

Mycobacterium florentinum sp. nov., isolated from humans

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Eight mycobacterial strains isolated during an 11 year period from the sputum of independent patients with various pulmonary disorders and, in one case, from a lymph node of a young girl, were found to present identical features. Phenotypic and genotypic characteristics revealed that the most closely related species to these test isolates were *Mycobacterium triplex* and *Mycobacterium lentiflavum*. However, the lipids of the cell wall of the test isolates differed from those of the latter species by TLC and presented unique profiles by both GC and HPLC. Genotypic analysis showed that they had unique 16S rRNA gene and internal transcribed spacer (ITS) sequences, and could be differentiated from all other mycobacterial strains by PCR restriction analysis of *hsp65*. The strains presented high resistance to antimycobacterial drugs. The name *Mycobacterium florentinum* sp. nov. is proposed for this taxon, with strain FI-93171^T (= DSM 44852^T = CIP 108409^T) as the type strain.

In recent years many novel mycobacterial species have been discovered and detailed genetic characterization has helped define new taxonomic groupings (Tortoli, 2003). A group genetically related to *Mycobacterium terrae* has been recognized within the slowly growing mycobacteria, and a group of thermotolerant mycobacteria has been characterized within the rapid growers (Tortoli, 2003). However, the more striking new taxonomic entity is the one related to *Mycobacterium simiae*, which presents genetic features

typical of rapid growers despite the fact that its members unquestionably belong to slowly growing species, requiring at least 2 weeks of incubation (Tortoli, 2003). The only species known initially to present such unique features was *M. simiae*; however, since 1993, this species has been joined by many others to embrace 14 taxa at present (Böttger *et al.*, 1993; Fanti *et al.*, 2004; Floyd *et al.*, 1996, 2000; Haas *et al.*, 1997; Levi *et al.*, 2003; Meier *et al.*, 1993; Selvarangan *et al.*, 2004; Springer *et al.*, 1993, 1996; Torkko *et al.*, 2002; Turenne *et al.*, 2004a, b).

Published online ahead of print on 9 December 2004 as DOI 10.1099/ijms.0.63485-0.

Abbreviations: ITS, internal transcribed spacer; PRA, PCR restriction analysis.

The GenBank/EMBL/DDBJ accession number of 16S rRNA gene and ITS region sequence of strain FI-93171^T is AJ616230.

Antimicrobial susceptibility data for three strains of *M. florentinum* are available as supplementary material in IJSEM Online.

Eight mycobacterial strains isolated from clinical human samples drew our attention as they presented an identical lipid profile by HPLC, which differed from known HPLC patterns. Genetic characterization showed that they belonged to a novel species characterized by the genetic properties of the *M. simiae* group. We propose the name *Mycobacterium florentinum* sp. nov. for this species.

METHODS

Strains. Eight independent strains were isolated in the last 11 years from eight patients (Table 1) hospitalized in Italy (seven cases) or in Finland (one case). Six strains were isolated, two or more times, from the sputum of elderly patients. The others were grown from the stools of an AIDS patient and from the excised cervical lymph node of a young girl. Media used for the primary growth varied according to the procedures adopted by different isolating laboratories. Four of the strains were grown on Löwenstein–Jensen medium and in broth (radiometric Bactec, MGIT or BacT Alert), three in liquid medium only and one on Löwenstein–Jensen medium only.

Cultural and biochemical tests. A large selection of biochemical and cultural tests was performed by using established methods and media (Vincent *et al.*, 2003). Biochemical tests included niacin accumulation, nitrate reduction, Tween 80 hydrolysis, urea hydrolysis, tellurite reduction, β -glucosidase, 3 day arylsulfatase, and semi-quantitative and thermostable catalase. Cultural tests were performed on Löwenstein–Jensen medium to investigate the growth rate, the ability to grow at different temperatures (25, 37 and 45 °C) and the possible production of pigmentation. The ability to grow on MacConkey's agar and the inhibitory effect of several substances [5% (w/v) NaCl, 1 μ g isoniazid ml⁻¹, 500 μ g hydroxylamine ml⁻¹, 250 μ g oleic acid ml⁻¹, 500 μ g *p*-nitrobenzoic acid ml⁻¹, 10 μ g thiacetazone ml⁻¹] were also tested.

Chromatographic investigation of the cell-wall lipids. The lipid composition was investigated with different chromatographic approaches. TLC was run on silica gel after methyl esterification (Minnikin *et al.*, 1984) and spots corresponding to mycolic acids were identified by comparison with those of authentic reference strains run in parallel.

Mycolic acid cleavage products, saturated and unsaturated fatty acids and secondary alcohols were investigated with GLC. The fatty acids were extracted as methyl esters by means of methanolysis and injected in a temperature-controlled column where the pyrolysis of mycolic acid occurred (Torkko *et al.*, 2003). GLC and TLC were performed on four strains only (FI-93171^T, FI-93188, FI-94029 and 1696).

All the test strains were also subjected to HPLC. Cell-wall mycolic acids, once saponified and chloroform-extracted, were derivatized to UV-adsorbing mycolic esters and then separated with a gradient of methanol and methylene chloride (Butler *et al.*, 1996).

Antimicrobial susceptibility testing. Antimicrobial susceptibility was investigated in three strains (FI-93171^T, FI-93199, FI-94029) using the microdilution radiometric method developed for *Mycobacterium avium* complex (MAC). This was made possible as the growth rate of the test strains, very similar to that of MAC, meant that all the requirements of the technique were fulfilled (Siddiqi *et al.*, 1993). Antimicrobial agents are listed in a supplementary table available in IJSEM Online.

Genetic sequencing and phylogenetic investigations. Complete sequences of the 16S rRNA gene and the internal transcribed spacer (ITS) were determined using primers and PCR protocols described previously (Kirschner *et al.*, 1993; Roth *et al.*, 1998). The PCR products were sequenced with an automated apparatus (ALFexpress DNA sequencer; Pharmacia Biotech) using the Thermo Sequenase fluorescent labelled primer cycle-sequencing kit with 7-deaza-dGTP and the Thermo Sequenase Cy5 dye terminator kit (Amersham Pharmacia Biotech). Both the forward and the reverse strands of each strain were sequenced.

The nucleotide sequences were compared with those present in the GenBank and RIDOM (Harmsen *et al.*, 1999) databases. Furthermore,

Table 1. Strains of *M. florentinum* sp. nov. included in this study

Isolate	Sex, age (years)	Source	Microscopy	Isolations	Clinical presentation	Underlying condition(s)	Clinical significance*	Isolation site	Isolation year
FI-93171 ^T	F, 6	Lymph node	Positive	Single	Lymphadenopathy	None	Yes	Italy 1	1993
FI-93188	F, 82	Sputum	Negative	Twice	Emphysema	Lung cancer	No	Italy 2	1993
FI-94029	M, 33	Stools	Negative	Single	Weight loss	AIDS	No	Italy 1	1993
FI-98295	F, 70	Sputum	Positive	Multiple	Pulmonary fibrosis, bronchiectasis	Ulcerative colitis	Yes	Italy 3	1998–2004
FI-04013	F, 93	Sputum, gastric aspirate	Positive	Twice	Haemoptysis, fever	Previous pulmonary tuberculosis, rheumatoid arthritis, senile dementia, surgically cured gall bladder cancer	Uncertain	Italy 4	2004
FI-04063	F, 84	Sputum	Negative	Twice	Haemoptysis, pleurisy	Hypertension	No	Italy 5	2004
FI-04276	M, 65	Sputum	Positive	Three times	Pneumonia	Multiple small nodules	Yes	Italy 6	2004
1696	F, 64	Sputum	Positive	Four times	Pneumonia	Lobectomy	Yes	Finland 2	2004
23†	F, 67	Sputum, bronchoalveolar lavage	Negative	Three times	Haemoptysis	Multiple small nodules	Yes	Finland 1	1995

*As defined by Wallace *et al.* (1997).

†From Suomalainen *et al.* (2001). The strain was initially identified tentatively as *M. triplex*.

they were aligned with sequences from all other species that were characterized by genetic relatedness to *M. simiae* by using the CLUSTAL W program (European Bioinformatics Institute; <http://www.ebi.ac.uk/cluster/>).

The neighbour-joining method was used for the construction of a phylogenetic tree (Felsenstein, 1993). The tree was rooted using *Mycobacterium tuberculosis* as the outgroup. Tree branches were reproduced by performing 100 bootstrap replicates.

PCR restriction analysis (PRA). PRA of the 65 kDa heat-shock protein gene (*hsp65*) was performed as previously reported using the enzymes *BstEII* and *HaeIII* and separating the digestion products by agarose gel electrophoresis (Telenti *et al.*, 1993). A third enzyme, *HhaI*, was used to differentiate species with overlapping PRA patterns.

RESULTS AND DISCUSSION

With minor exceptions, the overlapping of the phenotypic features of the test strains suggested that they could belong to a unique taxon, a hypothesis confirmed by the absolute identity of these strains at the genetic level. The strains undoubtedly belonged to the genus *Mycobacterium*, as inferred by the acid–alcohol–fastness of the bacilli, the presence of long-chain mycolic acids and, at the genetic level, by the presence of genus-specific regions within the 16S rRNA gene. The short helix 18 (a 12-nucleotide deletion near the end of the first third of the 16S rRNA gene) clearly assigned our strains to the group of *M. simiae*-related mycobacteria. The latter feature clearly distinguished them from other slow growers. The species presenting the best match with the test strains were *M. lentiflavum* and *M. triplex*.

Phenotypic features

The strains grew in about 2 weeks at 25 and 37 °C, but not at 45 °C and formed smooth, creamy, non-chromogenic colonies. They reduced tellurite and hydrolysed urea, were unable to accumulate niacin or to hydrolyse Tween 80 and did not possess β -glucosidase or arylsulfatase. Nitrate reduction was positive in five of the strains and negative or weakly positive in the others; catalase too was variable. Mycobacterial growth was inhibited on MacConkey's agar and on Löwenstein–Jensen medium supplemented with NaCl. No inhibition was observed on media containing isoniazid, hydroxylamine, oleic acid, *p*-nitrobenzoic acid or thiacetazone. The above phenotypic tests did not reveal features helpful in differentiating the organisms from *M. triplex* or (apart from colony morphology) from *M. lentiflavum*.

TLC revealed the presence of alpha-, methoxy- and keto-mycolates, a pattern very common within the genus *Mycobacterium*, presented also by *M. tuberculosis*.

GLC analysis revealed patterns close to those reported for *M. lentiflavum* and *M. triplex*. However, the patterns of the novel strains differed from those of both of these species in having the peak for 24:0 higher than that for 26:0, and

18:0 higher than that for tuberculostearic acid (10-Me-18:0, TBSA); they also differed from *M. triplex* in having 20:0 higher than 22:0 (Fig. 1).

The HPLC pattern fell within the three-clustered motif that characterizes most species genetically related to *M. simiae*. It differed from the latter, however, in the earlier emergence of single clusters and in the very limited height of the peaks in the central cluster (Fig. 2).

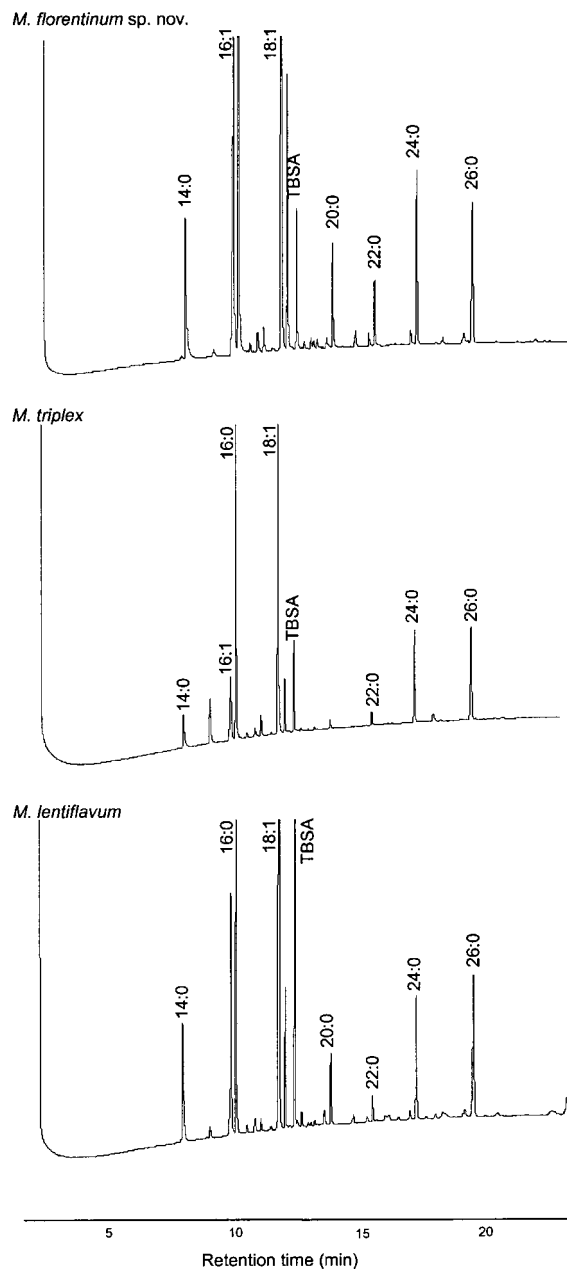


Fig. 1. GLC pattern of the type strain of *M. florentinum* sp. nov. in comparison with those of *M. triplex* and *M. lentiflavum*. TBSA, Tuberculostearic acid.

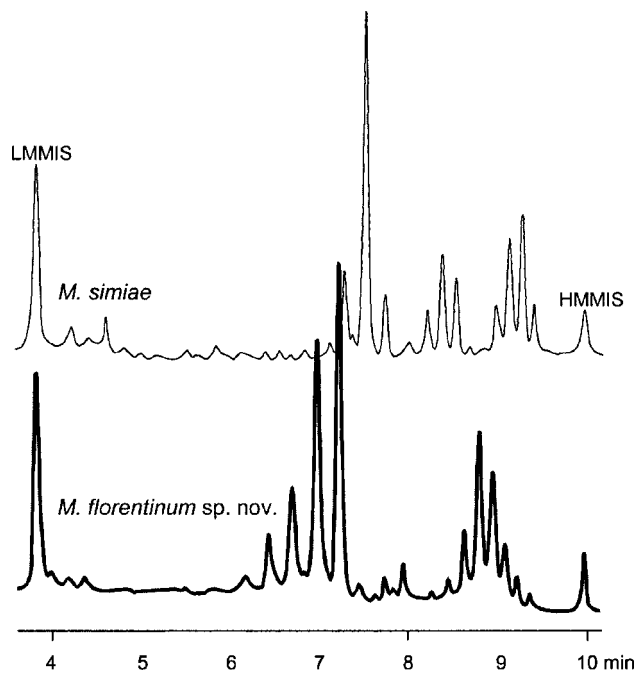


Fig. 2. HPLC profile of the type strain of *M. florentinum* sp. nov. compared with that of *M. simiae*. LMMIS, Low-molecular-mass internal standard; HMMIS, high-molecular-mass internal standard.

The three strains investigated presented quite high resistance, in particular to quinolones and rifampicin, with only clarithromycin and clofazimine being fully active *in vitro* (see Supplementary Table A in IJSEM Online).

Genotypic features

The number of nucleotide mismatches with the most closely related species, *M. triplex*, was 22 (1.2%) in the 1788 bp stretch including the 16S rRNA gene (8 mismatches) and the ITS (14 mismatches). Such a number of mismatches is clearly higher than that differentiating many of the other recognized species. Within the 16S rRNA gene, four mismatches occurred in the first third of the gene, one of them in hypervariable region A, thus allowing differentiation from all other species by studying this region alone.

In the RIDOM database, consisting of the 5' end of the 16S rRNA gene sequences (corresponding to positions 54–510 of the *Escherichia coli* 16S rRNA gene) of strictly controlled mycobacterial strains, the most closely related species was *M. triplex* (ATCC 700071^T) which differed by 4 bp. In GenBank, the complete sequence of the 16S rRNA gene, and the ITS as well, was 100% identical to that of a strain tentatively assigned to the species *M. triplex*, isolate 23 (Suomalainen *et al.*, 2001); however, the sequences of the latter strain and our novel strains differed by 22 bp from the reference strain of that species (ATCC 700071^T).

The position of our strains in the phylogenetic tree was close to *M. lentiflavum* and the recently described species

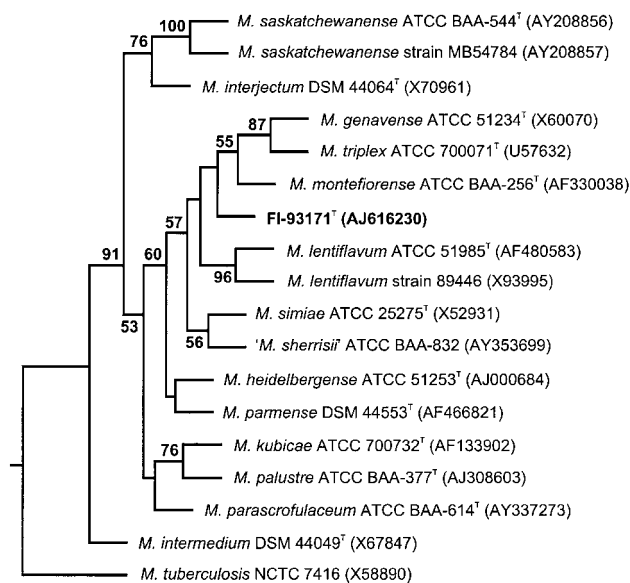


Fig. 3. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences including mycobacterial species genetically related to *M. simiae*. *M. tuberculosis* NCTC 7416 was used as an outgroup. Numbers on the dendrogram are percentages of occurrence in 100 bootstrapped trees (only values above 50% are shown).

Mycobacterium montefiorensis (Fig. 3). Interestingly, its position was closer to *M. montefiorensis* and *M. lentiflavum* than to *M. triplex*.

In the PRA, no digestion was produced by *Bst*EII. The use of only the conventionally employed enzymes revealed a pattern that was not distinguishable from *M. lentiflavum* but clearly different from *M. triplex*. However, differentiation from *M. lentiflavum* was possible when an additional enzyme, *Hha*I, was introduced (Table 2).

Three organisms related to *M. triplex* have been reported in the literature. Two of them are different from ours. The first, which was responsible for cervical lymphadenitis, differs in the ITS sequence and the HPLC profile (Hazra *et al.*, 2001). The second, responsible for disseminated infection in an

Table 2. PRA of *hsp65* fragments with different restriction enzymes from *M. florentinum* sp. nov. and the most closely related species

Sizes below 50 bp are given in parentheses as their recognition on the gel may be problematic.

Species	<i>Bst</i> EII	<i>Hae</i> II	<i>Hha</i> I
<i>M. florentinum</i> sp. nov.	441	148/130/(35)	235/105/65
<i>M. lentiflavum</i>	441	148/130/(35)	235/85/65
<i>M. triplex</i>	325/116	148/130/50/(35)	235/85/65

AIDS patient in France (Zeller *et al.*, 2003), presents four mismatches in a 229-nucleotide 16S rRNA gene fragment. However, the third, isolated in Finland and responsible for a pulmonary infection (Table 1), is identical to our strains, as strongly supported by the sequence identity of both the 16S rRNA gene and the ITS (Suomalainen *et al.*, 2001). This latter strain was retrieved and was tested for chromatographic patterns of cell-wall lipids by running in parallel with the test strains. Interestingly, it was found to be identical to the novel strains investigated here.

Description of *Mycobacterium florentinum* sp. nov.

Mycobacterium florentinum (flo'ren.ti.num. N.L. neut. adj. *florentinum* of the Italian city of Florence, where the majority of the strains were collected and investigated).

Non-motile, acid-fast bacillus devoid of spores or capsule. Grows in about 2 weeks at temperatures ranging from 25 to 37 °C, forming unpigmented colonies. The biochemical profile, characterized by positive urease and negative Tween 80 hydrolysis and 3 day arylsulfatase, is not suitable for unambiguous differentiation of strains from several other non-chromogenic species, in particular from *M. triplex* and *M. lentiflavum*. Among lipid investigations, GLC can differentiate *M. florentinum* sp. nov. from all other related species and the HPLC pattern is unique, although it roughly follows the three-clustered motif characteristic of many *M. simiae*-related species. *M. lentiflavum* is the only species that presents a PRA pattern that overlaps with that of *M. florentinum* sp. nov.; distinction is possible by digesting the DNA with *Hha*I, however. Finally, the sequences of both the 16S rRNA gene and of the ITS are unique. These data, in addition to its position in the phylogenetic tree, strongly support the novel species status of *M. florentinum* sp. nov.

The type strain, strain FI-93171^T (=DSM 44852^T=CIP 108409^T), was isolated from an excised lymph node of a young girl in Italy.

ACKNOWLEDGEMENTS

The work of L.R. was supported by grants from the Italian 'Istituto Superiore di Sanità' (National Research Program on AIDS) and from MIUR (PRIN 2002-04). We thank Dr S. Armitano, Dr G. L. Molinari and Dr S. Pierdomenico for providing microbiological and clinical information.

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Supplementary Table. Antimicrobial susceptibility of three strains of *Mycobacterium florentinum* sp. nov.

Antimicrobial agent	MIC ($\mu\text{g ml}^{-1}$)		
	FI-93171 ^T	FI-93188	FI-94029
Amikacin	2	0.5	4
Ciprofloxacin	4	128	128
Clarithromycin	2	2	2
Clofazimine	0.12	0.12	0.5
Ethambutol	8	4	8
Rifabutin	0.25	0.06	0.25
Rifampicin	16	8	4