Mycobacterium alsense sp. nov., a scotochromogenic slow grower isolated from clinical respiratory specimens

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The name ‘Mycobacterium alsiense’, although reported in 2007, has not been validly published. Polyphasic characterization of three available strains of this species led us to the conclusion that they represent a distinct species within the genus Mycobacterium. The proposed novel species grows slowly and presents pale yellow-pigmented colonies. Differentiation from other mycobacteria is not feasible on the basis of biochemical and cultural features alone while genetic analysis, extended to eight housekeeping genes and one spacer region, reveals its clear distinction from all other mycobacteria. Mycobacterium asiaticum is the most closely related species on the basis of 16S rRNA gene sequences (similarity 99.3 %); the average nucleotide identity between the genomes of the two species is 80.72 %, clearly below the suggested cut-off (95–96 %). The name Mycobacterium alsense sp. nov. is proposed here for the novel species and replaces the name ‘M. alsiense’, ex Richter et al. 2007, given at the time of isolation of the first strain. The type strain is TB 1906T (=DSM 45230T=CCUG 56586T).

Abbreviation: ANI, average nucleotide identity.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains TB 1906T and FI-02209 are AJ938169 and KT168286, respectively; those for hsp65 gene sequences are FM209298 and DQ381733, respectively; and those for the ITS sequences are AJ938170 and KT168286, respectively. Accession numbers for the rpoB gene sequences of TB 1906T and BF431DRS are KT168292 and KT168288, respectively; those for the gyrB gene sequences are KT168295 and KT168296, respectively; those for the sodA gene sequences are KT168300 and KT168301, respectively; those for dniA gene sequences are KT168303 and KT168304, respectively; those for the secA1 gene sequences are KT168307 and KT168309, respectively; and those for the secA2 gene sequences are KT168314 and KT168316, respectively. Accession numbers for the secA1 and rpoBC gene sequences of strain FI-02209 are KT168308 and KT168315, respectively. The accession number for the hsp65 gene sequence of strain BF431DRS is KT168293.

Nine supplementary figures are available with the online Supplementary Material.
In 2007 two strains of an uncommon mycobacterium were isolated in Europe from clinical specimens and named ‘Mycobacterium alsense’ (Richter et al., 2007). Since then, no other isolation has been reported and a description of the novel species has not been validly published. Following the recent isolation in Africa of an additional strain we performed an in-depth characterization of the three strains and we propose here the name Mycobacterium alsense sp. nov. to accommodate them.

The three mycobacterial strains were isolated from the sputum of patients with pulmonary disorders. The first strain (TB 19061) was repeatedly cultured, in Denmark, from a patient with bilateral lobe infiltrates; the second one (FI-02209) was grown in Italy from a patient with haemoptysis; and the third strain (BF431DRS) was isolated, in Burkina Faso, from a patient classified with drug-resistant tuberculosis on the basis of the persistent presence of acid-fast bacilli in the sputum, despite a completed cycle of first-line anti-tuberculosis treatment.

The three strains grew on solid media, both at 25 and at 37 °C, in about 3 weeks, developing tiny smooth colonies, pale yellow-pigmented, both in the light and in the dark. No growth was observed at 45 °C. All were positive at 68 °C for catalase but negative for other commonly tested biochemical features (niacin accumulation, nitrate reduction, semi-quantitative catalase, Tween 80 hydrolysis, 3 day aryl-sulfatase, urease and β-glucosidase) (Kent & Kubica, 1985). Growth was seen on media supplemented with thiophene-2-carboxylic acid hydrazide (1 μg ml⁻¹), p-nitrobenzoic acid (500 μg ml⁻¹), thiacetazone (10 μg ml⁻¹), isoniazid (1 μg ml⁻¹) and oleate (250 μg ml⁻¹) but not on MacConkey agar without crystal violet or on Lowenstein–Jensen medium supplemented with 5 % NaCl. As expected, due to the high number of species present in the genus Mycobacterium, this biochemical and cultural pattern was not unique and therefore cannot be used for the differentiation from other mycobacteria with similar characteristics. Of the genetically more closely related species Mycobacterium asiaticum and Mycobacterium interjectum, only the first, which is photochromogenic and positive for Tween 80 and semi-quantitative catalase, can be easily differentiated.

With a commercial line probe assay (GenoType CM; Hain Lifesciences) (Richter et al., 2006) the test strains hybridized with probes 10 and 11 and, weakly, with probe 9. The interpretation rules for this assay, recommending to take into account sharp hybridization lines only, do not provide details for such a pattern while they assign the combination of lines 9, 10 and 11 to the species M. interjectum.

HPLC analysis of cell-wall mycolic acids, performed as reported by the Centers for Disease Control (1996), produced a pattern characterized by a major cluster of peaks eluting after 8 min. A similar pattern is shared by a number of mycobacterial species with the closest similarity, as far as number and retention times of peaks are concerned, presented by Mycobacterium palustre (Fig. 1).

 MICs of drugs potentially active against slowly growing mycobacteria were determined in liquid medium (Clinical & Laboratory Standards Institute, 2011) using commercially available microdilution plates (SLOMYCOI; Sensititer). The strains were susceptible to amikacin, ciprofloxacin, clarithromycin, linezolid, moxifloxacin and rifabutin. Results variable among the strains were obtained for doxycycline, ethambutol, rifampicin, streptomycin and sulfamethoxazole.

dsDNA sequences from all the strains included in the study were determined using BigDye Terminator chemistry on an ABI 3730 DNA sequencer (Applied Biosystems) following the standard protocol of the supplier. The results included the almost-complete 16S rRNA gene (Kirschner et al., 1993), the complete internal transcribed spacer between 16S and 23S rRNA (ITS1) (Roth et al., 1993), and parts of genes encoding 65 kDa heat-shock protein (hsp65) (McNabb et al., 2006), RNA polymerase β subunit (rpoB) (Adékambi et al., 2003), RNA polymerase β’ subunit (rpoBC) (Dai et al., 2011), superoxide dismutase (sodA) (Zolg & Philippi-Schulz, 1994), DNA gyrase β subunit (gyrB) (Gomila et al., 2007), molecular chaperone DnaK (dnaK) and preprotein translocase subunit secA (secA1) (Dai et al., 2011). Microheterogeneity was detected in seven genetic targets with the strains presenting the same sequence in 16S rRNA and ITS1 only (Table 1). In the almost-complete 16S rRNA gene (1497 bp), M. asiaticum was the most closely related species. M. interjectum was, by contrast, the species with the highest similarity in four of the genes mentioned above (Table 2).

![Fig. 1. Representative mycolic acid patterns of strain TB19061 and M. palustre DSM 44572. LMMIS, low molecular mass internal standard; HMMIS, high molecular mass internal standard.](http://ijs.microbiologyresearch.org)
Table 1. Microheterogeneity presented by *M. alsense* sp. nov. in different genetic regions

The number of mismatches/number of sequenced base pairs (percentage similarity) is given for the other strains in comparison with the type strain, TB 1906\(^T\).

<table>
<thead>
<tr>
<th>Genetic region</th>
<th>Strain</th>
<th>FI-02209</th>
<th>BF431DRS</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td></td>
<td>0/1497 (100)</td>
<td>0/1497 (100)</td>
</tr>
<tr>
<td>ITS1</td>
<td></td>
<td>0/301 (100)</td>
<td>0/301 (100)</td>
</tr>
<tr>
<td>hsp65</td>
<td></td>
<td>0/371 (100)</td>
<td>2/371 (99.5)</td>
</tr>
<tr>
<td>rpoB</td>
<td></td>
<td>0/719 (100)</td>
<td>4/719 (99.4)</td>
</tr>
<tr>
<td>rpoBC</td>
<td></td>
<td>1/478 (99.8)</td>
<td>4/478 (99.2)</td>
</tr>
<tr>
<td>sodA</td>
<td></td>
<td>0/389 (100)</td>
<td>4/389 (99.0)</td>
</tr>
<tr>
<td>gyrB</td>
<td></td>
<td>0/281 (100)</td>
<td>10/281 (96.4)</td>
</tr>
<tr>
<td>dnaK</td>
<td></td>
<td>0/450 (100)</td>
<td>5/450 (98.9)</td>
</tr>
<tr>
<td>secA1</td>
<td></td>
<td>2/482 (99.6)</td>
<td>8/482 (98.3)</td>
</tr>
</tbody>
</table>

Due to high similarity (99.3%) in the 16S rRNA gene between the proposed type strain of the novel species and that of the most closely related species (*M. asiaticum*), the whole genomes of the respective type strains were sequenced to calculate the average nucleotide identity (ANI) between them. Paired-end libraries of read length 93 bp were prepared from strain TB 1906\(^T\) and *M. asiaticum* IP108 0001\(^T\) using Nextera XT DNA Sample Preparation kit and Nextera XT Index kit (Illumina) according to the manufacturer’s protocol. Libraries were then normalized to 2 nM, pooled for multiplexing in equal volumes and sequenced at 9 pM on an 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 VelvetOptimiser (https://github.com/tseemann/VelvetOptimiser) for optical setting of parameters. The resulting contigs (with N50 values (50% average length) of 46 190 and 23 719 bp, respectively) were used to calculate the ANI between the respective core genomes, using the software available at http://enve-omics.ce.gatech.edu/ani/ (Konstantinidis & Tiedje, 2005). The resulting value was 80.72%, clearly below the accepted cut-off 95–96% (Kim et al., 2014) (Fig. S1, available in the online Supplementary Material).

PCR restriction analysis patterns (Telenti et al., 1993) were deduced on the basis of restriction sites present in the hsp65 gene sequences. No restriction site for BstEII was detected in any of the three strains. By contrast, the restriction enzyme HaeIII produced fragments of 214 and 127 bp, a pattern not shared by any known mycobacterium (http://app.chuv.ch/prasite/index.html).

For phylogenetic analysis, the sequences of the type strains of the most closely related species were retrieved from the GenBank database, aligned using CLUSTAL W software (Thompson et al., 1994) and trimmed to start and finish at the same position. The analysis was conducted for each investigated genetic region and *Mycobacterium tuberculosis* H37Rv\(^T\) was used as an outgroup. The neighbour-joining method (Saitou & Nei, 1987), supported by the MEGA 6 software (Tamura et al., 2013), was used for the reconstruction of phylogenetic trees; 1000 bootstrap replications were implemented. All the phylogenetic trees confirmed that the three test strains belonged to a single, highly robust, branch. Most of the trees (Figs S2–S9) were characterized by low to moderate robustness. In four of them (Figs S2, S3, S4 and S6) the proposed novel species appeared more closely related to a member of the *Mycobacterium simiae* complex (Tortoli et al., 2011). In the others (Figs S5, S7, S8 and S9) the branch including the test strains was clearly separate from other mycobacteria. The tree reconstructed on the basis of the almost-complete 16S rRNA gene sequences (Fig. 2) had 57% of the nodes with bootstrap values >50, with *M. asiaticum* being the closest species.

A further phylogenetic tree was built by concatenating the sequences of eight of the nine investigated genetic regions in a single 4604 bp long string (Fig. 3); *gyrB* was excluded because of the low number of species for which sequences were available in GenBank. In this tree, *M. interjectum* was the species most closely related to the test strains.

On the basis of the data presented, the three strains are considered to represent a novel species of the genus *Mycobacterium*, for which the name *Mycobacterium alsense* sp. nov. is proposed.

Table 2. Highest sequence similarities between *M. alsense* sp. nov. and other *Mycobacterium* species in different genetic regions

<table>
<thead>
<tr>
<th>Genetic region</th>
<th>Closest species</th>
<th>Sequence similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td><em>M. asiaticum</em></td>
<td>99.3</td>
</tr>
<tr>
<td>ITS1</td>
<td><em>M. interjectum</em></td>
<td>89.9</td>
</tr>
<tr>
<td>hsp65</td>
<td><em>M. parmaense</em></td>
<td>97.3–97.8</td>
</tr>
<tr>
<td>rpoB</td>
<td><em>M. interjectum</em></td>
<td>94.3</td>
</tr>
<tr>
<td>rpoBC</td>
<td><em>M. interjectum</em></td>
<td>93.9–94.3</td>
</tr>
<tr>
<td>sodA</td>
<td><em>M. interjectum</em></td>
<td>91.2–91.5</td>
</tr>
<tr>
<td>gyrB</td>
<td><em>M. agri</em></td>
<td>90.0–91.8</td>
</tr>
<tr>
<td>dnaK</td>
<td><em>M. hiberniae</em></td>
<td>92.9–93.1</td>
</tr>
<tr>
<td>secA1</td>
<td><em>M. asiaticum</em></td>
<td>94.4–94.9</td>
</tr>
</tbody>
</table>

**Description of *Mycobacterium alsense* sp. nov.**

*Mycobacterium alsense* (als.en’se. N.L. neut. adj. alsense of, or belonging to, the isle of Als, Denmark, where the type strain was isolated).

Non-motile, non-spore-forming and acid-fast. Visible growth requires more than 2 weeks of incubation at 25–37 °C to develop. On solid media colonies are small, smooth and pale-yellow-pigmented both in the light and...
Fig. 2. Phylogenetic tree of representative species of the genus *Mycobacterium* based on 16S rRNA sequences, reconstructed using the neighbour-joining method bootstrapped 1000 times. Bootstrap values >50 are given at nodes. Bar, 0.005 substitutions per nucleotide position.
in the dark. Heat-stable catalase test is positive while nitrate reduction, semi-quantitative catalase, Tween 80 hydrolysis, 3 day ariysulfatase, urease and β-glucosidase are negative. Has a TLC pattern that is very common among mycobacteria, with χ-, keto- and methoxy-mycolic acids (Richter et al., 2007). The major lipid components, identified by GC/LC-MS, are C_{16:0}, C_{18:1}ω9c, tuberculosisaric acid and C_{18:0} (Richter et al., 2007). Most closely related (99.3 % similarity) to M. asiaticum based on the 16S rRNA gene; the ANI (80.72 %) between their core genomes is, however, clearly supportive of the status of independent species.

The type strain, isolated from a clinical sample in Denmark, is TB 1906^T (= DSM 45230^T = CCUG 56586^T).

References


