Mycobacterium litorale sp. nov., a rapidly growing mycobacterium from soil

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A Gram-positive, acid-fast and rapidly growing rod, designated F4^T, was isolated from a soil sample of Haikou in China. The isolate shared 98.2% 16S rRNA gene sequence similarity with *Mycobacterium monacense* B9-21-178^T, 96.2% *hsp65* sequence similarity with *M. monacense* FI-05352 and 79.6% 16S–23S rRNA internal transcribed spacer sequence similarity with *M. monacense* B9-21-178^T. DNA–DNA relatedness between the isolate and *M. monacense* DSM 44395^T was 43.5%. The morphological analysis and physiological tests also showed that the isolate differed from any strain reported to date. The mycolic acid profile and the cellular fatty acid composition were also determined. On the basis of phenotypic and chemotaxonomic characteristics and phylogenetic data, it was concluded that strain F4^T (=CGMCC 4.5724^T=JCM 17423^T) merited classification as the type strain of a novel species, for which the name *Mycobacterium litorale* sp. nov. is proposed.

The genus *Mycobacterium* is represented by a wide range of species. It is a heterogeneous group in terms of occurrence in clinical and environmental materials, complex phenotypic and genetic data and association with disease. The genus was first described by Lehmann and Neumann in 1896, with *Mycobacterium tuberculosis* as the type species (Skerman *et al.*, 1980). The genus comprises 149 recognized species and has two main divisions: slowly growing and rapidly growing species. Rapidly growing mycobacteria are increasingly recognized as a cause of human infections. This group of mycobacteria is heterogeneous in terms of epidemiology, clinical disease spectrum and drug susceptibility. Therefore, it is important to identify rapidly growing mycobacteria to the species level, but their identification by conventional biochemical methods is limited. In recent years, the development of molecular techniques has led to a more accurate description of the genus *Mycobacterium*. To overcome the limitations of conventional methods, several molecular analyses based on housekeeping genes, such as the 16S rRNA gene (Rogall *et al.*, 1990; Springer *et al.*, 1996), *hsp65* (Kim *et al.*, 2005) and the 16S–23S rRNA internal transcribed spacer (ITS) (Roth *et al.*, 1998), have been used for species identification.

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, *hsp65* and 16S–23S ITS sequences of strain F4^T are GU997640, JF431428 and JF431427, respectively.

A supplementary figure is available with the online version of this paper.

Strain F4^T was isolated from a soil sample collected from the seashore of Haikou, Hainan Province, China. Soil (1 g) was suspended in distilled water (100 ml), serially diluted, spread on ISP 2 (Shirling & Gottlieb, 1966) and incubated at 37 °C for 3 days. Strain F4^T was maintained on LB agar (Stanier *et al.*, 1966) at 4 °C and stored in 20% (v/v) glycerol at –20 °C.

Standard biochemical tests were performed. Cultures were grown on LB agar, Sauton's medium and tryptone soy agar (TSA) at 37 °C. The isolate was examined for colony morphology and pigmentation after 3 days on LB agar at 37 °C. Gram-staining was performed using the methods of Smibert & Krieg (1994). Acid fastness was studied using modified Kinyoun acid-fast stains (Berd, 1973). Hydrolysis of urea was determined in the following liquid medium by Goodfellow & Orchard (1974): 75 ml urea shake flask medium [10 g KH₂PO₄, 9.5 g Na₂HPO₄, 1 g yeast extract, with 0.04% (w/v) phenol red, 1000 ml distilled water, pH 7.2] and 15 ml of 15% urea. Resistance to antibiotics was examined on LB agar using impregnated filter-paper discs (Goodfellow & Orchard, 1974) containing cefalotin, amikacin, ciprofloxacin, clarithromycin, doxycycline, linezolid, tobramycin and sulfamethylisoxazole; results were recorded after incubation at 37 °C for up to 7 days. The phenotypic properties of strain F4^T are summarized in Table 1 and the species description.

Sequencing of the 16S rRNA gene, *hsp65* and the 16S–23S ITS sequences was carried out by standard procedures (Rainey *et al.*, 1996; Ringuet *et al.*, 1999; Roth *et al.*, 2000) using purified DNA as a template and an automated DNA sequencer (model 377; Applied Biosystems). A nearly

Table 1. Comparison of properties of strain F4^T and its closest neighbours in the genus *Mycobacterium*

Strains: 1, *Mycobacterium litorale* sp. nov. F4^T; 2, *M. monacense* DSM 44395^T (Reischl *et al.*, 2006); 3, *M. doricum* DSM 44339^T (Tortoli *et al.*, 2001); 4, *M. tusciae* DSM 44338^T (Tortoli *et al.*, 1999). All strains are positive for nitrate reduction. All strains are negative for β -glucosidase, arylsulfatase (3 days) and niacin accumulation. +, Positive; v, variable; –, negative; ND, no data available.

Characteristic	1	2	3	4
Temperature range (°C)	25–45	25–45	25–37	25–37
Growth with 5% NaCl	–	+	–	–
Growth on MacConkey agar	+	–	–	–
Visible growth in <7 days	+	+	–	–
Pigmentation	–	+	+	+
Colony morphology	Smooth	Smooth	Smooth	Rough
Tween 80 hydrolysis	+	+	–	+
Thermostable catalase	+	+	–	+
Catalase zone >45 mm	–	v	–	–
Urease	+	v	+	+
Tellurite reduction	+	ND	+	v

complete 16S rRNA gene sequence (1380 bp) and partial sequences of *hsp65* (403 bp) and the 16S–23S ITS (310 bp) were obtained. The 16S rRNA gene sequence was compared with those available in the GenBank database using BLAST to determine an approximate phylogenetic affiliation for strain F4^T. Multiple alignment was performed using CLUSTAL X

version 2.0 (Larkin *et al.*, 2007). Evolutionary distances were calculated using distance options according to Kimura's two-parameter model (Kimura, 1980). Phylogenetic trees were constructed using MEGA version 4.1 (Tamura *et al.*, 2007). The topologies of the resultant unrooted trees were evaluated by bootstrap analysis based on 1000 resamplings (Felsenstein, 1985) of the neighbour-joining dataset.

On the basis of the 16S rRNA gene sequence analysis, 28 members of the genus *Mycobacterium* were found to have a close relationship with the isolate. It is apparent from the neighbour-joining and maximum-parsimony phylogenetic trees (Fig. 1) that strain F4^T belonged to the genus *Mycobacterium*. Strain F4^T shared greatest similarity with *Mycobacterium monacense* B9-21-178^T, *Mycobacterium doricum* DSM 44339^T and *Mycobacterium tusciae* DSM 44338^T (98.2, 98.0 and 97.9% 16S rRNA gene sequence similarity, respectively). The analysis of the *hsp65* sequence included the hypervariable region of *hsp65*. The *hsp65* sequence obtained from strain F4^T was compared with sequences found in GenBank for 20 closely related members of the genus *Mycobacterium*. The phylogenetic tree based on *hsp65* sequences is shown in Fig. S1 (available in IJSEM Online). The result confirmed the close phylogenetic relationship between the isolate and *M. monacense*. The *hsp65* sequence most closely related to the isolate was from *M. monacense* FI-05352, which differed by 16 bp (96.2% *hsp65* sequence similarity). With the 16S–23S ITS sequence analysis, strain F4^T differed from the type strain of *M. monacense* by 63 bp (79.6% similarity) (Reischl *et al.*, 2006).

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences, showing the relationships between strain F4^T and representatives of some species of the genus *Mycobacterium*. Bootstrap values (>50%) based on 1000 resampled datasets are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in the maximum-parsimony tree. Bar, 0.005 substitutions per nucleotide position.

Fig. 2. Mycolic acid profiles of *M. monacense* DSM 44395^T (a) and strain F4^T (b) determined using HPLC. LMMIS, Low-molecular-mass internal standard; HMMIS, high-molecular-mass internal standard.

The low 16S–23S ITS sequence similarity may support the suggestion that the usefulness of the 16S–23S ITS for phylogenetic studies of rapidly growing mycobacteria is somewhat limited (Reischl *et al.*, 2006).

Biomass for chemotaxonomic studies was obtained after incubation at 37 °C for 2 days in shake flasks of tryptone soy broth (Oxoid). Alpha-mycolates, keto-mycolates and wax esters were detected in strain F4^T by two-dimensional TLC (Schröder *et al.*, 1997). Strain F4^T showed a mycolic acid pattern similar to that of *M. monacense* DSM 44395^T, which is a pattern shared by a large number of non-tuberculous mycobacteria.

For quantitative analysis of the cellular fatty acids, cell mass was collected and fatty acids were extracted, purified, methylated and identified using the Sherlock Microbial Identification System version 4.5 and the TSBA40 database (MIDI; Sasser, 1990; Kämpfer & Kroppenstedt, 1996). The major fatty acids of strain F4^T were C_{16:0} (23.1 %), C_{18:1}ω9c (20.7 %), C_{17:1}ω7c (14.1 %) and summed feature 3 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 12.5 %). Moderate amounts of 10-methyl C_{18:0} (9.5 %) and summed feature 7 (C_{19:0}ω10c cyclo and/or C_{19:0}ω6c; 8.9 %) and smaller amounts of C_{14:0} (3.0 %), C_{18:0} (2.9 %), C_{16:1}ω9c (0.9 %), iso-C_{13:0} 3-OH (0.8 %), C_{18:1}ω7c (0.7 %), iso-C_{15:1} F (0.7 %), C_{15:0} (0.4 %), C_{17:0} (0.3 %), anteiso-C_{15:1} A (0.3 %), 10-methyl C_{16:0} (0.2 %) and C_{20:0} (0.2 %) were also detected. The major components have also been found in other species of the genus *Mycobacterium* (Reischl *et al.*, 2006; Tortoli *et al.*, 2001).

HPLC analysis of mycolic acids was performed as described by Butler *et al.* (1996). Low- and high-molecular-mass

standards were included for peak identification. The results showed two well-separated clusters of peaks, the first including three major peaks eluting between 5 and 6 min and the second presenting two peaks eluting after a 2-min interval (Fig. 2). A thorough investigation of the HPLC mycobacterium library (available at <http://www.Mycobactoscana.it>) revealed that *M. doricum*, *Mycobacterium murale*, *Mycobacterium terrae* and *Mycobacterium tokaiense* present profiles that grossly resemble this pattern.

There are widely accepted criteria for delineating species in bacteriology, which state that strains with <70 % DNA–DNA relatedness or >3 % 16S rRNA gene sequence difference are considered to represent different species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002). DNA–DNA hybridization between strain F4^T and its closest neighbours, *M. monacense* DSM 44395^T, *M. doricum* DSM 44339^T and *M. tusciae* DSM 44338^T, was carried out applying the optical renaturation method (De Ley *et al.*, 1970; Huß *et al.*, 1983; Jahnke, 1992) under optimal conditions. The mean DNA–DNA relatedness values between strain F4^T and *M. monacense* DSM 44395^T, *M. doricum* DSM 44339^T and *M. tusciae* DSM 44338^T were 43.5, 40.2 and 36.5 %, respectively. These values are significantly lower than the threshold value of 70 % (Wayne *et al.*, 1987).

A range of phenotypic properties separated strain F4^T from its closest phylogenetic neighbours (Table 1). In particular, the isolate could be differentiated from *M. monacense* DSM 44395^T in that it was negative for pigment production, semiquantitative catalase and growth with 5 % NaCl.

On the basis of the data above, strain F4^T represents a novel species of the genus *Mycobacterium*, for which the name *Mycobacterium litorale* sp. nov. is proposed.

Description of *Mycobacterium litorale* sp. nov.

Mycobacterium litorale (li.to.ra'le. L. neut. adj. *litorale* of or belonging to the seashore).

Cells are aerobic, Gram-positive, acid-fast, short rods and do not form endospores or conidiospores. Grows well on LB agar, Sauton's medium and TSA at 37 °C. Grows on MacConkey agar. Diffusible pigments are not produced. After 24–36 h on LB agar at 37 °C, colonies are light yellow, round, translucent and 2.0–3.0 mm in diameter; edges are smooth. Positive for nitrate reduction, thermostable catalase, urease, tellurite reduction and Tween 80 hydrolysis, but negative for β-glucosidase, semiquantitative catalase, arylsulfatase (3 days), niacin accumulation and starch hydrolysis. Does not grow with 5 % NaCl. Susceptible to cefalotin, amikacin, ciprofloxacin, doxycycline, linezolid, tobramycin and sulfamethylisoxazole, but resistant to clarithromycin. The major fatty acids (>10 %) are C_{16:0}, C_{18:1}ω9c, C_{17:1}ω7c and summed feature 3 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH); other fatty acids are 10-methyl C_{18:0}, summed feature 7 (C_{19:0}ω10c cyclo and/or C_{19:0}ω6c), C_{14:0}, C_{18:0}, C_{16:1}ω9c, iso-C_{13:0} 3-OH, C_{18:1}ω7c, iso-C_{15:1} F, C_{15:0}, C_{17:0}, anteiso-C_{15:1} A,

10-methyl C_{16:0} and C_{20:0}. The HPLC mycolic acid pattern is characterized by two clusters of peaks that do not coincide with any previously reported mycobacterial profile.

The type strain, F4^T (=CGMCC 4.5724^T =JCM 17423^T), was isolated from a soil sample collected from the seashore of Haikou, Hainan Province, China.

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