Mycobacterium stomatepieae sp. nov., a slowly growing, non-chromogenic species isolated from fish

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Slowly growing, non-chromogenic mycobacteria were isolated from striped barombi mbo cichlids (*Stomatepia mariae*) maintained at the London Zoo Aquarium, UK. The isolates could be differentiated from other slowly growing, non-pigmented mycobacteria by a combination of phenotypic features including their inability to grow at 37 °C, positive tests for heat-stable catalase, tellurite reduction and arylsulfatase activity, and the absence of urease activity, Tween 80 hydrolysis, nitrate reductase, iron uptake and semiquantitative catalase. The almost full-length 16S rRNA gene sequence, together with partial sequences from the 65 kDa heat-shock protein (*hsp65*) and the β -subunit of the bacterial RNA polymerase (*rpoB*) genes and the 16S–23S internal transcribed spacer 1 (ITS 1) region were identical for all three novel strains, but distinct from those of all known mycobacterial species. Phylogenetic analysis based on 16S rRNA gene sequences placed the novel isolates within the slowly growing mycobacteria group in close proximity to *Mycobacterium florentinum*. Based on genotypic and phenotypic findings, it is proposed that these isolates represent a novel species of the genus *Mycobacterium*, for which the name *Mycobacterium stomatepieae* sp. nov. is proposed with strain T11^T (=DSM 45059^T=CIP 109275^T=NCIMB 14252^T) as the type strain.

In the past few years, the occurrence of fish mycobacteriosis has been increasingly reported (Kent *et al.*, 2004; Pate *et al.*, 2005; Sakai *et al.*, 2005; Whipps *et al.*, 2007b). Although three species, *Mycobacterium marinum*, *Mycobacterium fortuitum* and *Mycobacterium chelonae*, have been frequently cited as the main causative agents of infections in fish, several other mycobacterial species, including a number of novel species, have also been associated with mycobacteriosis (Levi *et al.*, 2003; Rhodes *et al.*, 2003, 2005; Whipps *et al.*, 2007a).

The majority of slowly growing mycobacteria contain a long helix 18 at position 430–500 (*Escherichia coli* 16S rRNA gene sequence numbering) in their 16S rRNA gene, which is absent in rapidly growing mycobacteria (Springer

et al., 1996a; Tortoli, 2003). There is a third group, with a short helix 18 (due to a deletion of 12 bp), and members of this group are also classified as slow growers (Tortoli, 2003). In this group, *Mycobacterium simiae*, a photochromogenic organism from monkeys, was the first species to be identified (Weiszfeiler *et al.*, 1981). *Mycobacterium intermedium* (Meier *et al.*, 1993) is another photochromogenic species related to *M. simiae*. Scotochromogenic species of the *M. simiae*-related (MSR) group consist of *Mycobacterium interjectum* (Springer *et al.*, 1993), *Mycobacterium kubicae* (Floyd *et al.*, 2000), *Mycobacterium palustre* (Torkko *et al.*, 2002), *Mycobacterium parascrofulaceum* (Turenne *et al.*, 2004a), *Mycobacterium parmense* (Fanti *et al.*, 2004) and *Mycobacterium saskatchewanense* (Turenne *et al.*, 2004b). Within the MSR group, there are also non-chromogenic species including *Mycobacterium genavense* (Böttger *et al.*, 1993), *Mycobacterium lentiflavum* (Springer *et al.*, 1996b), *Mycobacterium triplex* (Floyd *et al.*, 1996), *Mycobacterium heidelbergense* (Haas *et al.*, 1997), *Mycobacterium montefiorensense* (Levi *et al.*, 2003), ‘*Mycobacterium sherrisi*’ (Selvarangan *et al.*, 2004) and *Mycobacterium florentinum* (Tortoli *et al.*, 2005).

Of the MSR species, only *M. montefiorensense* and *M. triplex* have been isolated from fish (Herbst *et al.*, 2001; Rhodes *et al.*,

Abbreviations: ITS, internal transcribed spacer; MSR, *M. simiae*-related; PRA, PCR-restriction enzyme analysis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *hsp65* and *rpoB* gene sequences and ITS 1 region of *M. stomatepieae* sp. nov. T11^T are AM884331, AM902968, AM885873 and AM902938, respectively.

Neighbour-joining phylogenetic trees based on *hsp65* (Fig. S1) and *rpoB* (Fig. S2) gene sequences of *M. stomatepieae* sp. nov. and selected mycobacterial species, and the oligonucleotides used for 16S rRNA gene sequencing (Table S1) are available with the online version of this paper.

2004). Moreover, isolation of *M. simiae* from fish has been reported based on biochemical identification (Lansdell *et al.*, 1993). Recently, several MSR organisms have been proposed to be responsible for infections in a number of fish species based on DNA sequences obtained directly from tissue samples (Whipps *et al.*, 2003; Poort *et al.*, 2006). In this context, slowly growing, acid-fast, non-pigmented micro-organisms were isolated from kidney and spleen tissue samples of three diseased *Stomatepia mariae* (striped barombi mbo or nness; a cichlid fish endemic to Lake Barombi-Mbo, Cameroon). Specimens were collected at different times over a 7 month period. Polyphasic studies employing biochemical tests, whole-cell fatty acid and mycolic acid analysis, and sequencing of the 16S rRNA, 65 kDa heat-shock protein (*hsp65*) and polymerase β -subunit (*rpoB*) genes and the 16S–23S internal transcribed spacer 1 (ITS 1) region indicated that these isolates represent a single novel species, for which the name *Mycobacterium stomatepiae* sp. nov. is proposed.

All isolates ($n=3$) were collected by using an aseptic technique from specimens of *Stomatepia mariae* held at the London Zoo Aquarium, UK. Isolates T11^T and T3 were recovered from granulomatous spleen tissue samples and grown on Middlebrook 7H10 medium supplemented with 10% oleic acid-albumin-glucose-catalase (OADC; Becton Dickinson) and 0.5% glycerol (Sigma) for 4 weeks at 30 °C following decontamination with 2% NaOH (w/v) for 15 min. Strain T4 was isolated from kidney tissue, without NaOH decontamination, using the same conditions as the T11^T and T3 isolates. The spleen and liver of the fish from which strain T4 was recovered were fixed in 10% buffered formalin. Later, Ziehl–Neelsen staining of tissue sections showed multiple granulomas and a few acid-fast bacilli characteristic of fish mycobacteriosis.

Colony morphology, pigment production when incubated in the dark and following photoinduction, and the ability to grow at temperatures of 15–42 °C were examined during a 12 week incubation on two different media, Middlebrook 7H10 medium with OADC enrichment and Löwenstein–Jensen (LJ) medium.

Acid-alcohol-fastness was determined by Ziehl–Neelsen staining. The following biochemical features were investigated: tellurite reduction (tellurite reductase), nitrate reductase, 3 day arylsulfatase, semiquantitative catalase, iron uptake, Tween 80 hydrolysis and urease activity (Kent & Kubica, 1985). The ability to grow on MacConkey agar without crystal violet and on LJ medium in the presence of sodium chloride (5%) was also tested (Cooksey *et al.*, 2004).

Whole-cell fatty acid and mycolic acid analyses were performed commercially by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany, by GC and HPLC, respectively, as described previously (Butler *et al.*, 1992) using standard Microbial Identification System software (MIDI).

Sequence data were obtained from four distinct regions of the mycobacterial genome. Briefly, DNA was extracted

from cultures and, where indicated, from formalin-fixed tissue samples, using NucleoSpin columns (Macherey–Nagel) following the manufacturer's instructions. PCR amplicons were purified using Qiaquick purification columns (Qiagen) and sequenced in both directions using the original amplicon primers (and internal primers where necessary). Fluorescently labelled templates were generated using a GenomeLab Dye Terminator Cycle Sequencing Quick Start kit (Beckman Coulter) and read on a CEQ8800 automated capillary sequencer (Beckman Coulter). All primer sequences are given in 5'→3' orientation.

PCR amplifications of the 16S rRNA gene (1471 bp) were performed using primers pA (AGAGTTTGATCCTGGCTCAG) and pH^{*} (AAGGAGGTGATCCAGCCGCA) as described previously (Edwards *et al.*, 1989). Additional internal primers are given in Supplementary Table S1 (available in IJSEM Online). ITS 1 region amplicons (226 bp) were generated using primers Sp1 (ACCTCCTTTCTAAGGAGCACC) and Sp2 (GATGCTCGCAACCACTATCCA) as described previously (Roth *et al.*, 2000). An *rpoB* gene fragment (360 bp) was amplified using primers RPO5' and RPO3' as described by Lee *et al.* (2003). Finally, the DNAs extracted from three sources (fresh fish tissue, fixed fish tissue samples and pure cultures of T11^T, T4 and T3) were subjected to both PCR-restriction enzyme analysis (PRA) and sequence analysis of the *hsp65* gene. PRA of the *hsp65* gene was performed as described previously (Telenti *et al.*, 1993). Briefly, a 441 bp fragment of the *hsp65* gene was amplified by PCR and digested with *Bst*EII and *Hae*III restriction endonucleases. The restriction fragments were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The restriction pattern was compared with the published algorithm (Telenti *et al.*, 1993) and with available PRA profiles from PRASITE (<http://app.chuv.ch/prasite>). The remainder of the PCR product was sequenced using the forward and reverse PCR primers Tb11 (ACCAACGATGGTGTGTGCCAT) and Tb12 (CTTGTCGAACCGCATACCT). Primer Tb11 gave inconsistent sequencing results and was later replaced by primer F927 (GAGGACCCGTACGAGAAGAT; in-house design). Use of this primer, which is 39 bp further along the sequence than primer Tb11, resulted in greatly improved sequence data.

Both forward and reverse sequences were assembled and edited using the SEQMAN II module of LASERGENE v.6 (DNASTAR). The resulting consensus sequences were compared with published nucleotide sequences (in all cases BLASTN analyses were employed; nr database, default settings; NCBI website, <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>).

Phylogenetic trees were constructed within a group of related mycobacterial species using the neighbour-joining method in MEGA version 3.1 software (Kumar *et al.*, 2004) applying the Kimura two-parameter distance correction model. The tree inferred from the 16S rRNA gene sequences was rooted using *Mycobacterium tuberculosis*

NCTC 7416^T (GenBank accession no. X58890) as the outgroup. Statistical confidences in tree branches were generated by performing 1000 bootstrap replicates.

Strains T11^T and T3 were examined with a commercial probe kit (INNO-LiPA Mycobacteria version 2; Innogenetics), a PCR-based reverse hybridization assay targeting the ITS 1 region (Tortoli *et al.*, 2003; Lebrun *et al.*, 2005), following the manufacturer's instructions.

Growth characteristics, acid-fastness and multigene sequencing results were consistent with those of members of the genus *Mycobacterium*. Colonies were visible after 4 weeks incubation at 30 °C or 6 weeks at 22 °C on both Middlebrook 7H10 and LJ media. However, no growth was observed within a 12 week incubation period at 15, 37 or 42 °C on either medium. Colonies grown on Middlebrook 7H10 agar were smooth, off-white and non-chromogenic. They were negative for nitrate reductase, urease activity, iron uptake, semiquantitative catalase and Tween 80 hydrolysis and positive for heat-stable catalase, tellurite reduction and arylsulfatase activity (Table 1). Growth was inhibited on MacConkey agar and on LJ medium supplemented with 5 % NaCl.

The HPLC elution patterns for the three isolates appeared to be identical and fell within the triple-peak cluster that characterizes most MSR organisms (Tortoli *et al.*, 2005). The pattern was similar to that of *M. florentinum* in that it contained a three-clustered group of peaks emerging later than single peaks. The relative retention time of the main component of the triplet was also very similar in both species (Fig. 1). Major cellular fatty acids of strain T11^T were 18:1 ω 9c (50.8 %) and 16:0 (17.6 %); this combina-

tion clearly differentiated strain T11^T from other closely related species, as shown in Table 2.

The nearly complete 16S rRNA gene sequence, consisting of a continuous stretch of 1471 bp, corresponding to positions 28–1528 of the *E. coli* sequence (Brosius *et al.*, 1978), was determined for all isolates. All sequences were identical and contained the characteristic short helix 18 (region B) observed in *M. simiae* and other slowly growing MSR species. Using BLASTN against sequences from GenBank, EMBL and DDBJ, the closest match was with *M. florentinum* DSM 44852^T (GenBank accession no. AJ616230) with 4 bp mismatches (1467/1471 bp; 2 bp in hypervariable region A and 2 bp out of hypervariable regions). The phylogenetic tree confirmed the results of sequence analysis (Fig. 2).

All isolates revealed identical ITS 1 sequences. A BLAST search analysis in the GenBank database revealed closest similarity to *M. lentiflavum* ATCC 51985^T (GenBank accession no. AF318174), with a 98.4 % match (3 bp differences).

Sequences of the *rpoB* (321 bp) gene were identical in all three novel isolates. This sequence was unique; the most closely related sequence was identified in *Mycobacterium mageritense* CIP 104973^T (GenBank accession no. P000479; 91.9 % similarity, corresponding to 26 bp mismatches).

The *hsp65* gene of the all novel isolates contained a PCR-restriction fragment length polymorphism pattern that was compatible with that of *M. triplex*. Patterns consisted of three fragments of 145, 130 and 50 bp by *Bst*EII digestion and two fragments of 330 and 115 bp by *Hae*III digestion. All of the sequences obtained from DNA extracted from

Table 1. Phenotypic characteristics of *M. stomatepieae* sp. nov. and closely related species

Species: 1, *M. stomatepieae* sp. nov.; 2, *M. florentinum*; 3, *M. montefiorensis*; 4, *M. simiae*; 5, *M. genavense*; 6, *M. lentiflavum*; 7, *M. triplex*; 8, *M. heidelbergense*; 9, *M. intermedium*; 10, *M. interjectum*; 11, '*M. sherrisi*'. N, Non-chromogenic; P, photochromogenic; SC, scotochromogenic; S, smooth; –, negative; +, positive; +/–, variable; empty cell, no data available. Data for species other than *M. stomatepieae* are from Levi *et al.* (2003), Tortoli *et al.* (2005) and Tortoli (2006).

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Pigmentation	N	N	N	P	N	SC	N	N	P	SC	N
Colony morphology	S	S				S	S			S	S
Nitrate reduction	–	+ / –	–	–	–	–	+	–	–	–	–
Tween 80 hydrolysis	–	–	–	–	–	–	–	+	+	+	–
Tellurite reduction	+	+	+	+	–	–	–	–	–	–	–
Catalase (68 °C)	+	+ / –	–	+	+	+ / –	+	+	+	+	+
Semiquantitative catalase (37 °C)	–		+	+	+	+ / –	–	–	+	+	+ / –
Urease	–	+	–	+	+	–	+	+	+	+	+
Arylsulfatase	+	–	–	+	–	–	–	+	+	–	–
Growth on/at:											
5 % NaCl	–	–	–	–			–	–		–	
MacConkey agar without crystal violet	–	–									
30 °C	+	+	–	+	+	+	+	+	+	+	+
37 °C	–	+	–	+	+	+	+	+	+	+	+
42 °C	–	–	–	+	+	–	+	–	+	–	–

Fig. 1. Mycolic acid HPLC profiles of *M. stomatepiae* sp. nov. strain T11^T and *M. florentinum* (Tortoli *et al.*, 2005). HIS, High-molecular-mass internal standard; LIS, low-molecular-mass internal standard.

cultures and fixed and fresh tissues were identical. The sequence of the hypervariable region of the novel isolates (Ringuelet *et al.*, 1999) was significantly different from those

of other closely related slowly growing species. Phylogenetic analysis of 401 bp of this gene revealed that *M. triplex* CIP 106108^T (GenBank accession no. AF547882) had the highest similarity (98%; 8 bp mismatches). The neighbour-joining trees based on comparison of *hsp65* and *rpoB* gene sequences are shown in Supplementary Figs S1 and S2, respectively (available in IJSEM Online).

Strains T11^T and T3 were strongly reactive with the *Mycobacterium* genus-specific probe in the INNO-LiPA kit, whereas they did not hybridize with any of the species-specific probes included in the assay.

Evidence suggests that MSR species are difficult to culture *in vitro*. For example, based on 16S rRNA gene and ITS 1 sequence data from infected fish tissue, Whipps *et al.* (2003) characterized an MSR organism that was most closely related to *M. montefiorensis* (99.2% 16S rRNA gene sequence similarity). Similarly, from analysis of ethanol-fixed tissues from both shortfin molly (*Poecilia mexicana*) and green swordtail (*Xiphophorus hellerii*), Poort *et al.* (2006) detected a *Mycobacterium* species with a 16S rRNA gene sequence that was very similar to that of the proposed novel species described here. However, in both of these investigations, a live bacterium could not be isolated. Moreover, these results strengthen the hypothesis of Levi *et al.* (2003) who suggested that MSR and *Mycobacterium avium*-related organisms are common pathogens of aquatic animal environments.

The DNA–DNA hybridization method was not used in this study. The lack of a central database (particularly for mycobacterial species) and inherent technical difficulties in

Table 2. Microbial identification system whole-cell fatty acid analysis of *M. stomatepiae* sp. nov. and closely related species

Taxa: 1, *M. stomatepiae* sp. nov.; 2, '*M. sherrisi*'; 3, *M. simiae*; 4, *M. genavense*; 5, *M. lentiflavum*; 6, *M. triplex*; 7, *M. heidelbergense*; 8, *M. intermedium*; 9, *M. interjectum*. Data for taxa 2–8 are from Selvarangan *et al.* (2004). Values given are percentages; empty cells indicate that no data are available.

Fatty acid	1	2	3	4	5	6	7	8	9
12:0							1.0	1.2	
14:0	4.9	7.4	8.2	8.6	6.9	8.1	5.5	6.6	5.6
2-Me-14:0							1.3	1.5	
15:0	0.5	0.7	0.7	0.8	0.7	0.6	0.6	0.4	1.2
16:1 ω 9c	9.7	2.5	2.1	0.7	7.0	2.1	1.7	0.8	4.8
16:1 ω 7c	0.9	1.5	1.3			2.3	3.4	5.4	4.0
16:1 ω 6c	6.4			10.2	3.8	10.5	4.6	4.4	
16:1 ω 5c		2.8					0.5	0.6	
16:0	17.6	37.7	41.4	35.9	31.8	32.2	43.0	44.5	23.7
17:1 ω 8c	0.1	0.5							
17:0	0.7	0.8	0.9	0.6	0.9	0.7	0.6	0.5	1.2
18:2 ω 6,9c	2.4								
18:1 ω 9c	50.8	30.7	27.7	31.1	34.5	28.0	22.1	21.6	23.5
18:1 ω 7c	1.1								
18:0	2.6	3.2	5.4	2.3	4.3	3.1	5.1	4.1	7.9
10-Me-18 (TBSA)	1.8	10.8	7.2	8.6	8.4	10.8	9.1	7.1	15.6
20:0	0.7	1.0	1.0		1.1	0.7	0.7	0.5	0.4

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences including mycobacterial species genetically related to *M. simiae*. *M. tuberculosis* NCTC 7416^T was used as an outgroup. Numbers on the dendrogram are percentages of occurrence in 1000 bootstrapped trees (values >50 % are shown). Bar, 0.002 substitutions per site.

performing this test to a satisfactory and consistent standard (Short *et al.*, 2005; Adékambi *et al.*, 2006) limit its applicability. However, the application of polyphasic analyses for the formal identification of *Mycobacterium* species is a valid and well-recognized approach (Adékambi & Drancourt, 2004; Devulder *et al.*, 2005; Rhodes *et al.*, 2005; Adékambi *et al.*, 2006).

Description of *Mycobacterium stomatepiae* sp. nov.

Mycobacterium stomatepiae (sto.ma.tep'i.æ. N.L. gen. n. *stomatepiae* of *Stomatepia*, isolated from *Stomatepia mariæ*, the scientific name of the striped barombi mbo cichlid).

Cells are non-chromogenic, slowly growing, acid-fast bacilli. Smooth colonies are produced after 4–6 weeks on Middlebrook 7H10 medium at 22–30 °C, with optimal growth at 30 °C. No growth occurs at 37 °C or above. Does not grow on MacConkey agar without crystal violet or on LJ medium with 5 % NaCl. Tests for heat-stable catalase, tellurite reduction and arylsulfatase activity are positive, but those for nitrate reductase, urease activity, iron uptake, semiquantitative catalase and Tween 80 hydrolysis are negative. Partial sequences of the 16S rRNA (1471 bp), *rpoB* (321 bp), *hsp65* (401 bp) genes and ITS 1 (183 bp) sequences are substantially different from those of all currently recognized species of the genus *Mycobacterium*. The fatty acid composition profile is unique and can be used for differentiation from closely related species. Exhibits a mycolic acid profile (HPLC analysis) characterized by a pattern of triple-peak clusters that is typical of MSR species, particularly *M. florentinum*. Phylogenetic analysis using 16S rRNA gene sequences shows that *M. stomatepiae* belongs to the slowly growing mycobacteria and is closely related to *M. florentinum*.

The type strain, T11^T (=DSM 45059^T=CIP 109275^T=NCIMB 14252^T), was isolated from granulomatous lesions in spleen tissue from a striped barombi mbo cichlid.

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