Mycobacterium shinjukuense sp. nov., a slowly growing, non-chromogenic species isolated from human clinical specimens

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Seven isolates of a slowly growing, non-chromogenic *Mycobacterium* species were obtained from sputum and bronchial lavage fluid samples from elderly patients in different regions of Japan. These isolates were distinguished from related non-tuberculous species by colony morphology, positive results for Tween hydrolysis, catalase at 68 °C, nitrate reductase and pyrazinamidase and negative results for semi-quantitative catalase, urease and arylsulfatase. The mycolic acid pattern obtained by HPLC revealed a single cluster of late-eluting mycolic acids similar to but different from those of *Mycobacterium malmoeense* ATCC 29571^T. The 16S rRNA gene, 16S–23S internal transcribed spacer (ITS), *rpoB* and *hsp65* sequences were unique in comparison with those of other mycobacteria. Comparison of 16S rRNA gene sequences showed that the isolates were most closely related to *Mycobacterium tuberculosis* H37Rv^T (21 base differences in 1508 bp; 98.6 % 16S rRNA gene sequence similarity). A representative strain, GTC 2738^T, showed 91.9 % *rpoB* sequence similarity with *Mycobacterium marinum* strain M, 95 % *hsp65* sequence similarity with *Mycobacterium kansasii* CIP 104589^T and 81.1 % 16S–23S ITS sequence similarity with *Mycobacterium gordonae* ATCC 14470^T. Phylogenetic analysis of concatenated sequences of the 16S rRNA, *rpoB* and *hsp65* genes showed that strain GTC 2738^T was located on a distinct clade adjacent to *M. tuberculosis*, *M. ulcerans* and *M. marinum*, with bootstrap values of 81 %. DNA–DNA hybridization demonstrated less than 70 % reassociation with type strains of genetically related species and supported the novel species status of the isolates. On the basis of this evidence,

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Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, 16S–23S ITS, *rpoB* and *hsp65* sequences of strain GTC 2738^T are AB268503, AB551555, AB268504 and AB268505, respectively.

a novel species with the name *Mycobacterium shinjukuense* sp. nov. is proposed. The type strain, isolated from a sputum sample, is strain GTC 2738^T (=JCM 14233^T =CCUG 53584^T).

Non-tuberculous mycobacteria are widely distributed in the environment and some of them are pathogenic to humans and animals (Marras & Daley, 2002). The widespread use of sequence-based identification methods has increased our knowledge of the diversity within the genus *Mycobacterium* and has contributed to the discovery of novel taxa (Tortoli, 2003). Phylogenetic studies using the 16S rRNA gene, 16S–23S internal transcribed spacer (ITS), *rpoB* and *hsp65* sequences are often useful to clarify whether an organism is a member of a known taxon or represents a hitherto-unknown taxon (Springer *et al.*, 1996; Tortoli, 2003; van Ingen *et al.*, 2009a, b). This study describes seven isolates obtained from pulmonary samples which yielded 16S rRNA gene, 16S–23S ITS, *rpoB* and *hsp65* sequences that were unique when compared with other mycobacteria. A taxonomic examination, including DNA–DNA hybridization, revealed that these isolates belong to a novel species of the genus *Mycobacterium*.

Seven acid-fast isolates obtained from six sputum and one bronchial lavage fluid samples, previously identified as *Mycobacterium* sp., were submitted to our laboratory for species identification. These isolates were collected individually from five female and two male elderly patients, representing multiple geographical regions throughout Japan. The demographics of the isolates are summarized in Table 1. None of the patients had been diagnosed with any underlying immunocompromising disease. Of the seven cases, two (cases 1 and 2) satisfied the diagnostic criteria for pulmonary non-tuberculous mycobacterial disease proposed by the American Thoracic Society (Griffith *et al.*, 2007) and were considered clinically relevant in the patients from whom the strains were isolated. In the other five cases, their clinical relevance was not known because of a lack of medical information.

The bacterial morphology for each isolate was determined by acid-fast staining using the Ziehl–Neelsen staining method. Colony morphology, pigment production under dark and light conditions and the ability to grow at 25–45 °C were examined on Middlebrook 7H11 and 2% Ogawa egg medium. The following biochemical tests were performed as described by Kent & Kubica (1985): nitrate reductase, arylsulfatase on day 3, semi-quantitative catalase (45 mm), 68 °C catalase, Tween 80 hydrolysis, urease activity, tolerance of 5% NaCl and pyrazinamidase. All seven isolates exhibited the same results in the above biochemical and phenotypic identification tests. We also performed the above identification tests on four closely related reference strains (*Mycobacterium tuberculosis* ATCC 27294^T, *M. kansasii* ATCC 12478^T, *M. marinum* ATCC 927^T and *M. ulcerans* ATCC 19423^T). The results are given in the species description and Table 2.

Analysis of the cell-wall mycolic acid of a representative strain, GTC 2738^T, was performed by HPLC using methods described elsewhere (Butler & Guthertz, 2001). Strain GTC 2738^T was characterized by having a single cluster of mycolic acid peaks (Fig. 1). The pattern was compared at the Centers for Disease Control and Prevention (CDC) to those in a HPLC reference library for type strains and in an online image database at <http://www.MycobacToscana.it/>. The pattern was visually similar to that of *M. malmoeense* ATCC 29571^T; however, strain GTC 2738^T produced mycolic acid peaks that eluted with a retention time, relative to the high molecular mass standard, of 3.78–0.35 min, which differed from the relative retention time of 3.50–0.70 min for *M. malmoeense* ATCC 29571^T. In addition, strain GTC 2738^T demonstrated peaks with relative retention times of 0.60, 0.48 and 0.35 min, which were absent from

Table 1. Demographics of the clinical strains

BLF, Bronchial lavage fluid; F, female; M, male; TB, tuberculosis.

Case	Strain	Patient		Year of isolation	Source	Location	Symptoms
		Sex	Age				
1	GTC 2738 ^T	F	73	2004	Sputum	Tokyo	Cavitary lesion
2	UN-115	F	73	2005	Sputum	Hokkaido	Bronchiectasis, infiltrative shadow on middle lobe and lingula
3	UN-126	M	78	2005	Sputum	Aichi	Previous history of pneumoconiosis
4	UN-129	F	64	2006	BLF	Mie	Bronchiectasis
5	UN-130	F	89	2006	Sputum	Fukuoka	Pneumonia
6	UN-131	F	57	2006	Sputum	Tokyo	Cavitary lesion
7	UN-132	M	89	2006	Sputum	Kumamoto	Cavitary lesion, previous history of TB

Table 2. Phenotypic characteristics of strain GTC 2738^T and its closest phylogenetic neighbours

Strains: 1, *Mycobacterium shinjukuense* sp. nov. GTC 2738^T; 2, *M. tuberculosis* ATCC 27294^T; 3, *M. kansasii* ATCC 12478^T; 4, *M. marinum* ATCC 927^T; 5, *M. ulcerans* ATCC 19423^T. All data were obtained in this study. All strains grew at 30 °C, and none of the strains grew at 45 °C or with 5 % NaCl; all strains were negative for arylsulfatase activity at 3 days.

Characteristic	1	2	3	4	5
Colony morphology*	S	R	S/R	S/R	R
Pigmentation†	N	N	P	P	N
Growth at:					
25 °C	—	—	+	+	—
37 °C	+	+	+	—	+
42 °C	—	—	+	—	—
Tween 80 hydrolysis at 10 days	+	—	+	+	—
Urease	—	+	+	+	—
Nitrate reduction	+	+	+	—	—
Catalase >45 mm	—	—	+	—	+
Catalase 68 °C	+	—	+	—	+
Pyrazinamidase	+	+	—	+	—

R, Rough; S, smooth.

N, Non-chromogenic; P, photochromogenic.

M. malmoense ATCC 29571^T (not shown). Visually, the patterns did not match any of the existing HPLC patterns in the CDC library database or the online image website; thus, strain GTC 2738^T was considered to represent a novel species.

Fig. 1. Representative mycolic acid pattern of strain GTC 2738^T obtained by HPLC analysis.

Genomic DNA was extracted from the isolates using the Isoplant kit (Nippon Gene), according to the manufacturer's instructions. An almost-complete 16S rRNA gene sequence (1508 bp), the entire 16S–23S ITS sequence (286 bp) and partial *rpoB* (400 bp) and *hsp65* (401 bp) sequences were determined using methods described elsewhere (Telenti *et al.*, 1993; Roth *et al.*, 1998; Devulder *et al.*, 2005; Nakanaga *et al.*, 2007). The sequences corresponding to the primer binding sites were excluded manually from the data. The sequences obtained in this study were compared with sequences from GenBank using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

The seven isolates had identical 16S rRNA gene, ITS, *rpoB* and *hsp65* sequences, but the sequences were different from those available in GenBank. High 16S rRNA gene sequence similarity was observed between the isolates and *M. tuberculosis* H37Rv^T (GenBank accession no. BX842576; 98.6 % similarity across 1508 bp), which was possibly related to a cross-reaction between the isolates and commercial tuberculosis identification kits, as reported for *Mycobacterium celatum* (Somoskövi *et al.*, 2000) and *Mycobacterium riyadhense* (van Ingen *et al.*, 2009a). Aono *et al.* (2010) confirmed that the isolates gave positive cross-reactions for the TRC Rapid M.TB assay (Tosoh Bioscience) and AMPLIFIED MTD Test (Gen-Probe), while the COBAS AMPLICOR MTB (Roche Diagnostics), COBAS TaqMan MTB (Roche Diagnostics) and AccuProbe MTB (Gen-Probe) assays showed negative reactions. Strain GTC 2738^T exhibited the highest *rpoB* sequence similarities with *M. marinum* strain M (CP000854; 91.9 % *rpoB* sequence similarity across 395 bp) and *M. ulcerans* Agy99 (CP000325; 91.7 % across 399 bp) and the highest *hsp65* sequence similarity with *M. kansasii* CIP 104589^T (95.0 % *hsp65* sequence similarity across 401 bp). The highest ITS sequence similarity was observed between strain GTC 2738^T and *Mycobacterium gordonae* ATCC 14470^T (GenBank accession no. L42260); however, this similarity was low, with 230 matches out of 283 bp (81.1 %).

In an attempt to clarify the phylogenetic position of the isolates within the slowly growing mycobacteria, a concatenated phylogenetic tree using 16S rRNA gene (1341 bp), *rpoB* (398 bp) and *hsp65* (401 bp) sequences was constructed. The ITS sequence was not included in the analysis as the sequence is too variable to construct reliable trees. Phylogenetic trees were generated using CLUSTAL W version 1.83 (Thompson *et al.*, 1994) and displayed using TreeView (Page, 1996). Analyses were performed using the neighbour-joining method with Kimura's two-parameter distance model and 1000 bootstrap replications. Phylogenetic analysis of the 16S rRNA gene sequence (Fig. 2a) showed that strain GTC 2738^T was located in a clade adjacent to *M. tuberculosis* NCTC 7416^T, *M. ulcerans* Borstel 10564/70 and *M. marinum* DSM 44344^T, with 79.9 % bootstrap support. The bootstrap values in the phylogenetic trees of the *rpoB* (Fig. 2b) and *hsp65* (Fig. 2c) sequences were too low to indicate the phylogenetic position of the isolate. Analysis of the concatenated sequences (Fig. 2d) confirmed that strain GTC 2738^T formed a separate clade within the

Fig. 2. Neighbour-joining phylogenetic trees showing the positions of strain GTC 2738^T and closely related type strains of *Mycobacterium* species based upon comparison of sequences of 16S rRNA gene (a), *rpoB* (b) and *hsp65* (c) and a concatenation of 16S rRNA gene, *rpoB* and *hsp65* sequences (d). Bootstrap values (>500) based on 1000 replications are shown at branch nodes. *Nocardia asteroides* ATCC 19247^T was used as an outgroup. Bars, 1% sequence divergence.

Table 3. DNA–DNA relatedness between strain GTC 2738^T and members of the genus *Mycobacterium*

Strain	DNA–DNA hybridization (%) with labelled DNA from:		
	1	2	3
1. Strain GTC 2738 ^T	100	30	30
2. <i>M. tuberculosis</i> ATCC 27294 ^T	40	100	ND
3. <i>M. marinum</i> ATCC 927 ^T	20	ND	100
<i>M. bovis</i> ATCC 19210 ^T	61	111	ND
<i>M. microti</i> ATCC 19422 ^T	42	83	ND
<i>M. ulcerans</i> ATCC 19423 ^T	21	ND	81

ND, Not done.

cluster containing the type strains of *M. tuberculosis*, *M. ulcerans* and *M. marinum*.

Given the close phylogenetic position of strain GTC 2738^T to the *M. tuberculosis* complex, *M. marinum* and *M. ulcerans*, quantitative microplate DNA–DNA hybridization was carried out under optimal conditions as described by Ezaki *et al.* (1988, 1989). Strain GTC 2738^T exhibited <70 % DNA–DNA relatedness with members of the genus *Mycobacterium* (Table 3), below the suggested threshold for the delineation of species. This result provided further evidence of genetic divergence between strain GTC 2738^T and its closest phylogenetic neighbours.

On the basis of phenotypic, chemotaxonomic and phylogenetic analysis, strain GTC 2738^T is considered to represent a novel species in the genus *Mycobacterium*, for which the name *Mycobacterium shinjukuense* sp. nov. is proposed.

Description of *Mycobacterium shinjukuense* sp. nov.

Mycobacterium shinjukuense (shin.ju'ku.en'se. N.L. neut. adj. *shinjukuense* pertaining to Shinjuku ward, Tokyo, Japan, where the type strain was isolated).

Acid–alcohol-fast, rod-shaped bacilli. Grows to maturity in 21 days at 30–37 °C. Colonies on 2 % Ogawa egg medium are smooth and non-chromogenic. Positive in tests for Tween 80 hydrolysis, 68 °C catalase, nitrate reductase and pyrazinamidase. Negative in tests for semi-quantitative catalase, urease and 3-day arylsulfatase. Does not grow with 5 % NaCl. Identifiable by unique 16S rRNA gene, 16S–23S ITS, *hsp65* and *rpoB* sequences.

The type strain is GTC 2738^T (=JCM 14233^T =CCUG 53584^T), recovered from a sputum specimen.

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