Cells from cultures grown on Middlebrook 7H10 agar with OADC were saponified, extracted and derivatized according to the manufacturer's instructions and loaded onto an Agilent ChemStation HPLC (Agilent Technologies). Separation of the mycolic acids was achieved by using a gradient of methanol and 2-propanol. Results were analysed using MIDI Sherlock software (version 1.0).

The 16S rRNA genes of isolates L15^T and *Mycobacterium shottsii* M175^T were sequenced by using published methods (Lane, 1991; Turenne *et al.*, 2003); the BLASTN tool (http://www.ncbi.nlm.nih.gov/blast) was then used to compare the sequences with those in the GenBank database. The sequence for L15^T was submitted to the GenBank database under accession number AY570988. The 16S rRNA gene sequence for *Mycobacterium shottsii* was modified and the original sequence updated in GenBank (AY005147). Phylogenetic analysis of the 16S rRNA gene was performed by neighbour-joining based on the uncorrected (*P*) distances and by maximum parsimony and maximum likelihood using PAUP version 4.0b10 (Swofford, 2002). Parsimony was performed with the gaps treated as missing. The model for

the maximum-likelihood analyses was determined by MODELTEST 3.06 (Posada & Crandall, 1998) to determine the model which best fit the data based on hierarchical tests of models by calculating the likelihood ratio statistic for each model and its associated *P* value. We suspected a serious error at the 3′ end of the GenBank-deposited '*Mycobacterium chesapeaki*' sequence. An approximately 320 bp fragment of the 3′ end of the sequence is unlikely to be a valid *Mycobacterium* species sequence, but rather is corrupted by either severe errors in sequence determination or contamination by foreign DNA. Therefore, the distance and maximum-likelihood analyses were performed on a dataset of 1120 aligned nucleotide positions from the 5′ end of the *Mycobacterium* species 16S rRNA gene sequences.

The DNA sequence of the central region of the *erp* gene, corresponding to amino acid residues 81–174 (de Mendonça-Lima *et al.*, 2001), was obtained by sequencing PCR fragments generated from genomic DNA recovered from *Mycobacterium shottsii* M175^T and L1, L6 and L15^T as described previously (Pelicic *et al.*, 1996) but with minor modifications. The primers used for amplification by *Pfu* Turbo *Taq* polymerase (Stratagene) (30 cycles) were P1 (5′-atgaccgcgctgtcgcagggt-3′) and P2 (5′-ggggtcacccaggatcggata-3′); the annealing temperature was 55 °C. PCR fragments were sequenced and subsequently analysed with DNA Strider (Marck, 1988). The sequences were submitted to GenBank under the following accession numbers: *Mycobacterium shottsii* M175^T, AY496288; L1, AY496289; L6, AY496290; and L15^T, AY496291. The accession numbers of the *erp* gene sequences of other mycobacterial species used for the phylogenetic analyses were as follows: *Mycobacterium tuberculosis*, L38851; *Mycobacterium ulcerans*, AF213154; and *Mycobacterium marinum*, AF213153. Protein sequences were generated by virtual translations using MacVector version 7.2 (Accelrys) and aligned using the CLUSTAL W alignment program in the MacVector package. DNA sequences were also aligned using CLUSTAL W and gap positions were adjusted to correspond with those in the amino acid alignment. Phylogenetic analysis was performed as described above for the 16S rRNA gene except that parsimony was performed with the gap informative feature (gaps treated as a fifth nucleotide state).

DNA amplification of the *hsp65* gene was accomplished by using the method described by Telenti *et al.* (1993). Sequencing of the *hsp65* fragment was performed on an ABI Prism 310 or 3100 genetic analyser (Applied Biosystems) with the Big Dye Terminator Cycle Sequencing Ready Reaction kit, version 3.0 or version 3.1 (Applied Biosystems) as described previously, with the modification that 10 ng DNA was used with the Tb11 (forward) primer and 30 ng DNA was used with the Tb12 (reverse) primer in all sequencing reactions (Kapur *et al.*, 1995). *hsp65* sequences were determined for L1, L6, L15^T and *Mycobacterium shottsii* M175^T and compared with existing sequences in GenBank and the *hsp65* gene database of the British Columbia CDC Laboratory Services, Vancouver, Canada. The GenBank accession number for the *hsp65* sequence of L15^T is AY571788. Phylogenetic analysis was performed using PAUP*, version 4.0b10 (Swofford, 2002). Sequences were aligned and phylogenetic analyses were as described above for the 16S rRNA gene.

Isolate L15^T, *Mycobacterium shottsii* M175^T and *Mycobacterium ulcerans* ATCC 19423^T were evaluated by using PCR amplification and restriction endonuclease analysis of the 441 bp *hsp65* gene sequence as described previously (Steingrube *et al.*, 1995; Brown *et al.*, 1999; Telenti *et al.*, 1993). Cells were grown and harvested on Middlebrook 7H10 agar and ground cell supernatants were used as DNA templates (Steingrube *et al.*, 1995). Appropriate positive and negative controls, as recommended by Telenti *et al.* (1993), were included. Restriction endonucleases *Bst*EII and *Hae*III (Promega) were selected (Steingrube *et al.*, 1995) and used according to the manufacturer's recommendations. Restriction fragments were electrophoresed on 3 % Metaphor agarose (4 bp resolution; FMC Bioproducts) containing ethidium bromide in a Mini-Sub cell electrophoresis system (Bio-Rad) (Brown *et al.*, 1999). Fragment sizes (in bp) were estimated on a computerized Bio Image system (Millipore).

DNA amplification of the *Mycobacterium ulcerans*-specific insertion sequences, IS*2404* and IS*2606*, was performed with primer sets MU5/MU6 and MU7/MU8, respectively, as described by Stinear *et al.* (1999). DNA was extracted from cultures of L15^T, *Mycobacterium shottsii* and *Mycobacterium ulcerans* and PCR amplifications were performed as described previously (Trott *et al.*, 2004). PCRs were performed using a variety of DNA polymerases: Ampli*Taq* Gold DNA polymerase (Applied Biosystems), *Taq* DNA polymerase (Invitrogen), *Taq* DNA polymerase (Roche) and Platinum *Taq* DNA polymerase High Fidelity (Invitrogen).

Mycobacterial cells grown on Middlebrook 7H10 agar were acid-fast coccobacilli, small (approx. 0·4–0·6 μm by 0·8–1 μm) and tended to clump. Neither cell branching nor spores was observed. On Middlebrook 7H10 agar, isolates grew as rough colonies that were approximately 1–3 mm in diameter after incubation for 2 months at 23 °C and were not emulsifiable in water. Colonies were rough, initially flat with a slightly irregular margin, becoming umbonate upon continued incubation. Little or no growth occurred at 30 °C and none occurred at 37 °C. Colonies slowly became gold following exposure to light. Phenotypic features that varied between isolates are presented in Table 1. Other characteristics are listed in the species description below.

Mycobacterium shottsii M175^T and isolate L15^T mycolic acid patterns obtained by HPLC analysis using SMIS were similar but showed small quantitative differences (Fig. 1).

The BLASTN algorithm (http://www.ncbi.nlm.nih.gov/blast) was used to compare the 16S rRNA gene (1453 bp) of isolate L15^T with those of other mycobacterial sequences

Table 1. Characteristics that gave variable results for 19 similar isolates of *Mycobacterium pseudoshottsii* sp. nov.

The reaction of the type strain is indicated in parentheses.

Characteristic	Percent positive
Enzyme activities:	
Acid phosphatase	53 (−)
Catalase at 68 °C	50 (−)
Niacin production	95 (+)
Resistance to:	
Isoniazid (1 μg ml^{-1})	100
Isoniazid (10 μg ml^{-1})	5 (−)
p-Nitrobenzoic acid (500 μg ml^{-1})	84 (+)
Resistance (disc method) to:	
Ethambutol (5 μg ml^{-1})	16 (−)
Ethambutol (10 μg ml^{-1})	0
Ethionamide (5 μg ml^{-1})	33 (+)
p-Aminosalicylic acid (2 μg ml^{-1})	100
p-Aminosalicylic acid (10 μg ml^{-1})	95 (+)
Rifampicin (1 μg ml^{-1})	53 (+)
Rifampicin (5 μg ml^{-1})	0

deposited in GenBank. *Mycobacterium marinum* strains eilaticum DL240490 (GenBank/EMBL/DDBJ accession no. AF456238) and hellenicum DL045 (AF456241) differed by only 1 and 2 nt, respectively, resulting in 99·9 % similarity to isolate L15^{T}. '*Mycobacterium liflandii*' (AY500838), *Mycobacterium ulcerans* (X58954) and *Mycobacterium shottsii* M175^{T} (AY005147) had similarity values of 99·7, 99·5 and 99·4 %, respectively, with respect to isolate L15^{T}. There were eight nucleotide differences between L15^{T} and *Mycobacterium shottsii* with four of these (corresponding to *Escherichia coli* numbering positions 96, 104, 242 and 492) occurring near or within hypervariable regions A and B and the remainder occurring nearer the 3′ end (positions 973, 1012, 1220 and 1449). Both the hypervariable regions (Rogall *et al.*, 1990; Springer *et al.*, 1996) and differences near the 3′ end have been used to distinguish species (Tønjum, 1998; Portaels *et al.*, 1996). 16S rRNA gene sequences of closely related species may be identical or may differ by only a few nucleotides (Tortoli, 2003). In the phylogenetic analyses, trees of similar topology were obtained by neighbour-joining (Fig. 2), maximum parsimony and maximum likelihood. Although the neighbour-joining and maximum-likelihood analyses were done on a dataset with aligned truncated (approx. 320 bp shorter) sequences, the topology of the trees was similar to that when the complete dataset with the suspected corrupted portion of the '*Mycobacterium chesapeaki*' 16S sequence was included, except that branch length on the '*Mycobacterium chesapeaki*' sequence was considerably shorter with the revised dataset.

The DNA sequence of the gene encoding the Erp protein, a ubiquitous extracellular protein found in all mycobacterial species tested, has been shown to be species-specific (de Mendonça-Lima *et al.*, 2001, 2003). The central region of the Erp protein consists of tandem repeats of five amino acids that vary in terms of the numbers of repeats and the sequence of the repeat between mycobacterial species. In this study, sequence analysis of the central region of the *erp* gene revealed significant differences between isolates L1, L6 and L15^{T}, which were all identical to each other, and *Mycobacterium marinum*, *Mycobacterium shottsii* M175^{T} and *Mycobacterium ulcerans*. L1, L6 and L15^{T} had a reduced number of repeats (30 nt), as well as 13 substitutions, in comparison with *Mycobacterium shottsii* (*erp* sequences are available as a supplementary figure in IJSEM Online).

The *hsp65* gene, encoding heat-shock protein, has been used to distinguish between different mycobacterial species (Devallois *et al.*, 1997; Leclerc *et al.*, 2000). The sequences of the *hsp65* genes (401 nt, primers removed) from L1, L6 and L15^{T} were identical and differed from that of *Mycobacterium shottsii* M175^{T} by three nucleotides. When

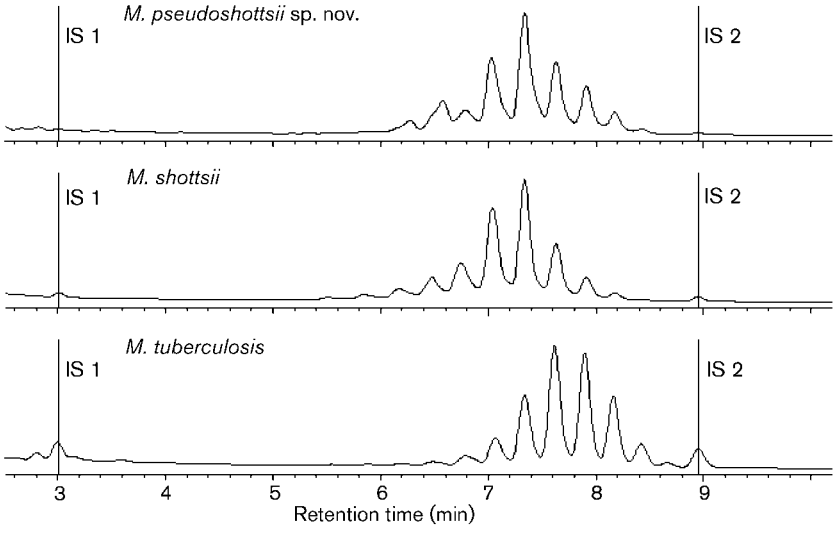

Fig. 1. Comparison of mycolic acid HPLC profiles for *Mycobacterium pseudoshottsii* sp. nov. (strain L15^{T}), *Mycobacterium shottsii* ATCC 700981^{T} and *Mycobacterium tuberculosis* ATCC 27294^{T}. IS, Internal standard.

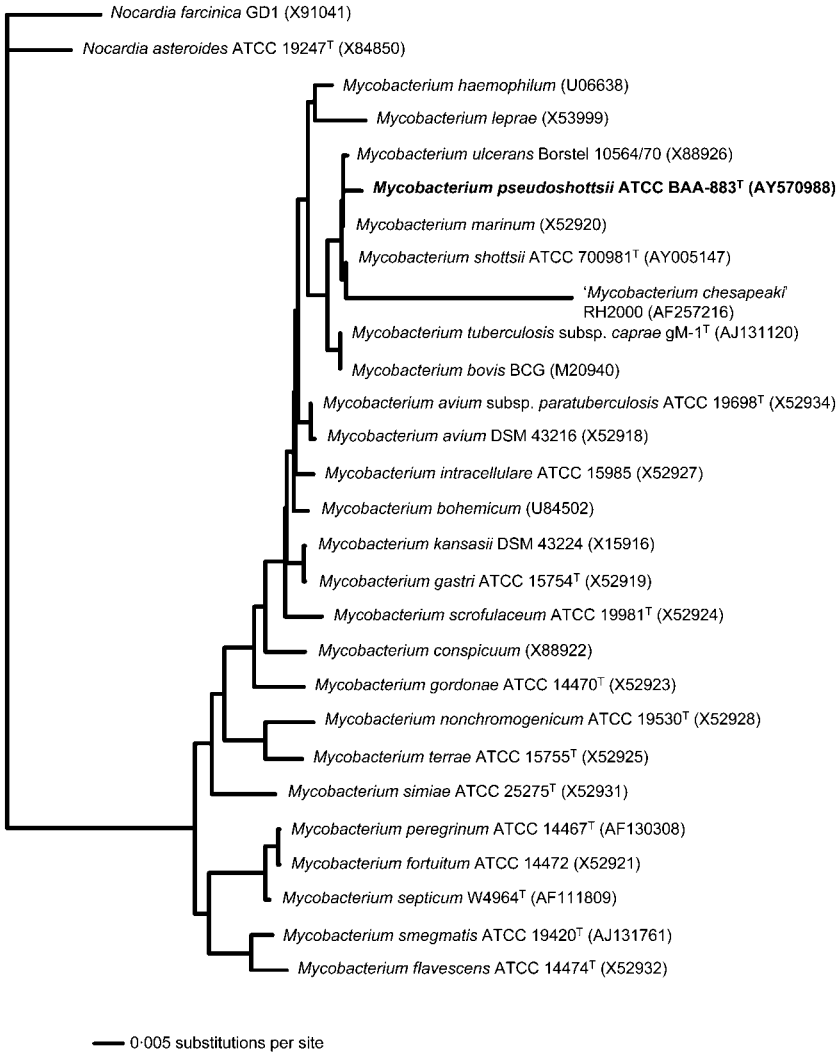

Fig. 2. Neighbour-joining tree, based on 16S rRNA gene sequences of *Mycobacterium* species, showing the relationship between *Mycobacterium pseudoshottsii* sp. nov. and other closely related *Mycobacterium* species. Sequence accession numbers (GenBank/EMBL/DDBJ) are given in parentheses. Note that the aligned dataset used for this analysis was truncated by approximately 320 nt positions to eliminate questionable '*Mycobacterium chesapeaki*' sequence from the analysis. Bar, 0·005 substitutions per site.

hsp65 gene sequence results were compared with those in the database at the British Columbia Centre for Disease Control, L15^T was found to have a sequence identical to that of '*Mycobacterium seriolae*' (Kusuda *et al.*, 1987). Sequences deposited in GenBank for a *Mycobacterium ulcerans*-like organism (AY500839) (Trott *et al.*, 2004) and *Mycobacterium marinum* strains eilaticum DL240490 (AF456468), hellenicum DL045 (AF456471) and cyprinum CC240299 (AF456469) (Ucko *et al.*, 2002) were identical to that of L15^T. *Mycobacterium ulcerans* (AY550209 and AF547888) differed by 1 or 3 nt and *Mycobacterium marinum* (AF547855) differed by 2 nt from L15^T. The topologies of the trees generated by neighbour-joining (Fig. 3) and parsimony were similar.

RFLP analysis of the PCR product of the *hsp65* gene is another approach frequently used to speciate mycobacteria, although sequence polymorphisms do occur among isolates of some species (Plikaytis *et al.*, 1992; Telenti *et al.*, 1993; Devallois *et al.*, 1997; Leclerc *et al.*, 2000; Brunello *et al.*, 2001). *Bst*EII digests of L15^T, *Mycobacterium shottsii* M175^T and *Mycobacterium ulcerans* ATCC 19423^T produced

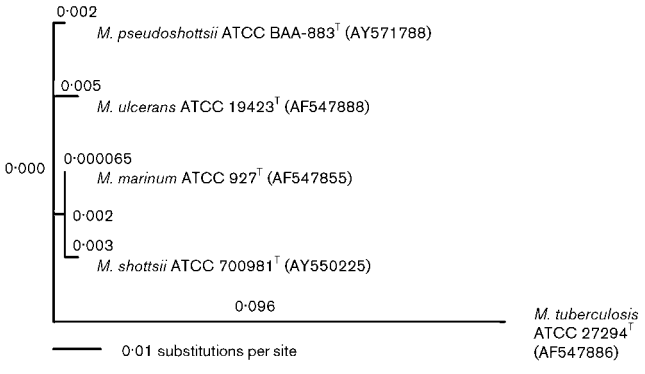

Fig. 3. Neighbour-joining tree, based on *hsp65* gene sequences of *Mycobacterium* species, showing the relationship between *Mycobacterium pseudoshottsii* sp. nov. and other closely related *Mycobacterium* species. The tree was rooted with *Mycobacterium tuberculosis*. Sequence accession numbers (GenBank/EMBL/DDBJ) are given in parentheses. The length of each branch is given. Bar, 0·01 substitutions per site.

identical restriction fragments of 235/210 nt. Digestion with *Hae*III yielded identical restriction fragments of 145/105/80 nt with *Mycobacterium ulcerans* and L15^T, while *Mycobacterium shottsii* yielded restriction fragments of 220/105 nt.

Mycobacterium ulcerans-specific insertion sequences, IS*2404* and IS*2606*, were detected in L1, L6, L15^T and *Mycobacterium ulcerans*, but only weakly or not at all in *Mycobacterium shottsii* M175^T when *Taq* DNA polymerase was used in PCRs. However, use of high-fidelity *Taq* DNA polymerase enhanced the signal for IS*2404* in *Mycobacterium shottsii* M175^T and yielded unequivocal positive results when tested independently in three of the laboratories participating in this study.

On the basis of 16S rRNA and *hsp65* gene sequences, L15^T is very similar to scotochromogenic species isolated from Mediterranean fish and described as strains of *Mycobacterium marinum* by Ucko *et al.* (2002), who postulated that *Mycobacterium marinum* isolates have genotypes that are unique and specific to a geographical region. The 401 nt sequence of the L15^T *hsp65* gene PCR product was identical to that of '*Mycobacterium seriolae*', a photochromogenic mycobacterium isolated from maricultured yellowtail (*Seriola quinqueradiata*) in Japan (Kusuda *et al.*, 1987), and a *Mycobacterium ulcerans*-like non-chromogenic mycobacterium isolated from a laboratory colony of frogs (*Xenopus tropicalis*) and which is also positive for IS*2404* and IS*2606* (Trott *et al.*, 2004). These results demonstrate the complexity of distinguishing interspecific polymorphisms from intraspecific variation, even when using a molecular approach. van Berkum *et al.* (2003) recently demonstrated that tree phylogenies based on different loci can be significantly different, i.e. 16S rRNA gene sequences versus those constructed with 23S rRNA gene sequences and internally transcribed spacer region sequences, and postulated that such differences could arise via lateral transfer and recombination. These observations underline the importance of examining different DNA loci to discriminate closely related species.

L15^T-like isolates, previously described as phenotypic Group F (Rhodes *et al.*, 2004), have been isolated from approximately 12 % of Chesapeake Bay striped bass sampled (n=24/192), but the pathogenicity of these isolates has not been assessed. Despite sharing certain phenotypic similarities and a common host, i.e. striped bass, L15^T has been clearly differentiated from *Mycobacterium shottsii*, in our study, on the basis of three genetic loci. The taxonomic relationship between L15^T and other mycobacteria reported in the literature as having different chromogenic characteristics but similar 16S rRNA and *hsp65* sequences is not known. It is possible that the aforementioned isolates, which have been described previously but whose names are not taxonomically valid, belong to the same taxon as the novel mycobacterium described in this study, for which we propose the name *Mycobacterium pseudoshottsii* sp. nov. These and previous observations (Chemlal *et al.*, 2002; Ucko *et al.*, 2002; Rhodes *et al.*, 2003) demonstrate the continuum of mycobacterial strains that are intermediate between *Mycobacterium marinum* and *Mycobacterium ulcerans*. Additional molecular studies, e.g. DNA–DNA homology and comparison of disease pathologies in human and animal hosts, are necessary for the taxonomic classification of species that are transitional between *Mycobacterium marinum* and *Mycobacterium ulcerans*.

Although IS*2606* has been reported in *Mycobacterium lentiflavum* (Stinear *et al.*, 1999), the presence of both IS*2404* and IS*2606* is considered specific to *Mycobacterium ulcerans*. Recently, both insertion sequences were identified in a mycobacterium isolated during an infection of laboratory-reared frogs that was characterized as being intermediate between *Mycobacterium ulcerans* and *Mycobacterium marinum* (Trott *et al.*, 2004). Chemlal *et al.* (2002) characterized a mycobacterium isolated from a human infection as resembling *Mycobacterium marinum* but having a low copy number for IS*2404*. Our finding of both insertion sequences in mycobacteria isolated from a euryhaline fish in a temperate climate implies that the use of IS*2404* and IS*2606* for the specific detection of *Mycobacterium ulcerans* in aquatic environments (Stinear *et al.*, 1999, 2000) may not be universally applicable. Sequencing of the PCR products generated by the primers designed for these insertion sequences would be informative regarding the similarity between human and environmental isolates.

Description of *Mycobacterium pseudoshottsii* sp. nov.

Mycobacterium pseudoshottsii (pseu.do.shott′si.i. Gr. adj. *pseudes* false; N.L. gen. n. *shottsii* name of a species; N.L. gen. n. *pseudoshottsii* a false *Mycobacterium shottsii*, not the true *Mycobacterium shottsii*).

Acid-fast coccobacillus (0·4–0·6 × 0·8–1 μm) that forms cell aggregates in culture. Spores and cell branching are not present. Colonies on Middlebrook 7H10 agar are rough, photochromogenic and, typically, flat with an irregular margin, becoming umbonate with age. A pale-yellow to gold pigment develops following exposure to light and intensifies with age. Visible colonies are observed from a dilute inoculum after 4–6 weeks incubation at 23 °C. Little or no growth occurs at 30 °C and none occurs at 37 °C. Isolates do not grow on MacConkey agar without crystal violet or on Löwenstein–Jensen medium with 5 % NaCl, are negative for arylsulfatase (at 3 and 14 days), β-galactosidase, nitrate reductase, pyrazinamidase (at 7 days), semi-quantitative catalase and Tween 80 hydrolysis, and have variable reactions for acid phosphatase and catalase at 68 °C. Positive pyrazinamidase reactions do not occur when incubation is extended to 14–21 days. Isolates are typically positive for urease production and niacin accumulation. These mycobacteria tolerate isoniazid at 1 μg ml^{-1} (but not at 10 μg ml^{-1}), thiacetazone and thiophene-2-carboxylic hydrazide. Growth is inhibited in media containing hydroxylamine and oleate. Isolates are

Table 2. Distinguishing characteristics of selected *Mycobacterium* species

1, *M. pseudoshottsii* sp. nov.; 2, *M. shottsii*; 3, *M. chesapeaki*; 4, *M. marinum*; 5, *M. ulcerans*; 6, *M. simiae*; 7, *M. tuberculosis*. Data for species other than *M. pseudoshottsii* are from Heckert *et al.* (2001) and Lévy-Frébault & Portaels (1992). All of the species were negative for resistance to 5 % NaCl. Abbreviations: N, non-pigmented; P, photochromogenic; S, scotochromogenic, +, at least 85 % strains positive; −, less than 15 % strains positive; M, 50–80 % strains positive; F, 15–49 % strains positive; NA, information not available.

Characteristic	1	2	3	4	5	6	7
Growth at:							
23–25 °C	+	+	+	+	+	+	NA
30 °C	−	F	+	+	+	+	+
37 °C	−	−	+	F	F	+	+
42 °C	−	−	NA	−	−	+	−
Pigmentation	P	N	N	P	N	P (M)	N
Resistance to:							
Isoniazid (1 μg ml^{-1})	+	+	NA	M	+	+	−
Isoniazid (10 μg ml^{-1})	−	−	NA	M	M	M	−
Thiophene-2-carboxylic hydrazide	+	+	NA	+	+	+	+
Hydroxylamine	−	−	NA	+	F	+	−
p-Nitrobenzoic acid	+/M	−	NA	M	−	+	−
Thiacetazone	+	+	NA	M	+	+	M
Oleate	−	−	NA	F	NA	+	M
Catalase activity:							
>45 mm column of bubbles	−	−	NA	F	−	+	−
Heat stable	M	F	−	+	−	+	−
Tween 80 hydrolysis	−	−	−	+	−	−	M
Urease	+	+	+	+	F	+	+
Niacin production	+	+	−	−	−	F	+
Nitrate reduction	−	−	−	−	−	−	+
Acid phosphatase	M	M	NA	+	−	−	M
Arylsulfatase (after 3 days)	−	−	−	F	NA	−	−
Pyrazinamidase (after 7 days)	−	−	+	M	NA	+	+

resistant to *p*-aminosalicylic acid and isoniazid but susceptible to ethambutol, kanamycin, rifampicin and streptomycin in disc-based antibiotic-susceptibility tests. The mycolic acid HPLC pattern consists of a single cluster of peaks resembling that of *Mycobacterium shottsii*. The 16S rRNA, *erp* and *hsp65* gene sequences are unique among recognized species of *Mycobacterium* and are most similar to those of *Mycobacterium shottsii*, *Mycobacterium ulcerans* and *Mycobacterium marinum*.

The type strain, L15^T (=ATCC BAA-883^T=NCTC 13318^T), was isolated from granulomatous lesions in splenic tissue from a striped bass (*Morone saxatilis*).

Slowly growing mycobacteria, which grow either poorly or not at all at 37 °C, or produce niacin, are compared with *Mycobacterium pseudoshottsii* in Table 2. Although the combined traits of photochromogenicity and niacin production distinguish *Mycobacterium pseudoshottsii* from other species that grow optimally at ⩽30 °C, expression of both features is dependent on sufficient incubation, i.e. 2–3 months. Other slowly growing mycobacteria that may accumulate niacin are differentiated from *Mycobacterium pseudoshottsii* by growth at 37 °C and by the profiles for resistance to inhibitory agents.

Acknowledgements

Funding was obtained, in part, from the Virginia Marine Resource Commission, Commonwealth of Virginia, the US Geological Survey Cooperative Research Agreement (01ERAG0015) and the Virginia Institute of Marine Science, College of William and Mary (contribution no. 2640 of the Virginia Institute of Marine Science). J.-M. R. and C. D. were funded by INSERM under the Avenir Programme. K. T. was funded by the NIH (GM66684) and the Biology Fellows Program at the University of California, Berkeley. The authors wish to thank Kathy Adie, Dana Booth, Christopher Crist, Tim Grammer, Karen Hudson, Luke Iwanowicz, Mustafa Khokha, Linda Mann, Julie Stubbs and Rebecca Wilson for their excellent technical assistance.

References

Belas, R., Fallon, P. & Hannaford, A. (1995). Potential applications of molecular biology to the study of fish mycobacteriosis. *Annu Rev Fish Dis* **5**, 133–173.

Brown, B. A., Springer, B., Steingrube, V. A. & 10 other authors (1999). *Mycobacterium wolinskyi* sp. nov. and *Mycobacterium goodii* sp. nov., two new rapidly growing species related to *Mycobacterium smegmatis* and associated with human wound infections: a cooperative study from the International Working Group on Mycobacterial Taxonomy. *Int J Syst Bacteriol* **49**, 1493–1511.

Brunello, F., Ligozzi, M., Cristelli, E., Bonora, S., Tortoli, E. & Fontana, R. (2001). Identification of 54 mycobacterial species by PCR-restriction fragment length polymorphism analysis of the *hsp*65 gene. *J Clin Microbiol* **39**, 2799–2806.

Chemlal, K., Huys, G., Laval, F. & 8 other authors (2002). Characterization of an unusual mycobacterium: a possible missing link between *Mycobacterium marinum* and *Mycobacterium ulcerans*. *J Clin Microbiol* **40**, 2370–2380.

de Mendonça-Lima, L., Picardeau, M., Raynaud, C. & 7 other authors (2001). Erp, an extracellular protein family specific to mycobacteria. *Microbiology* **147**, 2315–2320.

de Mendonça-Lima, L., Bordat, Y., Pivert, E., Recchi, C., Neyrolles, O., Maitournam, A., Gicquel, B. & Reyrat, J.-M. (2003). The allele encoding the mycobacterial Erp protein affects lung disease in mice. *Cell Microbiol* **5**, 65–73.

Devallois, A., Goh, K. S. & Rastogi, N. (1997). Rapid identification of mycobacteria to species level by PCR-restriction fragment length polymorphism analysis of the *hsp*65 gene and proposition of an algorithm to differentiate 34 mycobacterial species. *J Clin Microbiol* **35**, 2969–2973.

Dobos, K. M., Quinn, F. D., Ashford, D. A., Horsburgh, C. R. & King, C. H. (1999). Emergence of a unique group of necrotizing mycobacterial diseases. *Emerg Infect Dis* **5**, 367–378.

Floyd, M. M., Guthertz, L. S., Silcos, V. A., Duffey, P. S., Jang, Y., Desmond, E. P., Crawford, J. T. & Butler, W. R. (1996). Characterization of an SAV organism and proposal of *Mycobacterium triplex* sp. nov. *J Clin Microbiol* **34**, 2963–2967.

Goodfellow, M. & Magee, J. G. (1998). Taxonomy of mycobacteria. In *Mycobacteria I Basic Aspects*, pp. 1–71. Edited by P. R. J. Gangadharam & P. A. Jenkins. New York: Chapman Hall.

Hazra, R., Floyd, M. M., Sloutsky, A. & Husson, R. N. (2001). Novel mycobacterium related to *Mycobacterium triplex* as a cause of cervical lymphadenitis. *J Clin Microbiol* **39**, 1227–1230.

Heckert, R. A., Elankumaran, S., Milani, A. & Baya, A. (2001). Detection of a new *Mycobacterium* species in wild striped bass in the Chesapeake Bay. *J Clin Microbiol* **39**, 710–715.

Kapur, V., Li, L. L., Hamrick, M. R. & 10 other authors (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**, 131–138.

Kent, P. T. & Kubica, G. P. (1985). *Public Health Laboratory. A Guide for the Level III Laboratory*. US Department of Health and Human Services Publication no. (CDC) 86-8230. Atlanta: Centers for Disease Control.

Kusuda, R., Kawakami, K. & Kawai, K. (1987). A fish-pathogenic *Mycobacterium* sp. isolated from an epizootic of cultured yellowtail. *Nippon Suisan Gakkaishi* **53**, 1797–1804 (in Japanese).

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. New York: Wiley.

Leclerc, M.-L., Haddad, N., Moreau, R. & Thorel, M.-F. (2000). Molecular characterization of environmental *Mycobacterium* strains by PCR-restriction fragment length polymorphism of *hsp*65 and by sequencing of *hsp*65, and of 16S and ITS1 rDNA. *Res Microbiol* **151**, 629–638.

Levi, M. H., Bartell, J., Gandolfo, L., Smole, S. C., Costa, S. F., Weiss, L. M., Johnson, L. K., Osterhout, G. & Herbst, L. H. (2003). Characterization of *Mycobacterium montefiorense* sp. nov., a novel pathogenic mycobacterium from moray eels that is related to *Mycobacterium triplex*. *J Clin Microbiol* **41**, 2147–2152.

Lévy-Frébault, V. V. & Portaels, F. (1992). Proposed minimal standards for the genus *Mycobacterium* and for description of new slowly growing *Mycobacterium* species. *Int J Syst Bacteriol* **42**, 315–323.

Lutz, B. (1995). Section 3. Mycobacteriology. 3.12. Identification tests for mycobacteria. In *Clinical Microbiology Procedures Handbook*, vol. 1. Edited by H. D. Isenburg. Washington, DC: American Society for Microbiology.

Marck, C. (1988). 'DNA Strider': a 'C' program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. *Nucleic Acids Res* **16**, 1829–1836.

Pelicic, V., Reyrat, J.-M. & Gicquel, B. (1996). Generation of unmarked directed mutations in mycobacteria, using sucrose counter-selectable suicide vectors. *Mol Microbiol* **20**, 919–925.

Plikaytis, B. B., Plikaytis, B. D., Yakrus, M. A., Butler, W. R., Woodley, C. L., Silcox, V. A. & Shinnick, T. M. (1992). Differentiation of slowly growing *Mycobacterium* species, including *Mycobacterium tuberculosis*, by gene amplification and restriction fragment length polymorphism analysis. *J Clin Microbiol* **30**, 1815–1822.

Portaels, F., Fonteyne, P.-A., de Beenhouwer, H., de Rijk, P., Guédénon, A., Hayman, J. & Meyers, M. W. (1996). Variability in 3′ end of 16S rRNA sequence of *Mycobacterium ulcerans* is related to geographic origin of isolates. *J Clin Microbiol* **34**, 962–965.

Posada, D. & Crandall, K. A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.

Rhodes, M. W., Kator, H., Kotob, S. & 7 other authors (2003). *Mycobacterium shottsii* sp. nov., a slowly growing species isolated from Chesapeake Bay striped bass (*Morone saxatilis*). *Int J Syst Evol Microbiol* **53**, 421–424.

Rhodes, M. W., Kator, H., Kaattari, I., Gauthier, D., Vogelbein, W. & Ottinger, C. (2004). Isolation and characterization of mycobacteria from striped bass *Morone saxatilis* from the Chesapeake Bay. *Dis Aquat Organ* **61**, 41–51.

Rogall, T., Flohr, T. & Böttger, E. C. (1990). Differentiation of *Mycobacterium* species by direct sequencing of amplified DNA. *J Gen Microbiol* **136**, 1915–1920.

Springer, B., Stockman, L., Teschner, K., Roberts, G. D. & Bottger, E. C. (1996). Two-laboratory collaborative study on identification of mycobacteria: molecular versus phenotypic methods. *J Clin Microbiol* **34**, 296–303.

Steingrube, V. A., Gibson, J. L., Brown, B. A., Zhang, Y., Wilson, R. W., Rajagopalan, M. & Wallace, R. J., Jr (1995). PCR amplification and restriction endonuclease analysis of a 65-kilodalton heat shock protein gene sequence for taxonomic separation of rapidly growing mycobacteria. *J Clin Microbiol* **33**, 149–153; erratum **33**, 1686.

Stinear, T., Ross, B. C., Davies, J. K., Marino, L., Robins-Browne, R. M., Oppedisano, F., Sievers, A. & Johnson, P. D. (1999). Identification and characterization of IS*2404* and IS*2606*: two distinct repeated sequences for detection of *Mycobacterium ulcerans* by PCR. *J Clin Microbiol* **37**, 1018–1023.

Stinear, T., Davies, J. K., Jenkin, G. A., Hayman, J. A., Oppedisano, F. & Johnson, P. D. R. (2000). Identification of *Mycobacterium ulcerans* in the environment from regions in Southeast Australia in which it is endemic with sequence capture-PCR. *Appl Environ Microbiol* **66**, 3206–3213.

Swofford, D. L. (2002). PAUP* – Phylogenetic Analysis Using Parsimony (*and other methods), version 4.0b10. Sunderland, MA: Sinauer Associates.

Telenti, A., Marchesi, F., Balz, M., Bally, F., Böttger, E. C. & Bodmer, T. (1993). Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J Clin Microbiol* **31**, 175–178.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Tønjum, T., Welty, D. B., Jantzen, E. & Small, P. L. (1998). Differentiation of *Mycobacterium ulcerans*, *M. marinum*, and *M. haemophilum*: mapping of their relationships to *M. tuberculosis* by fatty acid profile analysis, DNA-DNA hybridization, and 16S rRNA gene sequence analysis. *J Clin Microbiol* **36**, 918–925.

Tortoli, E. (2003). Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. *Clin Microbiol Rev* **16**, 319–354.

Trott, K. A., Stacy, B. A., Lifland, B. D., Diggs, H. E., Harland, R. M., Khokha, M. K., Grammer, T. C. & Parker, J. M. (2004). Characterization of a *Mycobacterium ulcerans*-like infection in a colony of African tropical clawed frogs (*Xenopus tropicalis*). *Comp Med* **54**, 309–317.

Turenne, C. Y., Suchak, A. A., Wolfe, J. N., Kabani, A. & Nicolle, L. E. (2003). Soft tissue infection caused by a novel pigmented, rapidly growing *Mycobacterium* species. *J Clin Microbiol* **41**, 2779–2782.

Ucko, M., Colorni, A., Kvitt, H., Diamant, A., Zlotkin, A. & Knibb, W. R. (2002). Strain variation in *Mycobacterium marinum* fish isolates. *Appl Environ Microbiol* **68**, 5281–5287.

van Berkum, P., Terefework, Z., Paulin, L., Suomalainen, S., Lindström, K. & Eardly, B. D. (2003). Discordant phylogenies within the *rrn* loci of rhizobia. *J Bacteriol* **185**, 2988–2998.