

Mycobacterium monacense sp. nov.

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Four bacterial strains were isolated from independent clinical specimens in different countries and their genotypic and phenotypic characters support their classification in a novel species within the genus *Mycobacterium*. One strain was clearly responsible for a severe, post-traumatic wound infection in a healthy boy. The novel species, for which the name *Mycobacterium monacense* sp. nov. is proposed, is yellow-pigmented, non-photochromogenic and grows in less than a week on solid medium. Based on phenotypic investigations alone, distinction of these four strains from known scotochromogenic rapidly growing strains is problematic. However, the novel strains differ from any other mycobacterium in each of the molecular species markers investigated: the 16S rRNA gene, the 16S–23S rRNA gene internal transcribed spacer and the *hsp65* gene. Of the strains investigated, two different sequevars were detected for the *hsp65* region. Phylogenetic analysis revealed that these four strains were most closely related to *Mycobacterium doricum*. The type strain of *Mycobacterium monacense* sp. nov. is B9-21-178^T (= DSM 44395^T = CIP 109237^T).

The pathogenic role of rapidly growing non-tuberculous mycobacteria (NTM) has been underrated for many years, with only *Mycobacterium fortuitum* and *Mycobacterium chelonae* being considered to be potentially responsible for disease (Wolinsky, 1979). In the last few years, a new scenario has appeared, with many novel rapidly growing

species being described that are often responsible for disease in humans (Brown-Elliott & Wallace, 2002). The majority of such novel mycobacteria are not pigmented and have emerged from the splitting of the heterogeneous group known, until a few years ago, as the *M. fortuitum* complex (Wallace *et al.*, 2004; Adékambi *et al.*, 2006). Here, four strains of a scotochromogenic rapidly growing mycobacterium are described. Their unique phenotypic and genotypic features justify their classification in a novel species.

Abbreviations: ITS, internal transcribed spacer; NTM, non-tuberculous mycobacteria.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of B9-21-178^T is AF107039. The GenBank/EMBL/DDBJ accession numbers for the *hsp65* gene sequences of strains FI-00234, FI-03115 and FI-05352 are DQ381731, DQ381730 and DQ381732, respectively. Those for the ITS sequences of B9-21-178^T, FI-03115, the six sequence variants of FI-00234 and the seven sequence variants of FI-05352 are DQ473393, DQ381726, DQ466891–DQ466896 and DQ466897–DQ466903, respectively.

A phylogenetic tree based on *hsp65* gene sequences of selected mycobacterial species and a table giving the fatty acid contents of strain B9-21-178^T and related mycobacteria are available as supplementary material in IJSEM Online.

The first strain (B9-21-178^T) was isolated 1998 in Germany from a bronchial lavage of an 80-year-old patient hospitalized to treat multifocal lung carcinoma and insulin-dependent diabetes mellitus. Tuberculostatic treatment did not change the clinical symptoms. Three other strains were isolated between 2000 and 2005 from unrelated patients hospitalized in different cities in Italy. Of particular interest is strain FI-00234, which was isolated from the biopsy material of an 11-year-old boy presenting with a fistula on his right thigh as a consequence of accidental deep penetration of a screwdriver. Various minor surgical

operations had no effect until a complete emptying of the wound, which deepened to the bone, resolved the problem. The other two strains, of uncertain clinical significance, were FI-03115, grown from the sputum of a 31-year-old HIV-positive woman with bronchopneumonitis, and FI-05352, obtained from the sputum of an 82-year-old patient suspected to have lung cancer.

Strains were isolated from clinical specimens, subjected to standard *N*-acetyl-L-cysteine-NaOH decontamination, on Lowenstein–Jensen medium at 37 °C. Strains were maintained at –80 °C in Middlebrook 7H9 broth supplemented with 15 % glycerol.

Genetic sequencing was carried out using standard procedures (Reischl *et al.*, 1998; Roth *et al.*, 1998; McNabb *et al.*, 2004) on three different species markers. The almost complete 16S rRNA gene sequences (1450 bp) of the four investigated strains were identical and differed by 14 bp from the 16S rRNA gene sequence of *Mycobacterium doricum*, the most closely related species (99 % similarity). Of the bulk of sequences from as yet unnamed mycobacterial isolates deposited in GenBank so far, entry AF387804 (*Mycobacterium* sp. JLS; environmental isolate) was 100 % identical, whereas entries AY083217 and AF387803 (*Mycobacterium* sp. KMS and MCS, respectively; environmental isolates) differed by only 2 nt from the novel strains described herein.

In the sequence of the hypervariable region within the *hsp65* gene, determined as reported previously (McNabb *et al.*, 2004), two sequevars were detected that differed by 4 bp, with the first shared by FI-00234 and FI-05352 and the second by B9-21-178^T and FI-03115 (Fig. 1). Again, the sequence of *M. doricum* was the most closely related (13–15 bp diversity within the analysed region of 424 bp; 96.5–96.9 % similarity).

dsDNA sequencing of PCR amplicons obtained from the 16S–23S internal transcribed spacer (ITS) region revealed a number of ambiguous nucleotides at defined positions, which suggest the intragenomic presence of multiple *rrn* operons with different sequences, a feature shared by almost every rapidly growing species (Tortoli, 2003). To investigate nucleotide ambiguities within the ITS sequences of strains FI-00234 and FI-05352 in more detail, the corresponding ITS-derived amplicons were cloned into DH5 α cells using the TOPO-TA cloning kit for sequencing (Invitrogen). From each of the two cloning experiments (strains FI-00234

and FI-05352), 12 colonies were selected for plasmid preparation and subsequent DNA sequencing using BigDye Terminator chemistry and an AB3730 DNA sequencer (Applied Biosystems). Clear and unambiguous sequences were observed for every cloned ITS-derived amplicon. Of the 12 plasmid preparations investigated from each strain, seven different sequence variants were identified in strain FI-05352 and six sequence variants were found in strain FI-00234. These precisely determined ITS sequences of the four novel strains were clearly divergent from those of other mycobacterial species. Sequence alignments of the ITS region revealed *Mycobacterium vanbaalenii* as the most closely related species. From a practical point of view, the alignment process was only possible for two stretches of approximately 125 and 60 bp within the ITS sequence, whereas only weak similarity was observed with the ITS sequence of *M. vanbaalenii* and those of other mycobacterial species. Such data may support the suggestion that the usefulness of ITS sequencing for phylogenetic studies of rapidly growing mycobacteria is somewhat limited.

Phylogenetic analysis within a group of related mycobacterial species, selected on the basis of phenotypic and genotypic properties, was conducted using MEGA version 3.1 software (Kumar *et al.*, 2004) applying the Kimura two-parameter distance correction model. The neighbour-joining method (Saitou & Nei, 1987) was carried out with 1000 bootstrap samples on the largest 16S rRNA gene stretch available in GenBank (positions 49–1448 of *Escherichia coli* 16S rRNA gene) shared by the species involved. As depicted in Fig. 2, *M. doricum* was the most closely related species.

A similar phylogenetic analysis approach was applied to a 352 bp overlapping region within the *hsp65* sequence (starting at position 442 of the *Mycobacterium tuberculosis hsp65* gene; GenBank accession no. M15467) (Takewaki *et al.*, 1994) and the resulting phylogenetic tree (available as Supplementary Fig. S1 in IJSEM Online) confirmed the very close phylogenetic relationship between the test strains and *M. doricum*. Separation of the respective phyla is supported by a high bootstrap value in each of the determined phylogenetic trees.

Alpha-mycolates, keto-mycolates and wax ester mycolates were detected in B9-21-178^T by bi-dimensional TLC, performed as described previously (Schröder *et al.*, 1997). This is a very common mycolic acid pattern shared by a large number of NTM, but nevertheless differs from that of *M. doricum*, which presents methoxy-mycolates instead of keto-mycolates (Tortoli, 2003).

Major compounds revealed by GLC, determined using the Microbial Identification standard software package (Sasser, 1990), of B9-21-178^T included the methyl-branched fatty acid 10-methyl 18:0 and the alcohol 2-OH 18:0 (the full fatty acid content is available in Supplementary Table S1 in IJSEM Online).

	616	891	1025
FI-00234/FI-05352	CCGTCGAGAAGG	CCGGTATCT	CGGTCGAC
FI-03115/B9-21-178 ^TA..A....G....C....

Fig. 1. Alignment of nucleotide sequevars detected in the *hsp65* gene of the investigated strains; nucleotide positions according to *hsp65* of *M. tuberculosis* (GenBank accession no. M15467; Takewaki *et al.*, 1994).

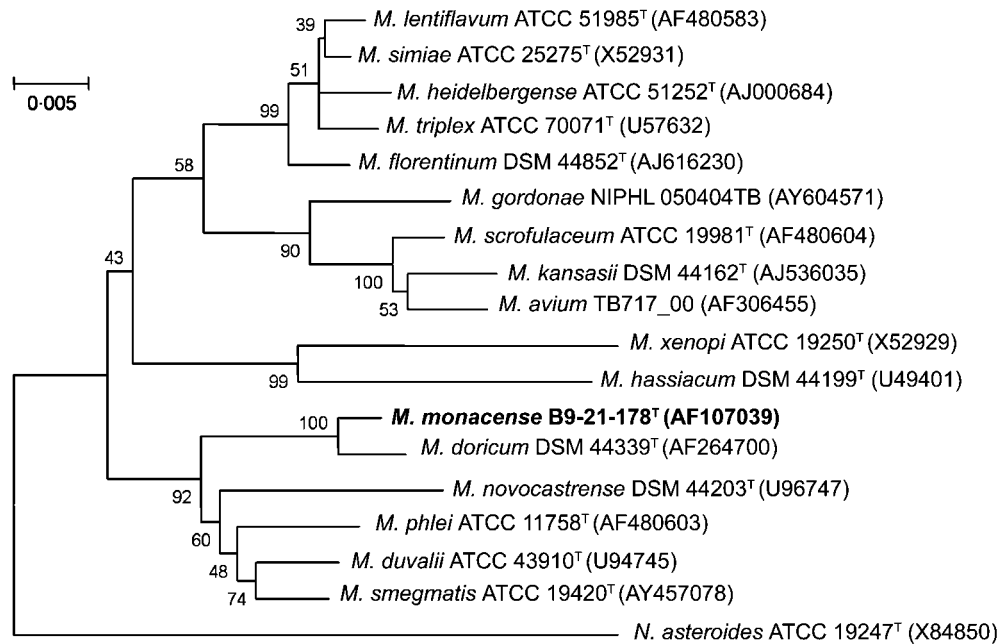


Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of selected mycobacterial species. *Nocardia asteroides* ATCC 19247^T was selected as an outgroup. Bar, 0.005 substitutions per site.

The HPLC pattern of mycolic acids, performed after esterification to bromophenacyl esters (Butler & Kilburn, 1988), was identical in the four investigated strains. It was characterized by the presence of two well-separated clusters of peaks, with the first including four major peaks eluting between 5 and 6 mins and the second, eluting after a 2 min interval, presenting three minor peaks (Fig. 3). Although characterized by a similar motif, the HPLC pattern of *M. doricum* clearly differed, mainly because of the later emergence of peaks of the first cluster (Fig. 3). A thorough

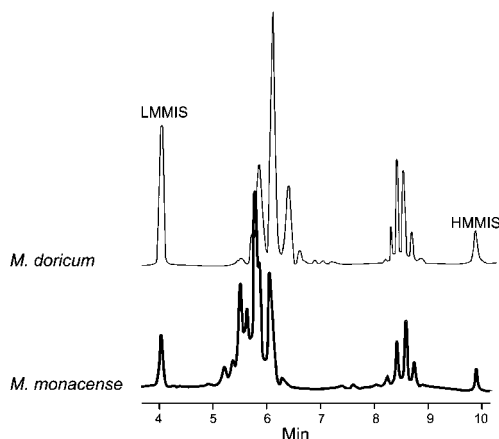


Fig. 3. Mycolic acid HPLC profiles of *M. monacense* sp. nov. and *M. doricum*. LMMIS, low molecular mass internal standard; HMMIS, high molecular mass internal standard.

investigation of our HPLC mycobacterium library (available online at <http://www.MycobacToscana.it>) revealed that *Mycobacterium conspicuum*, *Mycobacterium murale* and *Mycobacterium terrae* present profiles that grossly resemble this pattern.

Susceptibility testing, performed using the minimal inhibitory concentration method recommended by the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) for rapidly growing mycobacteria (NCCL, 2002), showed uniform susceptibility to amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline and linezolid and variable results for trimethoprim-sulfamethoxazole and imipenem (Table 1).

Table 1. Results of susceptibility testing of the four novel strains given in minimal inhibitory concentrations ($\mu\text{g ml}^{-1}$)

Drug	B9-21-178 ^T	FI-00234	FI-03115	FI-05352
Amikacin	1	<1	<1	<1
Cefoxitin	8	8	16	16
Doxycycline	0.25	<0.25	<0.25	<0.25
Imipenem	1	8	16	16
Co-trimoxazole	>64	4	4	4
Linezolid	4	<2	<2	<2
Clarithromycin	1	0.12	0.25	0.12
Ciprofloxacin	0.5	<0.125	<0.125	<0.125

Results of biochemical tests, performed on all four investigated strains according to standard procedures (Kent & Kubica, 1985), were positive for nitrate reduction, thermostable catalase and Tween 80 hydrolysis, whereas the strains were negative for niacin accumulation, 3-day arylsulfatase and β -glucosidase. Urease and semi-quantitative catalase activities were variable. All four strains grew in less than a week on solid medium, forming smooth yellow colonies at temperatures of 25–37 °C. Growth, although slower, did occur at 45 °C and on Lowenstein–Jensen medium supplemented with 5% NaCl, but not on MacConkey medium without crystal violet.

It is proposed that the four strains are representatives of a novel species of mycobacteria for which the name *Mycobacterium monacense* sp. nov. is proposed. The type strain is B9-21-178^T (= DSM 44395^T = CIP 109237^T).

Description of *Mycobacterium monacense* sp. nov.

Mycobacterium monacense (mo.na.ce'nse. L. neut. adj. *monacense* from Monacum, the Latin name of the German city Munich where the first strain was isolated).

Gram-positive, acid-fast, non-motile, does not form spores and produces smooth, yellow, scotochromogenic colonies within 7 days at 25–45 °C. Nitrate reduction and Tween 80 hydrolysis are positive. Neither TLC, which reveals the presence of alpha-mycolates, keto-mycolates and wax ester mycolates, nor GLC, whose major compounds include the methyl-branched fatty acid 10-methyl 18:0 and the alcohol 2-OH 18:0, are suitable for precise differentiation from other species. HPLC may be useful for differentiation as none of the known rapidly growing scotochromogenic strains presents a similar pattern. Susceptible *in vitro* to amikacin, cefoxitin, ciprofloxacin, doxycycline and linezolid. Differs from every other known mycobacterium in a substantial number of nucleotides in each of the most frequently sequenced regions, 16S rRNA gene, ITS and *hsp65*. The divergence of the ITS sequence from those of almost all other mycobacteria is noteworthy. Of the rapidly growing mycobacteria, most closely related to *M. doricum* based on phylogenetic analysis of the 16S rRNA gene sequence.

The type strain, B9-21-178^T (= DSM 44395^T = CIP 109237^T), was isolated in Germany from a bronchial lavage sample. The three other representatives of this species were isolated from clinical specimens and one was responsible for post-traumatic wound infection.

Acknowledgements

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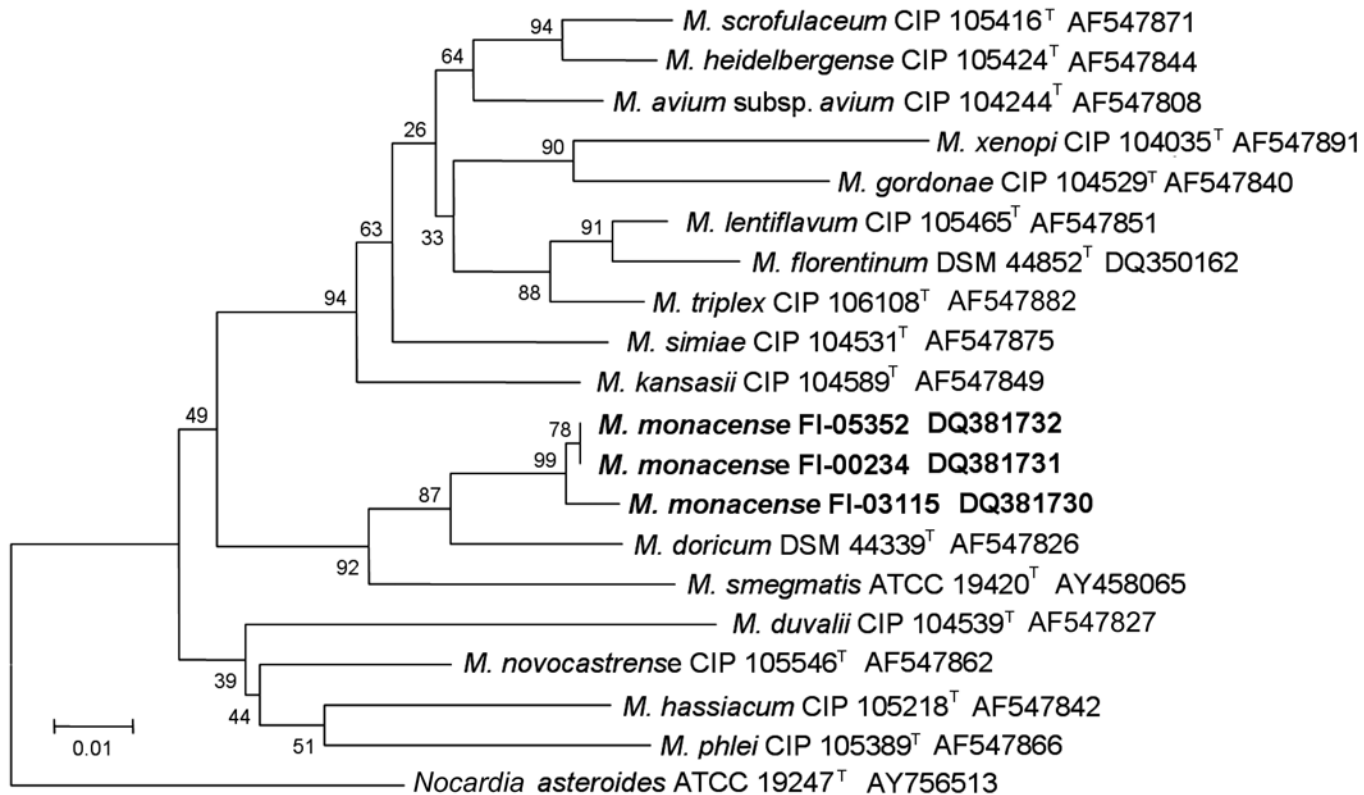
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Supplementary Table S1. Fatty acid methyl ester content (%) of strain B9-21-178^T and related mycobacteria

Fatty acid	B9-21-178 ^T	<i>M. doricum</i>	<i>M. duvalii</i>
14:0	1.83	2.37	2.68
15:0	0.38	–	–
16:1 <i>cis</i> -7	1.24	2.94	1.36
16:1 <i>cis</i> -9	1.55	0.75	0.92
16:1 <i>cis</i> -10	11.08	9.36	5.72
16:0	24.52	26.74	29.68
16:0 10-methyl	0.69	–	–
18:2 <i>cis</i> -9, 10	0.54	–	0.16
18:1 <i>cis</i> -9	21.79	25.91	24.30
18:0	3.52	0.85	6.02
18:0 10-methyl	12.45	16.49	7.12
20:0	0.52	0.44	0.56
18:0 alcohol	11.50	13.05	8.99
20:0 alcohol	0.52	1.10	12.49

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Supplementary Fig. S1. Phylogenetic tree based on *hsp65* sequences of selected mycobacterial species. *Nocardia asteroides* was selected as an outgroup. Bar, 0.01 substitutions per nucleotide position.

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