Mycobacterium fragae sp. nov., a non-chromogenic species isolated from human respiratory specimens

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Three isolates of a slow-growing, non-chromogenic *mycobacterium* were grown from three sputum samples of a patient from the north-eastern Ceará state in Brazil. Identification at species level could not be obtained with PCR restriction analysis of the *hsp65* gene. In order to characterize the isolates we carried out phenotypic and genotypic tests. We sequenced the nearly complete 16S rRNA gene and obtained partial sequences of the *hsp65* (encoding the hypervariable region of the 65 kDa heat-shock protein) and *rpoB* (encoding the beta-subunit of RNA polymerase) genes. The three isolates turned out to be identical and most closely related to the species *Mycobacterium celatum* and *Mycobacterium kyorinense*. The results, however, showed significant differences between these species and the isolates studied, which led us to consider them members of a novel species for which we propose the name *Mycobacterium fragae*. The type strain is HF8705^T (=Fiocruz-INCQS/CMRVS P4051^T=DSM 45731^T).

DNA sequencing is increasingly used in molecular taxonomy, especially of the 16S rRNA gene, which represents an important method for classification and identification of mycobacteria (Griffith *et al.*, 2007; Tortoli, 2010, 2012). The number of officially recognized non-tuberculous mycobacteria (NTM) species is nearly 150 and the extensive use of molecular techniques has led to the detection of many new NTM, most of which are clinically significant (Tortoli, 2006, 2010).

Abbreviations: NTM, non-tuberculous mycobacteria; TCH, thiophene-2-carboxylic acid hydrazide.

The GenBank/EMBL/DDBJ accession numbers for the gene sequences of *Mycobacterium fragae* HF8705^{TT} are: 16S rRNA (JQ898451), *hsp65* (JQ902012) and *rpoB* (JQ902013). Other depositions related to this study include: *Mycobacterium kyorinense* DSM 45166 *rpoB* (JN866833); *Mycobacterium celatum* (type 3) NCTC 12882 *hsp65* (JQ582663), 16S rRNA (JQ582665 and JQ582668) and *rpoB* (JN866834) and *Mycobacterium branderi* ATCC 51788 16S rRNA (JQ582664) and *rpoB* (JQ582668).

Three species have been described in the last 20 years which belong to the same phylogenetic branch: *Mycobacterium celatum*, of which three types have been reported (Bull *et al.*, 1995; Butler *et al.*, 1993), *Mycobacterium branderi* (Koukila-Kähkölä *et al.*, 1995) and *Mycobacterium kyorinense* (Okazaki *et al.*, 2009).

In this work, we characterized a mycobacterium isolated from three clinical specimens of a Brazilian patient with a lung infection, which presents close phylogenetic relationships to the three species noted above.

The isolates HF8703, HF8704 and HF8705^T were recovered over a period of one week from sputum (no acid-fast bacilli were seen in the smear) of a patient from Ceará state, Brazil in 2010. After being grown in Löwenstein–Jensen (LJ) medium, (Kent & Kubica, 1985), the cultures were sent to the Centro de Referência Professor Hélio Fraga, Escola Nacional de Saúde Pública, Fundação Oswaldo Cruz for identification.

Cultures presented smooth, unpigmented colonies which, in the smear, revealed short acid-fast rods. The isolates

were tested for pigment production and growth rate, at 30, 37 and 45 °C. Growth on MacConkey agar without crystal violet and on media containing thiophene-2-carboxylic acid hydrazide (TCH, 2 μg ml^{-1}), *p*-nitrobenzoic acid (500 μg ml^{-1}) and hydroxylamine (500 μg ml^{-1}) was tested as well. The following biochemical tests were performed: nitrate reduction, niacin production, heat-stable catalase (68 °C), semiquantitative catalase (>45 mm), arylsulfatase activity (at 3 days), tellurite reduction, Tween hydrolysis and urease (Kent & Kubica, 1985; Vincent & Gutierres, 2007). Isolates HF8703, HF8704 and HF8705^T produced identical results in all such tests; a comparison with the most closely related species is reported in Table 1.

Some biochemical tests allowed the isolates HF8703, HF8704 and HF8705^T to be distinguished from related species. They differed from *M. celatum* in growth at 45 °C and the arylsulfatase test; from *M. branderi* in the heat stable catalase test and growth at 45 °C and from *M. kyorinense* in the tellurite reduction test (Butler *et al.*, 1993; Koukila-Kähkölä *et al.*, 1995; Okazaki *et al.*, 2009; Vincent & Gutierres, 2007).

HPLC of cell-wall mycolic acids was carried out on isolates HF8703, HF8704 and HF8705^T and on type strains of *M. celatum* and *M. kyorinense* as well, after esterification to bromophenacyl esters as described previously (CDC, 1996).

The profiles of isolates were identical and presented three clusters of peaks: the first cluster, including the more prominent peaks, was clearly separated by the other two which were, in contrast, close and consisted of lower peaks (Fig. 1). An easy differentiation from *M. celatum* was possible mainly through the presence, in the latter, of two clusters of peaks only, roughly corresponding to the first and the third of the novel strains. The most distinctive feature from *M. kyorinense* was the presence, in the latter, of supplementary peaks close to the ones of the first cluster.

Table 1. Comparison of biochemical tests of *M. fragae* sp. nov., *M. kyorinense*, *M. celatum* and *M. branderi*

Taxa: 1, *M. fragae* HF8705^T; 2, *M. kyorinense*; 3, *M. celatum*; 4, *M. branderi*. +, Positive; −, negative; ND, no data. Data for reference species were taken from Okazaki *et al.* (2009), Butler *et al.* (1993), Vincent & Gutierres (2007) and Koukila-Kähkölä *et al.* (1995). For the following assays, results were the same for all species: growth at 37 °C, niacin accumulation, nitrate reduction, semiquantitative catalase, urease and Tween hydrolysis.

Test	1	2	3	4
Growth at 30 °C	+	+	+	ND
Growth at 45 °C	−	+ (42 °C)	+	+
Growth with TCH	+	ND	+	ND
Catalase (68 °C)	+	+	+	−
Arylsulfatase activity at 3 days	−	−	+	−
Tellurite reduction	+	−	+	ND

MICs of the isolates were investigated using Sensititer (TREK Diagnostics), a commercially available microdilution method, including the major drugs with potential activity against slow-growing NTM.

Isolates HF8703, HF8704 and HF8705^T presented low MICs to all antimicrobials tested: clarithromycin, 0.25 μg ml^{-1}; ethambutol, 4 μg ml^{-1}; amikacin, 2 μg ml^{-1}; linezolid, 4 μg ml^{-1}; ciprofloxacin, 0.25 μg ml^{-1}; streptomycin, 1 μg ml^{-1}; doxicyclin, 4 μg ml^{-1}; moxicyclin, $\leqslant$0.12 μg ml^{-1}; rifampicin, $\leqslant$0.12 μg ml^{-1} and sulfamethoxazole, $\leqslant$2.5 μg ml^{-1}.

Partial amplification of the 65 kDa heat-shock protein gene (*hsp65*) and β-subunit of RNA polymerase gene (*rpoB)* was performed as described by Telenti *et al.* (1993) and Adékambi *et al.* (2003). The nearly complete 16S rRNA gene was amplified with a MicroSEQ Full Gene 16S rRNA gene PCR kit (Applied Biosystems), as indicated by the manufacturer. The sequences of PCR products were obtained with an ABI3130 sequencer (Applied Biosystems) using standard protocols. The sequences were then checked using Chromas Pro version 1.42.

Isolates HF8703, HF8704 and HF8705^T showed identical sequences of *hsp65*, *rpoB* and 16S rRNA genes. All the sequences were compared with those of the closest reference strains present in GenBank using the BLAST software (Altschul *et al.*, 1997).

The BLAST search showed that, for the 16S rRNA gene, the sequences with highest similarity were those of *M. celatum* ATCC 51131^T (type 1) with 25 base mismatches out of 1394 bp (98.2 % identity) and *M. celatum* NCTC 12882 (type 3) with 26 base mismatches out of 1442 bp (98.2 % identity). These were followed by *M. celatum* ATCC 51130 (type 2) with 28 base mismatches out of 1417 bp (98.0 % identity), *M. kyorinense* KUM 060204^T with 33 base mismatches out of 1410 bp (97.7 % identity) and *M. branderi* ATCC 51789^T with 41 base mismatches out of 1411 bp (97.1 % identity).

For the 401 bp fragment of the *hsp65* gene, the highest similarity was with *M. kyorinense* KUM 060204^T and *M. celatum* type 2 (96.0 % identity), which are characterized by identical sequences. The next closest species were *M. branderi* ATCC 51789^T (95.8 % similarity) and *M. celatum* types 1 and 3 (95.3 % similarity).

Alignment of the *rpoB* sequence showed highest similarity with *M. kyorinense* KUM 060204^T and *M. celatum* type 2 (93.2 % identity), which share identical sequence, followed by *M. celatum* types 1 and 3 (92.3 % identity) and *M. branderi* ATCC 51789^T (91.7 % identity).

The phylogenetic analysis was conducted using both the 16S rRNA gene and the concatenated sequences of 16S rRNA, *hsp65* and *rpoB* genes (Devulder *et al.* 2005, Stackebrandt *et al.*, 2002). The neighbour-joining trees were reconstructed using MEGA software version 5 (Tamura *et al.*, 2011) under the Kimura two-parameter substitution

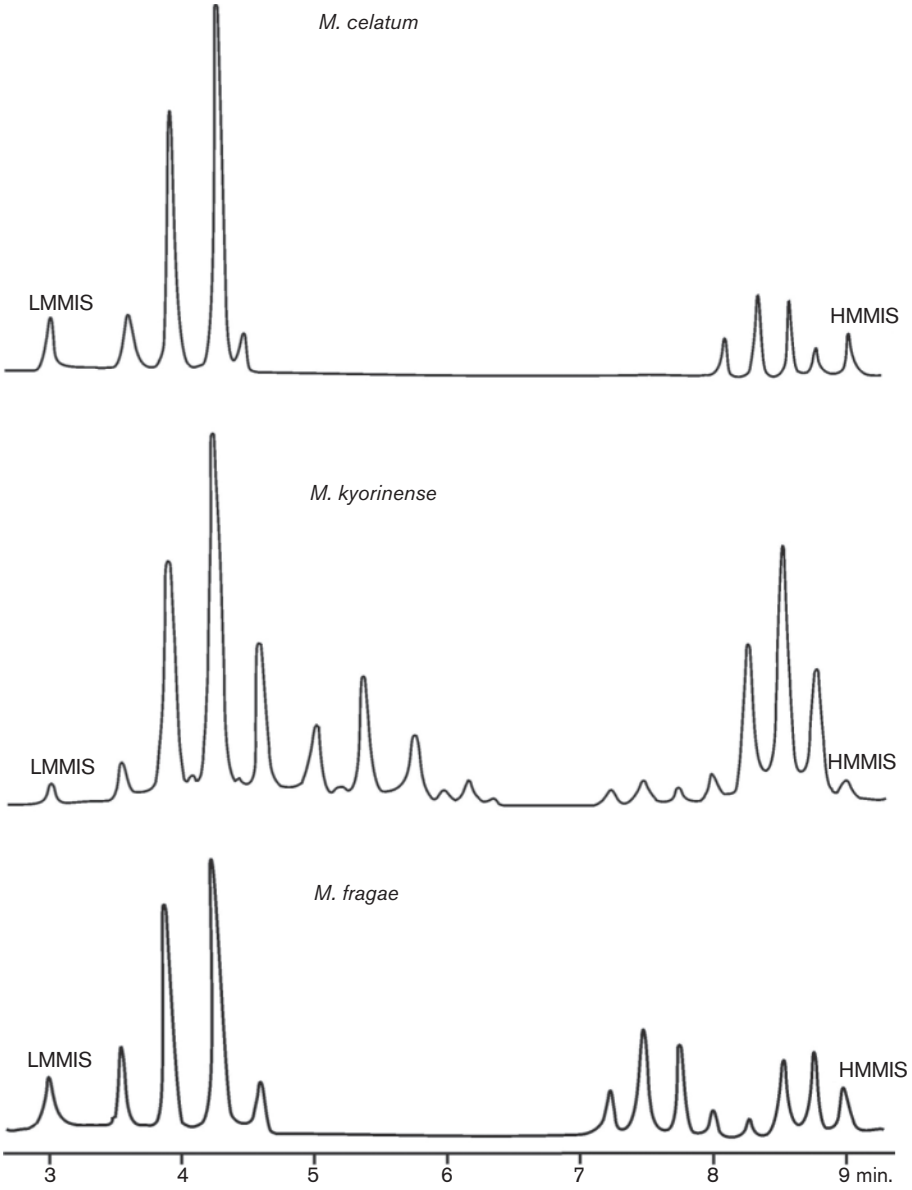

Fig. 1. Representative HPLC patterns of cell wall mycolic acids of *M. celatum* ATCC 51131^T, *M. kyorinense* KUM 060204^T and *M. fragae* sp. nov. HF8705^T. LMMIS, Low-molecular-mass internal standard; HMMIS, high-molecular-mass internal standard.

model (Kimura 1980) and were evaluated by bootstrap based on 1000 random samplings. *Nocardia farcinica* ATCC 3318^T was used as an outgroup in both trees. The sequences of different species were trimmed to start and finish at the same positions.

The phylogenetic trees based on 16S rRNA and concatenated sequences both located the isolates investigated here in the branch including *M. celatum* (types 1, 2 and 3), *M. kyorinense* and *M. branderi* (Figs 2 and 3), supporting their monophyletic origin, clearly distinct from all other slow- or rapid-growing species.

Surprisingly, in the concatenated tree, *M. celatum* type 2 is grouped in the same clade with *M. kyorinense* KUM 060204^T. The high bootstrap value (99 %) of this topology suggests a revision of the taxonomic assignment of strain ATCC 51130 to the species *M. celatum*. The *hsp65* PCR products of the isolates, when cleaved with *Bst*EII, produced fragments whose exact sizes, inferred by the position of the restriction sites in the sequence were 231, 131 and 79 bp and, when cleaved with *Hae*III, 127, 103, 78, 51, 40, 23 and 19 bp. This profile turned out to be unique in the PRAsite database (http://app.chuv.ch/prasite/), more closely matching with the one of *Mycobacterium*

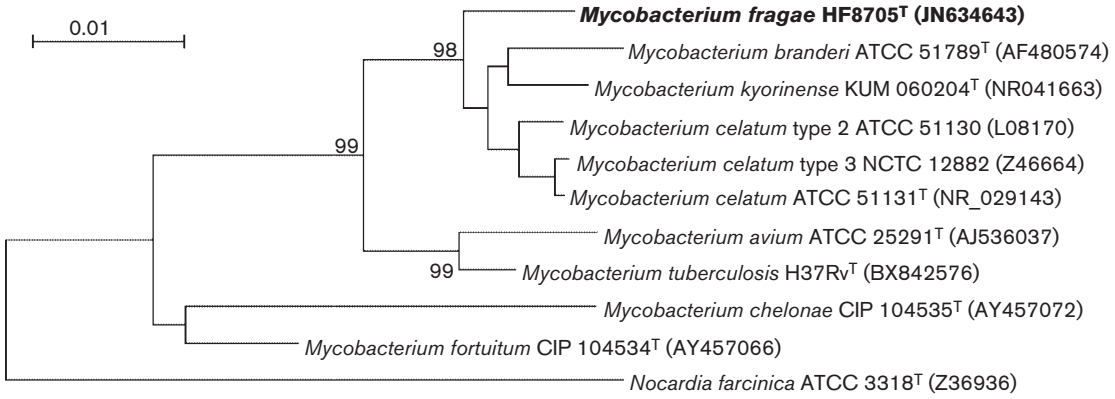

Fig. 2. Phylogenetic tree computed from the 16S rRNA gene sequences by the neighbour-joining method and Kimura's two-parameter substitution model. The significance of branches is indicated by bootstrap values calculated from 1000 replicates. The sequence of *Nocardia farcinica* ATCC 3318^T was used as the outgroup. Bar, 0.01 nt substitutions per position.

tuberculosis complex type 1 (*Bst*EII: 235/120/85, *Hae*III: 150/130/70).

Description of *Mycobacterium fragae* sp. nov.

Mycobacterium fragae sp. nov. (fra′ga.e. N.L. masc. gen. n. *fragae* of Fraga, referring to the doctor and researcher after whom the Centro de Referência Professor Hélio Fraga, Escola Nacional de Saúde Pública, Fundação Oswaldo Cruz was named and where this species was characterized and described).

Cells appear as short rods, typically acid–alcohol-fast and not motile. Colonies are smooth and unpigmentated. Growth is obtained in LJ in 3–4 weeks at temperatures between 30 and 37 °C. The biochemical profile is characterized by positivity for tellurite reduction and heat-stable catalase (68 °C) and negativity for niacin accumulation, nitrate reduction, semiquantitative catalase (>45 mm), urease, arylsulfatase activity at 3 days and Tween 80 hydrolysis. Growth does not occur on MacConkey agar without crystal violet and no inhibition is observed on media containing TCH, *p*-nitrobenzoic acid or hydroxylamine. The antimicrobial pattern is characterized by susceptibility to clarithromycin, ethambutol, amikacin, linezolid, ciprofloxacin, streptomycin, doxicyclin, moxicyclin, rifampicin and sulfamethoxazole. The HPLC of mycolic acids allows a clear distinction from closely related species. The sequences of 16S rRNA, *hsp65* and *rpoB* genes are unique.

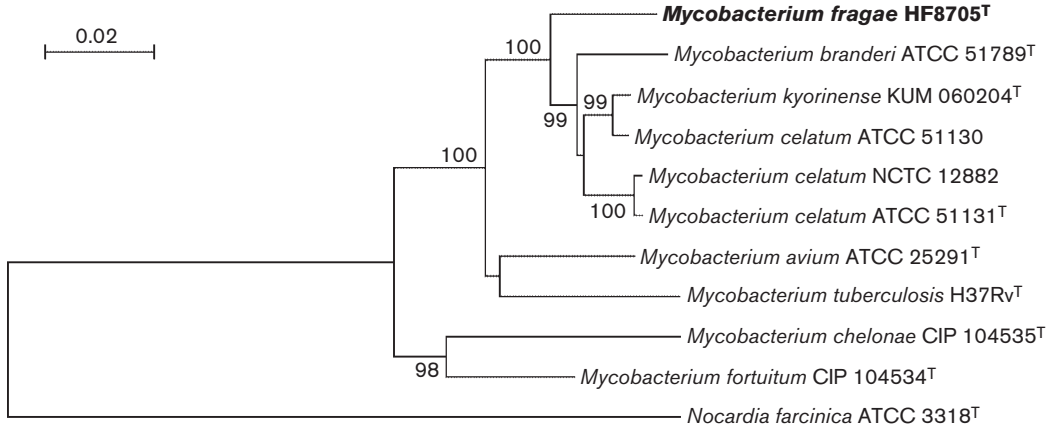

Fig. 3. Phylogenetic tree computed from the concatenation of 16S rRNA gene, *rpoB* and *hsp65* sequences by the neighbour-joining method and Kimura's two-parameter substitution model. The significance of branches is indicated by bootstrap values calculated from 1000 replicates. The sequence of *N. farcinica* ATCC 3318^T was used as the outgroup. Bar, 0.02 nt substitutions per position.

The type strain is HF8705^T (=Fiocruz-INCQS/CMRVS P4051^T=DSM 45731^T).

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