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

Yabo Zhang,1 Jianli Zhang,1 Caiyuan Fang,1 Huancheng Pang2 and Jinping Fan1

1School of Life Science, Beijing Institute of Technology, Beijing 100081, PR China
2Institute of Agri-resources and Regional Planning, CAAS, Beijing 100081, PR China

A Gram-positive, acid-fast and rapidly growing rod, designated F4T, was isolated from a soil sample of Haikou in China. The isolate shared 98.2 % 16S rRNA gene sequence similarity with Mycobacterium monacense B9-21-178T, 96.2 % hsp65 sequence similarity with M. monacense FI-05352 and 79.6 % 16S–23S rRNA internal transcribed spacer sequence similarity with M. monacense B9-21-178T. DNA–DNA relatedness between the isolate and M. monacense DSM 44395T 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 F4T (=CGMCC 4.5724T=JCM 17423T) 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.

Strain F4T 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 F4T 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 flasks 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 sulfamethoxazole; results were recorded after incubation at 37 °C for up to 7 days. The phenotypic properties of strain F4T 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...
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 F4T. 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 F4T belonged to the genus Mycobacterium. Strain F4T shared greatest similarity with Mycobacterium monacense B9-21-178T, Mycobacterium doricum DSM 44339T and Mycobacterium tusciae DSM 44338T (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 F4T 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 F4T differed from the type strain of M. monacense by 63 bp (79.6 % similarity) (Reischl et al., 2006).

### Table 1. Comparison of properties of strain F4T and its closest neighbours in the genus Mycobacterium

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature range (°C)</td>
<td>25–45</td>
<td>25–45</td>
<td>25–37</td>
<td>25–37</td>
</tr>
<tr>
<td>Growth with 5% NaCl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Growth on MacConkey agar</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Visible growth in &lt;7 days</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Pigmentation</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Colony morphology</td>
<td>Smooth</td>
<td>Smooth</td>
<td>Smooth</td>
<td>Rough</td>
</tr>
<tr>
<td>Tween 80 hydrolysis</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Thermostable catalase</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Catalase zone &gt;45 mm</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tellurite reduction</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>V</td>
</tr>
</tbody>
</table>

Table 1. Comparison of properties of strain F4T and its closest neighbours in the genus Mycobacterium

Strains: 1, *Mycobacterium litorale* sp. nov. F4T; 2, *M. monacense* DSM 44395T (Reischl et al., 2006); 3, *M. doricum* DSM 44339T (Tortoli et al., 2001); 4, *M. tusciae* DSM 44338T (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.
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 F4T by two-dimensional TLC (Schroeder et al., 1997). Strain F4T showed a mycolic acid pattern similar to that of Mycobacterium doricum DSM 44395T, 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 F4T were C16:0 (23.1%), C18:1ω9c (20.7%), C17:0ω7c (14.1%) and summed feature 3 (comprising C16:1ω7c and/or iso-C15:0 2-OH; 12.5%). Moderate amounts of 10-methyl C18:0 (9.5%) and summed feature 7 (C19:0ω10c cyclo and/or C19:0ω6c; 8.9%) and smaller amounts of C14:0 (3.0%), C18:0 (2.9%), C16:1ω9c (0.9%), iso-C13:0 3-OH (0.8%), C18:1ω7c (0.7%), iso-C15:0 F (0.7%), C15:0 (0.4%), C17:0 (0.3%), anteiso-C15:0 2-OH (0.3%), 10-methyl C16:0 (0.2%) and C20:0 (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.Mycobacteriumtosca.ne.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 F4T and its closest neighbours, M. monacense DSM 44395T, M. doricum DSM 44339T and M. tusciae DSM 44338T, 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 F4T and M. monacense DSM 44395T, M. doricum DSM 44339T and M. tusciae DSM 44338T were 43.5, 40.2 and 36.5%, respectively. These values are significantly lower than the threshold value of 70% (Wayne et al., 1987).

Fig. 2. Mycolic acid profiles of M. monacense DSM 44395T (a) and strain F4T (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 F4T by two-dimensional TLC (Schroeder et al., 1997). Strain F4T showed a mycolic acid pattern similar to that of M. monacense DSM 44395T, 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 F4T were C16:0 (23.1%), C18:1ω9c (20.7%), C17:0ω7c (14.1%) and summed feature 3 (comprising C16:1ω7c and/or iso-C15:0 2-OH; 12.5%). Moderate amounts of 10-methyl C18:0 (9.5%) and summed feature 7 (C19:0ω10c cyclo and/or C19:0ω6c; 8.9%) and smaller amounts of C14:0 (3.0%), C18:0 (2.9%), C16:1ω9c (0.9%), iso-C13:0 3-OH (0.8%), C18:1ω7c (0.7%), iso-C15:0 F (0.7%), C15:0 (0.4%), C17:0 (0.3%), anteiso-C15:0 A (0.3%), 10-methyl C16:0 (0.2%) and C20:0 (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.Mycobacteriumtosca.ne.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 F4T and its closest neighbours, M. monacense DSM 44395T, M. doricum DSM 44339T and M. tusciae DSM 44338T, 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 F4T and M. monacense DSM 44395T, M. doricum DSM 44339T and M. tusciae DSM 44338T 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 F4T from its closest phylogenetic neighbours (Table 1). In particular, the isolate could be differentiated from M. monacense DSM 44395T in that it was negative for pigmentation, semiquantitative catalase and growth with 5% NaCl.

On the basis of the data above, strain F4T 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 sulfamethoxazole, but resistant to clarithromycin. The major fatty acids (>10%) are C16:0, C18:1ω9c, C17:0ω7c and summed feature 3 (comprising C16:1ω7c and/or iso-C15:0 2-OH); other fatty acids are 10-methyl C18:0 summed feature 7 (C19:0ω10c cyclo and/or C19:0ω6c), C14:0, C18:0, C16:1ω9c, iso-C13:0 3-OH, C18:1ω7c, iso-C15:1 F, C15:0, C17:0, anteiso-C15:1 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.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (grant numbers 30970009, 31070002), by the Special Fund for Public Welfare Industrial (Agriculture) Research of China (grant number 200903001) and by the Basic Research Foundation of Beijing Institute of Technology (grant number 20091642006). The authors are grateful to Dr Yafang Tan for carrying out the quantitative fatty acid analysis.

References


