Mycobacterium paraseoulense sp. nov., a slowly growing, scotochromogenic species related genetically to *Mycobacterium seoulense*

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A previously unidentified, slowly growing, scotochromogenic *Mycobacterium* species, represented by strain 31118^T, was discovered during *hsp65* sequence-based reidentification of Korean clinical isolates that had been previously identified as *Mycobacterium scrofulaceum* by conventional biochemical tests. Although the 16S rRNA gene sequence of strain 31118^T was identical to that of the recently described *Mycobacterium seoulense*, phylogenetic analyses based on three independent alternative targets (*rpoB*, *hsp65* and the 16S–23S internal transcribed spacer) showed that it was closely related to *M. seoulense* but was a distinct phylogenetic entity. Furthermore, the phenetic characteristics of this strain were more similar to those of *M. scrofulaceum* than to those of *M. seoulense*. Taken together, these results support the conclusion that this strain represents a novel mycobacterium species, for which the name *Mycobacterium paraseoulense* sp. nov. is proposed. The type strain is 31118^T (=DSM 45000^T =KCTC 19145^T).

Recently, the application of molecular techniques to the taxonomy and identification of isolates from environmental sources and clinical specimens has led to increased awareness of the diversity within the genus *Mycobacterium* (Kirschner *et al.*, 1993). Currently, most clinical laboratories use conventional biochemical assays to identify suspected mycobacterial pathogens. However, with the increasing number of newly defined taxa and the recognition of ‘difficult-to-identify’ variants of known species, these assays sometimes fail to provide precise identification because of their lack of discriminatory power

(Kirschner *et al.*, 1993). 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), *rpoB* (Kim *et al.*, 1999) and *hsp65* (Kim *et al.*, 2005) and the 16S–23S rRNA gene internal transcribed spacer (ITS) (Roth *et al.*, 1998) have been used for species identification. The combination of molecular assays and conventional methods should provide conclusive identification for differentiation of infrequently encountered species as well as the delimitation of novel taxa.

Abbreviations: EMB, ethambutol; ITS, internal transcribed spacer; PNB, *p*-nitrobenzoate; SGM, slowly growing mycobacteria; TCH, thiophene-2-carboxylic acid hydrazide.

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA gene, *hsp65*, *rpoB* and ITS sequences of strain 31118^T are DQ536404, DQ536402, DQ536406 and FJ042510, respectively.

Mycolic acid profiles of strain 31118^T and *M. seoulense* DSM 44998^T and alignments and neighbour-joining trees generated from ITS, *hsp65* and *rpoB* sequences are available as supplementary material with the online version of this paper.

Recently, some novel scotochromogenic, slowly growing *Mycobacterium* (SGM) species have been described on the basis of chronometer-targeting sequence analysis (Mohamed *et al.*, 2004; Turenne *et al.*, 2004; Mun *et al.*, 2007, 2008). However, sequence-based delimitation among scotochromogenic strains, whose separation by conventional phenetic approaches is sometimes impossible, has rarely been performed. In the present study, a novel scotochromogenic SGM is described. This was discovered

during reidentification of 43 Korean clinical isolates, which had been previously identified as *Mycobacterium scrofulaceum* by conventional biochemical methods, using *hsp65* sequencing methods (Kim *et al.*, 2005).

The strain used in the study was one of the Korean clinical isolates identified as *M. scrofulaceum* by conventional methods at the Korean Institute of Tuberculosis. This strain was isolated from sputa of a patient who had experienced general pulmonary symptoms. This strain was recovered from -70°C storage in the deep-freezer by subculturing to Lowenstein–Jensen (LJ) medium and Middlebrook 7H9 broth incubated at 37°C .

The phenetic characteristics of strain 31118^T and eight reference mycobacteria were analysed and compared (Table 1). Colony morphology, pigment production in the dark, photoinduction and the ability to grow at temperatures ranging from 25 to 45°C were examined during a 6-week incubation on LJ medium and Middlebrook 7H10 agar. Acid–alcohol-fastness was deter-

mined by Ziehl–Neelsen and auramine O staining. The following biochemical features (Kent & Kubica, 1985) were investigated: niacin accumulation, nitrate reductase, aryl-sulfatase on days 3 and 14, heat-stable catalase (pH 7, 68°C), tellurite reductase, Tween 80 hydrolysis, urease and pyrazinamidase. Inhibition tests included tolerance of thiophene-2-carboxylic acid hydrazide (TCH), *p*-nitrobenzoate (PNB), 5% sodium chloride, ethambutol (EMB) and picric acid and the ability to grow on MacConkey agar without crystal violet.

HPLC was used to analyse mycolic acids from strain 31118^T and the type strain of the most phenotypically similar species, *Mycobacterium seoulense* DSM 44998^T, as described by Butler *et al.* (1992). Low- and high-molecular-mass standards (Ribi ImmunoChem) were added for peak identification. To identify and quantify mycolic acids and to assign these to *Mycobacterium* species based on mycolic acid patterns, the Microbial Identification system (MIDI Inc.) was used.

Table 1. Cultural and biochemical characteristics that differentiate strain 31118^T from other closely related SGM species

Strains: 1, 31118^T; 2, *M. seoulense* DSM 44998^T; 3, *M. nebraskense* ATCC BAA-837^T; 4, *M. scrofulaceum* ATCC 19981^T; 5, *M. avium* ATCC 25291^T; 6, *M. kansasii* ATCC 12478^T; 7, *M. phlei* ATCC 11758^T; 8, *M. goodii* ATCC 14470^T; 9, *M. triviale* ATCC 23292^T. +++, Strong growth; ++, good growth; +, positive/growth; –, negative/no growth; ±, variable. All strains showed negative results in niacin and EMB tests and positive results in catalase and pyrazinamidase tests. All strains showed strong growth at 37°C .

Characteristic	1	2	3	4	5	6	7	8	9
Growth at:									
25 °C	+++	+++	+++	+++	++	+++	++	+++	+++
45 °C	–	–	–	–	+	–	+++	–	–
Visible growth in:									
<7 days	–	–	±	–	–	–	+	–	–
>7 days	+	+	++	+	+	+	++	+	+
Morphology*	SO	SO	IWY	SY	SWY	IW	RWY	SY	RW
Pigmentation†	S	S	N	S	N	P	N	S	N
Nitrate reductase	+	–	+	–	–	+	+	–	+
Arylsulfatase									
3 days	–	–	–	–	–	±	–	–	+
14 days	–	–	–	–	–	+	–	–	+
Tellurite reductase	+	+	+	±	±	+	+	–	–
Tween hydrolysis									
<5 days	–	–	+	–	–	+	±	+	+
<10 days	–	–	+	–	+	+	+	+	+
Urease	–	+	–	+	–	+	+	–	–
Growth with:									
10 mg TCH ml ⁻¹	+++	+++	+++	+++	++	+++	++	+++	+++
500 mg PNB ml ⁻¹	++	++	+++	++	++	+++	+++	+++	+++
5% NaCl	–	–	–	–	–	–	+	–	+++
Growth on:									
MacConkey agar	–	–	–	–	–	–	+	–	++
Picric acid	–	–	–	–	–	–	+	–	–

*R, Rough; S, smooth; I, intermediate in roughness; O, orange; W, white; Y, yellow.

†P, Photochromogenic; S, scotochromogenic; N, non-photochromogenic.

Chromosomal DNA for molecular taxonomy was extracted using the bead beater/phenol extraction method as reported previously (Kim *et al.*, 1999). The purified DNA was used as a template for PCR amplifications of four independent targets: the 16S rRNA gene, heat-shock protein 65 (*hsp65*), RNA polymerase (*rpoB*) and the ITS. The nearly complete 16S rRNA gene (1523 bp), the partial *hsp65* gene (644 bp), the partial *rpoB* gene (352 bp) and the full-length ITS (372 bp) were amplified as described previously (Springer *et al.*, 1996; Roth *et al.*, 1998; Kim *et al.*, 1999; Kim *et al.*, 2005). PCR amplicons of all target genes were cloned directly using Topo TA cloning kits (Invitrogen) and sequenced (Kim *et al.*, 2005). The four target sequences of 31118^T obtained in the present study were compared with information from GenBank using BLAST analysis (<http://www.ncbi.nlm.nih.gov/blast/>).

Multiple alignments of *hsp65*, *rpoB* and ITS sequences of strain 31118^T and reference strains of a wide range of both slowly and rapidly growing mycobacteria were created using the multiple-alignment algorithm in MEGALIGN as described previously (Kim *et al.*, 1999; Roth *et al.*, 1998; Turenne *et al.*, 2001; Kim *et al.*, 2005). Phylogenetic analysis from concatenation of partial *rpoB* (306 bp), *hsp65* (603 bp), 16S rRNA gene (552 bp) and ITS1 (279 bp) sequences was also performed. All four trees were inferred by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods using *Tsukamurella paurometabola* KCTC 9821^T (*hsp65*), *Rhodococcus equi* IMSNU 20114^T (*rpoB*), *Mycobacterium smegmatis* ATCC 19980^T (ITS) or *Mycobacterium abscessus* ATCC 19977^T (concatenation) as outgroups. Evolutionary distance matrices were generated according to the model described by Jukes & Cantor (1969). The neighbour-joining and maximum-parsimony methods were carried out using MEGA version 4.1 (Kumar *et al.*, 2008). The resulting trees and topologies were evaluated by bootstrap analyses based on 1000 resamplings (Felsenstein, 1985).

Microscopy showed generally rod-shaped acid-fast bacilli, with frequent curved shapes. Occasional coccoid forms were noted. Spores and filaments were not present. The optimal growth temperature was 37 °C. No growth was observed at 45 °C. On Middlebrook 7H10 medium, mature growth developed in 3 weeks at 25 and 37 °C. Microcolonies developed in 2 weeks at the same temperatures. However, 4 weeks or more was required to form mature colonies on LJ medium. Colonies grown on Middlebrook 7H10 agar were usually larger, smooth, although occasionally rough, and always orange in appearance under both dark and photoinduction conditions. Cells on LJ medium grew in a film-like manner and produced an orange pigment. No growth was observed on MacConkey agar, after the addition of 5% NaCl to the culture medium or after adding 5 mg EMB or picric acid ml⁻¹ to the medium. However, the strain was tolerant of TCH at 10 mg ml⁻¹ and PNB at 500 mg ml⁻¹. The strain was negative for nitrate reductase, arylsulfatase, niacin accumulation and Tween 80 hydrolysis and it was positive

for urease activity, heat-stable catalase, pyrazinamidase and tellurite reductase. Generally, the biochemical profile of 31118^T was most like those of another scotochromogenic strain, *Mycobacterium scrofulaceum* ATCC 19981^T. However, distinct differences were found from the most genotypically related type strain, *M. seoulense* DSM 44998^T, in activities of urease and nitrate reductase. These tests might be used to differentiate the two strains. Cultural and biochemical characteristics that differentiated 31118^T from other closely related SGM species are shown in Table 1.

In HPLC analyses of mycolic acids, the profile for strain 31118^T showed two clusters of peaks. The closest match was with *Mycobacterium gordonae* subgroup II, with a similarity index of 0.005. In a comparison with the mycolic acids of *M. seoulense* DSM 44998^T, differences were found in terms of the relative heights of the acid peaks as follows: peak 1, 1.35% (strain 31118^T) and 2.70% (*M. seoulense* DSM 44998^T); peak 2, 1.12 and 1.53%; peak 3, 5.75 and 11.16%; peak 4, 3.15 and 5.67%; peak 5, 7.40 and 9.74%; peak 6, 17.82 and 11.74%; peak 7, 27.23 and 14.88%; peak 8, 8.92 and 4.48%; peak 9, 7.09 and 8.79%; and peak 10, 9.39 and 11.76% (Supplementary Fig. S1, available in IJSEM Online). These differences in HPLC peak heights have been reported to be species-specific for mycobacteria (CDC, 1999; Duffey *et al.*, 1996; Floyd *et al.*, 1996). Furthermore, two unique peaks (retention times 3.900 and 4.482 min) distinct from those of *M. seoulense* were present in the profile of strain 31118^T.

A BLAST search of the GenBank database using the 16S rRNA gene sequence of strain 31118^T gave a completely identical match to *M. seoulense* DSM 44998^T (GenBank accession no. DQ536403) that was reported by Mun *et al.* (2007). Although the 16S rRNA gene sequence-based genetic approach is considered the 'gold standard' molecular method for identification of mycobacteria, it has limitations for species delimitations between some strains in the genus. Thus, other chronometer-based genetic approaches are required for exact delimitation among strains in the genus *Mycobacterium*.

BLAST search analysis using the complete ITS1 sequence (279 bp) of strain 31118^T revealed the closest similarities with various sequences designated for *Mycobacterium parascrofulaceum*. The highest similarity (95.0%) was for *M. parascrofulaceum* ATCC BAA-614^T (GenBank accession no. AY337279), with 12 mismatches (4 gaps and 8 substitutions). Compared with the sequence of *M. seoulense* DSM 44998^T determined in our laboratory (GenBank accession no. FJ041521), 6 mismatches (1 gap and 5 substitutions) among 279 bp (97.8%) were detected (Supplementary Fig. S2a). BLAST search analyses using partial *hsp65* (603 bp) and *rpoB* (306 bp) sequences for strain 31118^T also revealed closest similarity with *M. seoulense* DSM 44998^T (GenBank accession nos DQ536401 and DQ53640, respectively), which showed 9 (98.5%) and 5 (98.4%) mismatches, respectively, without gaps. Of the three target sequences, the greatest sequence variation

between strain 31118^T and *M. seoulense* DSM 44998^T was observed in the ITS region. These sequence variations in the ITS region might be used effectively for differentiation between these two closely related strains.

Phylogenetic analyses based on the ITS (Supplementary Fig. S3a), *hsp65* (Supplementary Fig. S3b) and *rpoB* (Supplementary Fig. S3c) sequences and a concatenation of these four sequences (Fig. 1) showed that 31118^T is most closely related to *M. seoulense* DSM 44998^T, but also has distinct sequence characteristics. This grouping was strongly supported by high bootstrap values and recovery in maximum-parsimony trees (Fig. 1 and Supplementary Fig. S3). Although 31118^T is phenotypically most similar to *M. scrofulaceum* (Table 1), phylogenetic analyses showed that these two species are genetically distinct. Sequence alignments for the complete ITS sequence and partial *hsp65* and *rpoB* gene sequences for strain 31118^T and closely related species are shown in Supplementary Fig. S2.

The distinct sequence characteristics of these four independent targets, together with its unique mycolic acid

profile and phenetic traits, confirm the taxonomic status of 31118^T as a member of a novel *Mycobacterium* species rather than a variant of a previously described species. These results stress the importance of polyphasic taxonomic approaches for mycobacterial taxonomy.

Description of *Mycobacterium paraseoulense* sp. nov.

Mycobacterium paraseoulense (pa.ra.se.oul.en'se. Gr. prep. para like; N.L. neut. adj. seoulense specific epithet of a bacterial species; N.L. neut. adj. paraseoulense like seoulense, referring to the genotypic resemblance of the type strain to *Mycobacterium seoulense*).

The bacillus stains acid–alcohol-fast. Cells are generally rod-shaped, with frequent curved cells. Spores and filaments are not present. The optimal growth temperature is 37 °C. No growth is observed at 45 °C. On Middlebrook 7H10 medium, mature growth develops in 3 weeks at 25 and 37 °C. Microcolonies develop in 2 weeks at the same temperatures. However, 4 weeks or more is required to form mature colonies on LJ medium. Colonies grown on Middlebrook 7H10 agar are usually larger, smooth, although occasionally rough, but always orange in appearance under both dark and photoinduction conditions. Cells on LJ medium grow in a film-like manner and produce an orange pigment. No growth is observed on MacConkey agar or on culture medium containing 5% NaCl or 5 mg EMB ml⁻¹. Negative for nitrate reductase, arylsulfatase, niacin accumulation and Tween 80 hydrolysis and positive for urease activity, heat-stable catalase, pyrazinamidase and tellurite reductase. Morphology and biochemical profiles are not distinguishable from those of *M. scrofulaceum*. HPLC analysis shows a unique mycolic acid profile. Genetically, although the 16S rRNA gene sequence is identical to that of *M. seoulense*, it has sequences unique within *Mycobacterium* for *hsp65*, *rpoB* and the ITS region. Phylogenetic analysis based on these three independent targets shows that *M. paraseoulense* belongs to the SGM and is closely related to the scotochromogenic species *M. seoulense*.

The type strain is 31118^T (=DSM 45000^T =KCTC 19145^T), which was isolated from a human sputum sample.

Fig. 1. Phylogenetic relationships of strain 31118^T among other *Mycobacterium* species. The phylogenetic tree of the genus *Mycobacterium* was computed from the concatenation of partial *rpoB* (306 bp), *hsp65* (604 bp), 16S rRNA gene (552 bp) and ITS1 (279 bp) sequences by the neighbour-joining method. The tree includes 28 strains and was rooted using *M. abscessus* ATCC 19977^T. Percentages at nodes represent bootstrap levels supported by 1000 resampled datasets; values <50% are not shown. Solid circles indicate that the corresponding nodes (groupings) were also recovered in maximum-parsimony trees. Species between the horizontal lines are scotochromogenic species. Bar, 0.01 substitutions per nucleotide position.

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