Mycobacterium franklinii sp. nov., a species closely related to members of the Mycobacterium chelonae-Mycobacterium abscessus group

Christiane Lourenço Nogueira,1 Keith E. Simmon,2 Erica Chimara,3 Margo Cnockaert,4 Juan Carlos Palomino,4 Anandi Martin,4 Peter Vandamme,4 Barbara A. Brown-Elliott,5 Richard J. Wallace Jr5,6 and Sylvia Cardoso Leão1

1Departamento de Microbiologia, Imunologia e Parasitologia, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brazil
2Department of Biomedical Informatics, University of Utah School of Medicine, Salt Lake City, UT, USA
3Núcleo de Tuberculose e Micobacterioses, Instituto Adolfo Lutz, São Paulo, SP, Brazil
4Laboratory of Microbiology, Faculty of Sciences, Ghent University, Ghent, Belgium
5University of Texas Health Science Center at Tyler, Mycobacteria/Nocardia Laboratory, Tyler, TX, USA
6University of Texas Health Science Center at Tyler, Department of Medicine, Tyler, TX, USA

Two isolates from water, D16Q19 and D16R27, were shown to be highly similar in their 16S rRNA, 16S–23S internal transcribed spacer (ITS), hsp65 and rpoB gene sequences to ‘Mycobacterium franklinii’ DSM 45524, described in 2011 but with the name not validly published. They are all nonpigmented rapid growers and are related phenotypically and genetically to the Mycobacterium chelonae-Mycobacterium abscessus group. Extensive characterization by phenotypic analysis, biochemical tests, drug susceptibility testing, PCR restriction enzyme analysis of the hsp65 gene and ITS, DNA sequencing of housekeeping genes and DNA–DNA hybridization demonstrated that ‘M. franklinii’ DSM 45524, D16Q19 and D16R27 belong to a single species that is separated from other members of the M. chelonae–M. abscessus group. On the basis of these results we propose the formal recognition of Mycobacterium franklinii sp. nov. Strain DSM 45524T (=ATCC BAA-2149T) is the type strain.

The Mycobacterium chelonae–Mycobacterium abscessus group comprises closely related rapidly growing mycobacteria. They are widespread saprophytes that can cause several opportunistic infections in humans, ranging from localized abscesses to pulmonary and disseminated disease (Brown-Elliott & Wallace, 2002; Wallace et al., 1983). Skin and soft-tissue infections caused by members of the M. chelonae–M. abscessus group have been reported increasingly in recent years, representing a serious public health problem in some settings. The ubiquitous distribution of these organisms facilitates the contamination of medical equipment and solutions, which may be associated with the growing number of therapeutic interventions leading to nosocomial infections and outbreaks (Brown-Elliott & Wallace, 2012; Tortoli, 2009).

Until 2007, the M. chelonae–M. abscessus group was composed of Mycobacterium chelonae, Mycobacterium abscessus (Kusunoki & Ezaki, 1992), Mycobacterium immunogenum (Wilson et al., 2001), Mycobacterium massiliense (Adékambi et al., 2004, 2006b), Mycobacterium bolletii (Adékambi et al., 2006a) and Mycobacterium salmonophilum

Abbreviations: ITS, internal transcribed spacer; PNB, para-nitrobenzoic acid

The GenBank/EMBL/DDJB accession numbers for the partial 16S rRNA, hsp65, rpoB and ITS sequences of isolate D16R27 are KM392061, KM392060, KM392057 and KM392062, respectively. Those for the partial hsp65 and rpoB gene sequences of M. franklinii DSM 45524T are KM392059 and KM392056, respectively, and that for the partial rpoB gene sequence of M. salmonophilum ATCC 13758T is KM392058.

Three supplementary tables are available with the online Supplementary Material.
(Whipps et al., 2007). Recently, this group has undergone taxonomic changes, including the unification of *M. abscessus*, *M. massiliense* and *M. bolletii* in a single species (*M. abscessus*) with two subspecies (*M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*). Strains formerly representing *M. massiliense* were reclassified as *M. abscessus* subsp. *bolletii* (Leão et al., 2009, 2011). Although beyond the scope of this paper, whole genomic sequencing has recently provided evidence that there are indeed three taxonomic groups within *M. abscessus*. It is not clear yet how this will affect the 2011 reclassification (Tettelin et al., 2014).

In 2011, Simmon & Brown-Elliott et al. studied a group of clinical isolates from Pennsylvania, and primarily the north-eastern USA, that were initially misidentified as *M. chelonae* with an atypical antimicrobial drug susceptibility profile (Simmon et al., 2011). This study included the proposal of *Mycobacterium franklinii* as a new member of the *M. chelonae–M. abscessus* group, but this name has not been validly published.

During taxonomic studies of isolates from the collection of Professor Françoise Portaels (Antwerp, Belgium), two isolates (D16Q19 and D16R27) showed high similarity with *M. franklinii* by sequencing of 16S rRNA, *hsp65* and *rpoB* genes and the 16S–23S internal transcribed spacer (ITS). Strains D16Q19 and D16R27 were isolated in Germany by Professor Roland Schulze-Röhbecke (Düsseldorf, Germany) from water works and tap water, respectively.

The aim of the present study was to examine the taxonomic position of these two isolates and *‘M. franklinii’* DSM 45524 using phenotypic and genotypic tests. The results were compared with those displayed by other members of the *M. chelonae–M. abscessus* group, *M. abscessus* subsp. *abscessus* ATCC 19977T, *M. abscessus* subsp. *bolletii* CCUG 50184T, *M. chelonae* ATCC 35752T, *M. immunogenum* ATCC 700505T and *M. salmoniphilum* ATCC 13758T.

Cultures were grown on solid-phase media including Middlebrook 7H10 (Becton Dickinson Co, Sparks, MD, USA) and Luria–Bertani agar and in liquid medium including Mueller–Hinton (Becton Dickinson) and Luria–Bertani broth with 0.1 % Tween 80. All cultures were incubated at 28–30 °C. Microscopic examination of isolated colonies by Ziehl–Neelsen staining showed that isolates D16Q19, D16R27 and DSM 45524T were acid-fast bacilli. Pigment production, single-source carbon utilization (mannitol, inositol and citrate), growth at 26 °C and 37 °C and the ability to grow in the presence of 5 % NaCl, 0.2 % picric acid, para-nitrobenzoic acid (PNB) and nitrite were performed on 7H10 medium. Nitrate reduction and Tween 80 hydrolysis were also examined. The results of these tests were read after 7 and 14 days of incubation at 28 °C, except for Tween 80 hydrolysis, which was read after 5 and 10 days, as described in standard protocols for biochemical testing of mycobacteria (Kent & Kubica, 1985; Leão et al., 2004; Tsukamura, 1984). Isolates D16Q19, D16R27 and DSM 45524T exhibited indistinguishable phenotypic and biochemical characteristics, which are indicated in Table S1 (available in the online Supplementary Material). Colonies that appeared on Middlebrook 7H10 and Luria–Bertani agar after aerobic incubation were nonpigmented. On these solid media, visible growth required 3 to 5 days at 25 °C to 37 °C; optimum growth occurred at 28 °C. The bacterial cells showed tendency to form large clumps when grown in liquid media and the use of 0.1 % Tween 80 helped to obtain dispersed growth. They grew in the presence of 5 % NaCl, picric acid, PNB and nitrite. They were negative for nitrate reduction and Tween hydrolysis and growth did not occur when mannitol or inositol was used as a single-source of carbon. D16Q19, D16R27, *M. chelonae* ATCC 35752T and *M. immunogenum* ATCC 700505T grew in the presence of citrate but not strain DSM 45524T or the other type strains of the *M. chelonae–M. abscessus* group. These phenotypic and biochemical tests were not useful for distinguishing D16Q19, D16R27 and DSM 45524T from other members of the *M. chelonae–M. abscessus* group.

Antimicrobial susceptibility testing was performed in cation-supplemented Mueller–Hinton broth by the microdilution method, according to the recommendations of the Clinical and Laboratory Standards Institute for rapidly growing mycobacteria (CLSI, 2011). The antimicrobials tested were amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline, minocycline, moxifloxacin and tobramycin. According to CLSI interpretative standards, isolates D16Q19, D16R27 and DSM 45524T were susceptible to amikacin, ciprofloxacin, doxycycline, minocycline and moxifloxacin. D16Q19 and D16R27 were susceptible to clarithromycin at 3 days of incubation but extended clarithromycin MICs (14 days of incubation) was not performed. Susceptibility of D16Q19 and D16R27 to cefoxitin was intermediate and they were resistant or intermediate to tobramycin while DSM 45524T was susceptible to both drugs (Table 1). The results obtained with cefoxitin were in agreement with results obtained by Simmon & Brown-Elliott et al., who reported intermediate and susceptible rates of 88 % and 22 %, respectively, for *‘M. franklinii’* isolates (Simmon et al., 2011). D16Q19, D16R27 and DSM 45524T were more susceptible to antimicrobial drugs than the other members of the *M. chelonae–M. abscessus* group, but studies of larger strain numbers have shown greater MIC variability than exhibited by these reference type strains (Table 1).

GenoType Mycobacterium common mycobacteria (CM) and additional species (AS) assays (Hain Lifescience), two commercial DNA strip assays for mycobacteria identification, were performed according to manufacturer’s instructions. Using GenoType CM, isolates D16Q19, D16R27 and DSM 45524T showed the *M. chelonae* profile
Isolates/strains: 1, D16Q19; 2, D16R27; 3, Mycobacterium franklinii sp. nov. DSM 45524T; that were analyzed by Simmon & Brown-Elliott in a previous study (Simmon et al., 2011) 4, range of results of 25 CV* isolates; 5, M. abscessus subsp. abscessus ATCC 19977T; 6, M. abscessus subsp. bolletii CCUG 50184T; 7, M. chelonae ATCC 35752T; 8, M. immunogenum ATCC 700505T; 9, M. salmoniphilum ATCC 13758T. The antimicrobial susceptibility breakpoints were those established by CLSI (2011) for rapidly growing mycobacteria. ND, Not determined; n, number of isolates tested by Simmon & Brown-Elliott et al. (Simmon et al., 2011).

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC (µg ml⁻¹)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td></td>
<td>8</td>
<td>16</td>
<td>≤4</td>
<td>8–32</td>
<td>8</td>
<td>16</td>
<td>8</td>
<td>≤4</td>
<td>16</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td></td>
<td>≤0.25</td>
<td>≤0.25</td>
<td>0.5</td>
<td>≤0.25–8</td>
<td>4</td>
<td>8</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Clarithromycin (3 days)</td>
<td></td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>≤0.12–1</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>≤0.5</td>
</tr>
<tr>
<td>Clarithromycin (14 days)</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>&lt;2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Doxycycline</td>
<td></td>
<td>≤0.25</td>
<td>≤0.5</td>
<td>≤0.25</td>
<td>≤0.25–&gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>4</td>
<td>&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td></td>
<td>64</td>
<td>64</td>
<td>16</td>
<td>16–64</td>
<td>64</td>
<td>32</td>
<td>512</td>
<td>256</td>
<td>512</td>
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<td>Tobramycin</td>
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<td>4</td>
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<td>2</td>
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<td>8</td>
<td>16</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Minocycline</td>
<td></td>
<td>1</td>
<td>≥0.25</td>
<td>≥0.25</td>
<td>≥0.5–16xs</td>
<td>8</td>
<td>16</td>
<td>2</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td></td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>0.5–8</td>
<td>8</td>
<td>8</td>
<td>≤0.25</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

*Simmon & Brown-Elliott et al. labelled 'M. franklinii' isolates as CV indicating M. chelonae variant (Simmon et al., 2011).

(hybridization with probes 5 and 10). They showed the same GenoType AS profile, hybridization with probe 12, which assigns the isolates to a species of mycobacteria different from the 18 species identifiable with the AS strip.

For molecular identification by PCR restriction enzyme analysis (PRA) of the hsp65 gene and ITS, DNA was prepared by boiling one loopful of bacteria for 10 min in 300 µl TET (10 mM Tris, 1 mM EDTA, 1% Triton X-100, pH 8.0) followed by centrifugation at 14,000 g for 2 min. For PRA-hsp65, a 441 bp fragment of the hsp65 gene was amplified using primers Tb11 and Tb12 (Table S2), as described by Telenti et al. (1993). The amplicons were digested in two separate tubes with BseEII and HaeIII restriction enzymes. For PRA-ITS, the ITS fragment was amplified using primers Sp1 and Sp2 (Table S2), as described by Roth et al. (2000). ITS amplicons were digested with TaqI restriction enzyme. The digestion products of both PRA-hsp65 and PRA-ITS were visualized after electrophoresis in 3% agarose gels stained with ethidium bromide, using 50 bp ladder as the molecular size standard. The restriction fragment sizes were estimated using the BioNumerics program version 5.1 (Applied Maths) and compared with the patterns included in the PRASITE (http://app.chuv.ch/prasite/index.html) for PRA-hsp65 and published by Roth et al. (2000) for PRA-ITS.

Isolates D16Q19, D16R27 and DSM 45524T showed indistinguishable PRA-hsp65 patterns – BseEII (320 and 130 bp) and HaeIII (200, 60 and 55 bp). They showed the same PRA-ITS pattern – TaqI (225 and 30 bp), which is common to M. abscessus. Both assays, GenoType and PRA, were not useful for distinguishing D16Q19, D16R27 and DSM 45524T from other members of the M. chelonae–M. abscessus group (data not shown).

Partial sequences of the 16S rRNA gene (Adékambi & Drancourt, 2004; Gomila et al., 2007; Harmsen et al., 2003), hsp65 (Telenti et al., 1993), rpoB (Adékambi et al., 2003) and ITS (Roth et al., 2000) were obtained with isolates D16Q19 and D16R27. The primers used for PCR amplification and sequencing are listed in Table S2. PCR products were purified using a QIAquick PCR purification kit (Qiagen). Dideoxy sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and run in an ABI PRISM 3100 DNA Analyser (Applied Biosystems). Sequences from both isolates D16Q19 and D16R27 shared 100% identity and only the sequences from isolate D16R27 were deposited in the GenBank database. The corresponding sequences of the M. chelonae–M. abscessus group type strains were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). The hsp65 and rpoB genes of DSM 45524T and...
the rpoB gene of *M. salmoniphilum* ATCC 13758<sup>T</sup> were resequenced, as the sequences deposited in the GenBank database were incomplete. All sequences of isolates D16R27 and D16Q19 showed the highest sequence similarity with the corresponding sequences of DSM 45524<sup>T</sup>, suggesting that they belong to the same taxon (Table S3). Sequence alignments and phylogenetic trees were reconstructed by using the neighbour-joining method with the Kimura two-parameter distance correction model and 1000 bootstrap replications in MEGA6 (Tamura et al., 2013) (Fig. 1).

Isolate D16R27 was selected to perform DNA–DNA hybridization (DDH) experiments. High molecular mass DNA was prepared from 2 g cell mass using the protocol described by Pitcher et al. (1989) with modifications. After centrifugation, cells were inactivated at 90 °C for 30 min and resuspended in 3 ml of lysis buffer containing 200 μg RNase ml<sup>-1</sup>, 25 mg fresh lysozyme ml<sup>-1</sup> and 100 U mutanolysine ml<sup>-1</sup>. The suspensions were incubated overnight at 37 °C. Chloroform/isoamyl alcohol extraction, RNase treatment and ethanol precipitation were performed as described by Marmur (1961). DDH was performed with photobiotin-labelled probes in microplate wells, as described by Ezaki et al. (1989), using an HTS7000 Bio Assay Reader (Perkin-Elmer) for fluorescence measurements. The hybridization temperature was 50 °C, calculated on the basis of the DNA G+C contents of the isolates and type strains, estimated as described by Mesbah & Whitman (1989). The DNA G+C content of isolates D16R27 and D16Q19 was 64.2 mol%. The DNA G+C content of *M. abscessus* subsp. *abscessus* ATCC 19977<sup>T</sup>, *M. chelonae* ATCC 35752<sup>T</sup>, *M. immunogenum* ATCC 700505<sup>T</sup>, *M. salmoniphilum* ATCC 13758<sup>T</sup> and DSM 45524<sup>T</sup> was 64.0, 64.1, 64.2, 64.3 and 64.1 mol%, respectively. These values are consistent with the DNA G+C contents of this genus, between 59 and 66 mol% (Devulder et al., 2005). DDH values are presented as means of reciprocal experiments, performed in quadruplicate hybridization reactions (Table 2). The mean DNA–DNA hybridization value between D16R27 and DSM 45524<sup>T</sup> was higher than the accepted cut-off value of 70 % and confirmed that both belong to the same species. The mean hybridization values of D16R27 with *M. abscessus* subsp. *abscessus* ATCC 19977<sup>T</sup>, *M. chelonae* ATCC 35752<sup>T</sup>, *M. immunogenum* ATCC 700505<sup>T</sup> and *M. salmoniphilum* ATCC 13758<sup>T</sup> were all below 70 %, further confirming that isolate D16R27 represents a

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**Table 2. DNA–DNA hybridization values of D16R27 against the type strains of the *M. chelonae–M. abscessus* group**

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>DNA–DNA relatedness (%)&lt;sup&gt;*&lt;/sup&gt;</th>
<th>Reciprocal values</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mycobacterium franklinii</em> sp. nov.</td>
<td>DSM 45524&lt;sup&gt;T&lt;/sup&gt;</td>
<td>78.5 (2.5)</td>
<td>76; 81</td>
</tr>
<tr>
<td><em>M. abscessus</em> subsp. <em>abscessus</em></td>
<td>ATCC 19977&lt;sup&gt;T&lt;/sup&gt;</td>
<td>50.5 (4.5)</td>
<td>46; 55</td>
</tr>
<tr>
<td><em>M. chelonae</em></td>
<td>ATCC 35752&lt;sup&gt;T&lt;/sup&gt;</td>
<td>55 (8)</td>
<td>47; 63</td>
</tr>
<tr>
<td><em>M. immunogenum</em></td>
<td>ATCC 700505&lt;sup&gt;T&lt;/sup&gt;</td>
<td>45.5 (4.5)</td>
<td>41; 50</td>
</tr>
<tr>
<td><em>M. salmoniphilum</em></td>
<td>ATCC 13758&lt;sup&gt;T&lt;/sup&gt;</td>
<td>51 (10)</td>
<td>41; 61</td>
</tr>
</tbody>
</table>

<sup>*</sup>Values are means with SD.
distinct species of the *M. chelonae–M. abscessus* group (Table 2).

In conclusion, phenotypic and genotypic characteristics of isolates D16R27, D16Q19 and DSM 45524\(^T\) indicate that these isolates belong to the *M. chelonae–M. abscessus* group. In addition, several results clearly indicate that D16R27, D16Q19 and DSM 45524\(^T\) form a homogeneous group separated from other members of the *M. chelonae–M. abscessus* group. Therefore, we propose to formally name this new member of the *M. chelonae–M. abscessus* group *M. franklinii* sp. nov., which was originally described by Simmon & Brown-Elliott *et al.* but the name was not validly published (Simmon *et al.*, 2011).

**Description of Mycobacterium franklinii** sp. nov.

*Mycobacterium franklinii* (frank.li’ni.i i, N.L. masc. gen. n. *franklinii* of Franklin, pertaining to Benjamin Franklin, famous USA statesman and scientist from Pennsylvania where the first isolates originated).

Cells are acid-fast bacilli and visible growth on solid media requires 3–5 days of incubation at 28 °C. Colonies are nonpigmented and growth occurs in the presence of 5% NaCl, picric acid, PNB and nitrite. Growth is not observed when mannitol or inositol is used as single-source of carbon. The type strain does not grow when citrate is used as single-source of carbon. Negative reactions are observed for nitrate reduction and Tween 80 hydrolysis. Conventional biochemical tests cannot distinguish *M. franklinii* from other members of the *M. chelonae–M. abscessus* group and should not be used as confirmatory tests for identification of this species. Susceptible to clarithromycin at 3 days. Eight of the original 25 isolates have clarithromycin MICs at ≤2 at 14 days. Susceptible to amikacin, ciprofloxacin, moxifloxacin, doxycycline and minocycline. Variable susceptibility to tobramycin and cefoxitin (see Table 1). Partial sequencing of *rpoB* gene can distinguish *M. franklinii* from other members of the *M. chelonae–M. abscessus* group – the *M. franklinii* sequence has a 726 bp fragment length while the corresponding sequences of the type strains of the *M. chelonae–M. abscessus* group have a 711 bp fragment length. The PRA-hsp65 pattern that characterizes *M. franklinii* is BstEII (320 and 130 bp) and *Hae*III (200, 70, 60 and 55 bp); these fragment sizes are closely related to the pattern of *M. immunogenum* type 2.

The type strain is DSM 45524\(^T\) (=ATCC BAA-2149\(^T\)).

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**References**


suggests that revision of the taxonomic status of members of the Mycobacterium chelonae-M. abscessus group is needed. J Clin Microbiol 47, 2691–2698.


