Mycobacterium angelicum sp. nov., a non-chromogenic, slow-growing species isolated from fish and related to Mycobacterium szulgai

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The name ‘Mycobacterium angelicum’ dates back to 2003 when it was suggested for a slowly growing mycobacterium isolated from freshwater angelfish. This name is revived here and the novel species is proposed on the basis of the polyphasic characterization of four strains including the original one. The four strains presented 100 % 16S rRNA gene sequence similarity with Mycobacterium szulgai but clearly differed from M. szulgai for the milky white aspect of the colonies. The sequence similarity with the type strain of M. szulgai ranged, in eight additionally investigated genetic targets, from 78.9 to 94.3 %, an evident contrast with the close relatedness that emerged at the level of 16S rRNA gene. The average nucleotide identity between the genomes of M. szulgai DSM 44166T and strain 126/5/03T (type strain of the novel species) was 92.92 %, and supported the status of independent species. The confirmation of the name Mycobacterium angelicum sp. nov. is proposed, with strain 126/5/03T (=CIP 109313T = DSM 45057T) as the type strain.

A close connection exists between mycobacteria and water, both fresh and salt, and this makes infection of aquatic animals quite common. In addition to Mycobacterium marinum, the best-known fish and zoonotic pathogen, a number of other species of the genus Mycobacterium have been reported which are epidemiologically linked to fish (Gauthier & Rhodes, 2009). Mycobacterium shottsii (Rhodes et al., 2003), Mycobacterium montefioreense (Levi et al., 2003), Mycobacterium pseudoshottsii (Rhodes et al., 2005), Mycobacterium salmoniphilum (Whipps et al., 2007) and Mycobacterium stomatopiae (Pourahmad et al., 2008) have all been described following isolation from fish.

The name Mycobacterium angelicum was proposed in 2003 for one strain isolated in Slovenia from a freshwater angelfish (Pterophyllum scalare). Several characteristics suggested

Abbreviations: ANI, average nucleotide identity; ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, hsp65, rpoB, sodA, gyrB, dnaK, secA1, rpoBC and ITS1 sequences of strain 126/5/03T are AM884328, AM902962, KT168289, KT168302, KT168298, KT168305, KT168310, KT168313 and AM902930, respectively. The GenBank/EMBL/DDBJ accession numbers for the hsp65 and gyrB gene sequences of strain JCM 18266 are KC481266 and KT168297, respectively, and those for the dnaK and secA1 gene sequences of strain JCM 18267 are KT168306 and KC481266, respectively. The GenBank/EMBL/DDBJ accession numbers for the rpoB, gyrB and ITS1 sequences of Mycobacterium szulgai DSM 44166T are KT168290, KT168299 and KT168285, respectively.

Nine supplementary figures are available with the online Supplementary Material.
the strain belonged to a not previously reported species, but an official description was not made, mainly because of the firm convincement that the sound description of a novel species cannot be based on a 'single' strain (Christensen et al., 2001). In subsequent years, two additional strains tentatively identified as *M. angelicum* have been reported (Davies et al., 2012; Durnez et al., 2010).

For characterization, the following strains were selected: the original strain isolated from angelfish (126/5/03T), and two strains (JCM 18266 and JCM 18267) isolated in Japan from gut homogenates of freshwater fish (*Corydoras sterbai*). These latter two strains, identified as *Mycobacterium szulgai* (Marumo et al., 2010), were selected due to suggestive similarity of their sequences, available in the GenBank database, with *M. angelicum*. Finally, one strain of our collection (FI-14310), regarded as a curiosity and trivially called 'white *M. szulgai*', was also added to the panel of test strains. Strain FI-14310 had been isolated in 2014 in Italy from the filter of a tank harbouring aquatic tortoises (*Trachemys scripta*).

All strains grew on solid media in about 2–3 weeks at temperatures ranging from 25 to 37 °C while no growth was obtained at 42 °C. The colonies were milky white in the four strains and presented round and smooth, though in one of them (JCM 18266) were mixed with large and fringed ones. Among biochemical tests (Kent & Kubica, 1985), nitrate reduction, urease and tellurite reduction were positive and the semi-quantitative catalase was >45 mm. Niacin accumulation, β-glucosidase, Tween 80 hydrolysis and catalase activity at 68 °C were negative. Growth of the strains was inhibited on media containing 5 % NaCl, hydroxylamine, isoniazid and p-nitrobenzoic acid, while it was supported in presence of thiacetazone. No growth was observed on MacConkey agar without crystal violet. As expected, due to the high number of species within the genus *Mycobacterium*, the pattern of phenotypic tests were not helpful in univocally differentiating the test strains. The large majority of the above-mentioned test results were shared by the closely related *M. szulgai* DSM 44166T. However, *M. szulgai* DSM 44166T did differ from the test strains in catalase activity at 68 °C and, more importantly, in the yellow scotochromogenic pigmentation of the colonies.

The commercial reverse hybridization test GenoType (Hain Lifescience) (Tortoli et al., 2010) assigned the four test strains to the species *M. szulgai*; the pattern shared by *M. szulgai* and *Mycobacterium intermedium* was actually produced with GenoType AS but *M. intermedium* was excluded by GenoType CM.

The HPLC profile of cell-wall mycolic acids (CDC, 1996) of strain 126/5/03T was characterized by a cluster of seven major peaks eluting between 7.5 and 9 min. A similar profile is shared, among the others, by *M. szulgai* DSM 44166T (Fig. 1).

Antimicrobial susceptibility was investigated in liquid medium by means of minimum inhibitory concentrations determination (CLSI, 2011) using commercially available microdilution plates designed for slowly growing mycobacteria (SLOMYCOI; Sensititre). One of the strains (JCM 18266) could not be tested because of failure to grow in broth in two weeks; the other strains were fully susceptible to amikacin, clarithromycin, ethambutol, linezolid, moxifloxacin and rifabutin, and moderately susceptible to doxycycline. Contrasting results were shown by different strains for ciprofloxacin, rifampicin, streptomycin and sulfamethoxazole susceptibility.

Nine genetic regions were investigated by double-strand sequencing using BigDye Terminator chemistry on an ABI 3730 DNA sequencer (Applied Biosystems) following the standard protocol of the manufacturer. In addition to the almost-complete 16S rRNA gene (Kirschner et al., 1993), partial sequences of seven housekeeping genes were determined: 65 kDa heat-shock protein (*hsp65*) (McNabb et al., 2004), preprotein translocase subunit secA (*secA1*), molecular chaperone DnaK (*dnaK*), RNA polymerase β′ subunit (*rpoBC*)...
Dai et al., 2011), DNA gyrase \( \beta \) subunit (gyr\(B \)) (Gomila et al., 2007), superoxide dismutase (sod\(A \)) (Zolg & Philippi-Schulz, 1994) and RNA polymerase \( \beta \) subunit (rpo\(B \)) (Adékambi et al., 2003). Furthermore, the complete internal transcribed spacer (ITS1) between 16S and 23S rRNA was sequenced (Roth et al., 1998). Difficulties were met sequencing the rpo\(B \) gene, which despite multiple attempts, could be established only for one strain (126/5/03\(T \)).

\(M. szulgai\) was the most closely related species in all the genetic regions, with the exception of hsp65 and sod\(A \) where Mycobacterium avium and Mycobacterium palustre, respectively, were the species presenting the best resemblance. While in the 16S rRNA gene sequence (1489 bp) the four test strains presented 100 % identity with \(M. szulgai\), in other genetic regions the similarity ranged from 94.3 to 98.5 % (Table 1). Interestingly, the sod\(A \) gene sequence similarity with \(M. szulgai\) was lower than 80 %. Moderate microheterogeneity was present, with three strains presenting identical sequences and strain JCM 18266 differing from them by 1 nt in hsp65, dna\(K \) and sec\(A \) genes and 5 nt in gyr\(B \).

PCR restriction analysis patterns (Telenti et al., 1993) were inferred on the basis of restriction sites present in the hsp65 gene sequences. No restriction site was present for Bst\(E II \) enzyme while Hae\(III \) had the potential of producing two major fragments of 127 and 112 bp (fragments <50 bp were not taken into account). No similar pattern is present in the online database at http://app.chuv.ch/prasite/index.html.

Phylogenetic molecular evolutionary analyses were conducted according to the neighbour-joining method (Saitou & Nei, 1987) bootstrapped 1000 times, using MEGA 6 software (Tamura et al., 2013). For each of the nine genetic regions, the sequences of the test strains were aligned, using CLUSTAL W software (Thompson et al., 1994), with those of the more closely related type strains retrieved from the GenBank database and trimmed to start and finish at the same position. Mycobacterium tuberculosis H37R\(v T \) was used as an outgroup. The test strains closely clustered in each phylogenetic tree and mostly belonged to the same evolutionary branch as the type strain of \(M. szulgai\) (Figs S1, S3–S6 and S8, available in the online Supplementary Material). This was however not true for the trees based on hsp65 (Fig. S2) and sod\(A \) sequences (Fig. S7), where \(M. szulgai\) and the proposed novel species appeared to have followed different evolutionary pathways. In the tree based on 16S rRNA gene sequences (Fig. 2), as expected due to the sequence identity, the proposed novel species and \(M. szulgai\) overlapped. A further evolutionary tree was reconstructed on the basis of the concatenated sequences (3788 bp) of seven genetic regions (gyr\(B \) was not included because of the limited number of entries present in the GenBank database, and rpo\(B \) because this gene could be sequenced for only one of the test strains). In this tree, \(M. szulgai\) was again the species most closely related to the test strains (Fig. 3).

To cast light on the taxonomic position of the test strains, made complicated by contrasting results emerging from the 16S rRNA gene and the other genetic targets, the whole genomes of \(M. szulgai\) DSM 44166\(T \) and strain 126/5/03\(T \) were sequenced. For this purpose, paired-end libraries of read length 150 bp were prepared using Nextera XT DNA Sample Preparation and Nextera XT Index kits (Illumina) according to the manufacturer’s protocol. Libraries, normalized to 2 nM and pooled for multiplexing in equal volumes, were sequenced at 9 pM on Illumina HiSeq 2500 platform, high output run mode, with 93 nt paired end reads to achieve a coverage >100 per base. Read tags were processed and assembled with Velvet software (Zerbino & Birney, 2008) supported by VelvetOptimizer (https://github.com/tssemann/VelvetOptimiser) for optimal setting of parameters. The resulting contigs were used to calculate the average nucleotide identity (ANI), using the software available at http://envo-omics.cce.gatech.edu/ani/ (Konstantinidis & Tiedje, 2005). The ANI was 92.92 % (Fig. S9), below the accepted cut-off of 95–96 % (Kim et al., 2014), and supported the legitimacy of the status of independent species.

Take together, these combined data support the status of an independent species of the genus Mycobacterium represented by the four tested strains, for which the name Mycobacterium angeli.cum sp. nov. is proposed.

**Description of Mycobacterium angeli.cum sp. nov.**

Mycobacterium angeli.cum (an.ge‘li.cum. N.L. neut. adj. angeli.cum isolated from angelfish, a freshwater aquarium fish).

The cells are non-motile, non-spore-forming and acid-fast. Visible growth requires about 2 weeks of incubation at

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**Table 1. Sequence similarity of the four test strains with M. szulgai in different genetic regions**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>1489/1489 (100)</td>
</tr>
<tr>
<td>ITS1</td>
<td>283/300 (94.3)</td>
</tr>
<tr>
<td>dnaK</td>
<td>427–428/450 (94.9–95.1)</td>
</tr>
<tr>
<td>gyr(B )</td>
<td>265–266/281 (94.3–94.7)</td>
</tr>
<tr>
<td>hsp65(\dagger )</td>
<td>380–383/401 (94.8–95.5)</td>
</tr>
<tr>
<td>rpo(B )</td>
<td>656/685 (95.8)</td>
</tr>
<tr>
<td>rpo(BC )</td>
<td>475/482 (98.5)</td>
</tr>
<tr>
<td>sec(A )</td>
<td>451–452/468 (96.4–96.6)</td>
</tr>
<tr>
<td>sod(A )</td>
<td>297/389 (78.9)</td>
</tr>
</tbody>
</table>

\*The closest species was \(M. avium\) (similarity 96.0 %).
\dagger The proposed type strain only was sequenced in this region.
\‡ The closest species was \(M. palustre\) (similarity 97.7 %).

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25–37 °C to develop. Mature colonies are 3–4 mm in diameter, smooth and milky white in the light and in the dark. Tests for reduction of nitrate and tellurite and for urease are positive; semi-quantitative catalase is 45 mm. Niacin, Tween 80 hydrolysis, 68°C catalase and β-glucosidase are negative. Susceptible to amikacin, clarithromycin, ethambutol, linezolid, moxifloxacin and rifabutin. Phylogenetically, the species is most closely related to *Mycobacterium szulgai*, with which it shares identical sequence in the 16S rRNA gene; however the ANI between the type strains of these two species is 92.92%.

The type strain 126/5/03T (=CIP 109313T = DSM 45057T), was isolated from a freshwater fish in Slovenia. Three additional strains of the species are JCM 18266, JCM 18267 and FI-14310.
Fig. 3. Phylogenetic tree based on concatenated sequences of 16S rRNA gene, ITS1, hsp65, rpoBC, secA1, sodA and dnaK, of representative species of the genus *Mycobacterium*, reconstructed using the neighbour-joining method bootstrapped 1000 times. Bootstrap values >50 are given at nodes. Bar, 0.01 substitutions per nucleotide position.

References


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Supplementary figure S1. Phylogenetic tree based on ITS1 sequences, constructed using the neighbor-joining method bootstrapped 1000 times. Bootstrap values >50 are given at nodes. Bar, 0.01 substitutions per nucleotide position.

Supplementary figure S2. Phylogenetic tree based on hsp65 sequences, constructed using the neighbor-joining method bootstrapped 1000 times. Bootstrap values >50 are given at nodes. Bar, 0.01 substitutions per nucleotide position.
**Supplementary figure S3.** Phylogenetic tree based on \( rpoB \) sequences, constructed using the neighbor-joining method bootstrapped 1000 times. Bootstrap values >50 are given at nodes. Bar, 0.01 substitutions per nucleotide position.

**Supplementary figure S4.** Phylogenetic tree based on \( rpoBC \) sequences, constructed using the neighbor-joining method bootstrapped 1000 times. Bootstrap values >50 are given at nodes. Bar, 0.01 substitutions per nucleotide position.
Supplementary figure S5. Phylogenetic tree based on dnaK sequences, constructed using the neighbor-joining method bootstrapped 1000 times. Bootstrap values >50 are given at nodes. Bar, 0.01 substitutions per nucleotide position.

Supplementary figure S6. Phylogenetic tree based on secA1 sequences, constructed using the neighbor-joining method bootstrapped 1000 times. Bootstrap values >50 are given at nodes. Bar, 0.01 substitutions per nucleotide position.
**Supplementary figure S7.** Phylogenetic tree based on sodA sequences, constructed using the neighbor-joining method bootstrapped 1000 times. Bootstrap values >50 are given at nodes. Bar, 0.01 substitutions per nucleotide position.

**Supplementary figure S8.** Phylogenetic tree based on gyrB sequences, constructed using the neighbor-joining method bootstrapped 1000 times. Bootstrap values >50 are given at nodes. Bar, 0.01 substitutions per nucleotide position.
Supplementary figure S9. Average nucleotide identity between 126/5/03$^T$ and *Mycobacterium szulgai*$^T$. The distribution of ANI is calculated using draft genomes obtained by assembly of short read tags.