Mycobacterium seoulense sp. nov., a slowly growing scotochromogenic species

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A previously undescribed, slowly growing, scotochromogenic mycobacterium was isolated from a patient with symptomatic pulmonary infection during hsp65 sequence-based identification of Korean clinical isolates. Phenetic characteristics of this strain were generally similar to those of Mycobacterium nebraskense and Mycobacterium scrofulaceum. However, some phenetic characteristics differentiated it from these two species. Its 16S rRNA gene sequences were unique and phylogenetic analysis based on 16S rRNA gene sequences placed the organism in the slowly growing Mycobacterium group close to M. nebraskense and M. scrofulaceum. Its unique mycolic acid profiles and the results of phylogenetic analysis based on two independent alternative chronometer molecules, hsp65 and rpoB, confirmed the taxonomic status of this strain as representing a novel species. These data support the conclusion that this strain represents a novel mycobacterial species, for which the name Mycobacterium seoulense sp. nov. is proposed. The type strain is strain 03-19T (=DSM 44998T = KCTC 19146T).

Mycobacteria are widely distributed in the environment; some are pathogenic to humans and animals and, of these, a number are saprophytes. Recently, the application of molecular techniques to the taxonomy and identification of isolates from environmental sources and clinical specimens has led to an increased awareness of the diversity within the genus Mycobacterium (Kirschner et al., 1993). Conventional biochemical tests have been performed extensively to detect and identify Mycobacterium tuberculosis and clinically important non-tuberculous mycobacteria (Goodfellow & Magee, 1998; Wayne & Kubica, 1986). However, because of the increasing number of newly defined taxa and the recognition of ‘difficult-to-identify’ variants of known species, tests sometimes fail to provide precise identification (Kirschner et al., 1993). To overcome the limitations of conventional methods, sequencing methods targeting several chronometer molecules have been developed (Kim et al., 1999, 2005; Roth et al., 1998; Stahl & Urbance, 1990; Stone et al., 1995). Moreover, the combination of molecular assays and conventional methods provides conclusive identification of infrequently encountered species and allows the delimitation of novel taxa. In the present study, a novel non-tuberculous mycobacterial species was isolated from a patient with general symptoms of pulmonary infection. The organism was discovered during an exercise designed to identify Korean slowly growing mycobacteria (SGM) clinical isolates using the recently developed hsp65 sequencing method (Kim et al., 2005).

The subject strain of the present study, strain 03-19T, was one of the ‘difficult-to-identify’ isolates submitted to the Korean Institute of Tuberculosis by mycobacteriology laboratories in Korea during 2003. This strain was isolated from sputum samples of a 52-year-old female who had been experiencing general pulmonary symptoms. The same strain was isolated from sputum specimens obtained from this patient on three successive occasions; no other mycobacterium was observed.

The phenetic characteristics of strain 03-19T and eight mycobacteria reference strains 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 Lowenstein–Jensen (LJ) medium and Middlebrook 7H10 agar. Acid–alcohol-fastness was
HPLC was used to analyse mycolic acids from strain 03-19\textsuperscript{T} and the most phenotypically similar strain, Mycobacterium nebraskense ATCC BAA-837\textsuperscript{T}, as described by Butler et al. (1992) or as described in the guidelines of the CDC National Center for HIV, STD, and TB Prevention Division of Tuberculosis Elimination (http://www.cdc.gov/nchstp/tb/Laboratory_Services/Liquid_Chroma.htm). Low- and high-molecular-mass standards (Ribi ImmunoChem) were added for peak identification. To identify and quantify mycolic acids and assign these to Mycobacterium species based on mycolic acid patterns, the Microbial Identification system (MIDI Inc.) was used.

Chromosomal DNA for molecular taxonomy was extracted using the bead-beater phenol extraction method as reported previously (Kim et al., 2005). Purified DNA was used as a template for PCR amplifications of three independent genes, the 16S rRNA gene, hsp65 (encoding heat-shock protein 65) and rpoB (encoding a subunit of RNA polymerase). The nearly complete 16S rRNA gene sequence (1523 bp) and partial sequences of hsp65 (644 bp) and rpoB (352 bp) were amplified as described previously (Springer et al., 1996; Kim et al., 1999, 2005). PCR amplicons of all target genes were cloned directly using Topo TA cloning kits (Invitrogen) and sequenced (Kim et al., 2005). To obtain sequence information on the rpoB and hsp65 genes of M. nebraskense, which were not available in GenBank, these sequences were also analysed from M. nebraskense ATCC BAA-837\textsuperscript{T}, purchased from the ATCC. The 16S rRNA gene sequence of 03-19\textsuperscript{T}.

Table 1. Cultural and biochemical characteristics that differentiate 03-19\textsuperscript{T} from other closely related SGM species

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*I, Intermediate in roughness; R, rough; S, smooth; O, orange; W, white; Y, yellow.
†N, Non-photochromogenic; P, photochromogenic; S, scotochromogenic.

determined by Ziehl–Neelsen and auramine O staining. The following biochemical features (Kent & Kubica, 1985) were investigated: niacin accumulation, nitrate reductase, arylsulfatase 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 ability to grow on MacConkey agar without crystal violet.

http://ijs.sgmjournals.org
obtained in the present study was compared with sequences
from GenBank using the BLAST analysis program (http://

Multiple alignments of sequences of the three genes of 03-
19T 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, 2005). All three trees were
inferred by neighbour joining (Saitou & Nei, 1987) and
maximum parsimony (Fitch, 1971) using Tsukamurella
paurometabola strain NCTC 10741 (16S rRNA gene) or
strain KCTC 9821T (hsp65) or Rhodococcus equi ATCC
10146T (rpoB) as an outgroup. 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 2.1 (Kumar et al., 2001) and the resulting trees
and topologies were evaluated by bootstrap analyses
(Felsenstein, 1985) based on 1000 resamplings.

Acid-fast microscopy showed generally rod-shaped and
frequently bent acid-fast bacilli. Occasional coccid 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 temperature. However,
4 weeks or more was required for mature colonies to form
on LJ medium. Colonies grown on Middlebrook 7H10 agar
were usually larger, smooth, occasionally rough, and always
orange in appearance under both dark and photoinduction
conditions. Cells on LJ medium were film-like 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 ml⁻¹ or picric acid to the
medium. However, strain 03-19T showed tolerance against
10 mg TCH ml⁻¹ and against 500 mg PNB ml⁻¹. The
strain was negative for urease activity, arylsulfatase, niacin
accumulation and Tween 80 hydrolysis and positive for
nitrate reductase, heat-stable catalase, pyrazinamidase and
tellurite reductase. Generally, the biochemical profile of
03-19T was most like those of M. nebraskense, another
scotochromogenic species. However, since a difference was
found between these two species in terms of tolerance
against TCH and PNB, such tests might be used to
differentiate the two taxa. Cultural and biochemical
characteristics that differentiate 03-19T from other closely
related SGM species are shown in Table 1.

In HPLC analysis of mycolic acids, the profile of strain 03-
19T showed two clusters of peaks that did not overlap with
any previously reported mycobacterial profile. The closest
match was with the Mycobacterium avium/intracellulare/
scrofulaceum complex, with a similarity index of 0.052. In a
comparison with the mycolic acids of M. nebraskense ATCC
BAA-837T (Fig. 1), differences were found in the relative
heights of peaks as follows: peak 1, 2.70 % (strain 03-19T)
and 10.45 % (M. nebraskense ATCC BAA-837T); peak 2, 1.53
and 6.64 %; peak 3, 11.16 and 14.21 %; peak 4, 5.67 and
22.20 %; peak 5, 9.74 and 6.23 %; peak 6, 11.74 and 5.06 %,
peak 7, 14.88 and 0.84 %; peak 8, 4.48 and 2.27 %; peak 9,
8.79 and 6.64 %, and peak 10, 11.76 and 3.08 %. Such
differences in HPLC peak heights have been reported to be
species-specific for mycobacteria (CDC, 1996, 1999; Duffey

Fig. 1. Mycolic acid patterns of strain 03-19T (a) and M.
nebraskense ATCC BAA-837T (b) obtained by HPLC analysis.
The relative retention time is indicated for each peak. LMMS,
Low-molecular-mass standard; HMMS, high-molecular-mass
standard. Asterisks (*) indicate peaks specific to either 03-19T
or M. nebraskense ATCC BAA-837T.
et al., 1996; Floyd et al., 1996). Furthermore, three unique peaks (retention times 4.834, 4.977 and 6.881 min) distinguished strain 03-19 $^T$ from *M. nebraskense* ATCC BAA-837 $^T$.

A BLAST search of the GenBank database using 16S rRNA gene sequences of strain 03-19 $^T$ gave a closest match (99%) to *Mycobacterium* sp. IWGM 90160 and the second best match to *M. nebraskense* UNMC-MY 1349 $^T$ (= ATCC BAA-837 $^T$) (99%). The 16S rRNA gene of 03-19 $^T$ differed from that of *M. nebraskense* UNMC-MY 1349 $^T$ by one deletion and eight substitutions and from that of *M. nebraskense* UNMC-MY 1349 $^T$ and *Mycobacterium* sp. IWGM 90160. In hypervariable region 'A' of the 16S rRNA gene, five and four differences were observed, respectively, between 03-19 $^T$ and *M. nebraskense* UNMC-MY 1349 $^T$ and *Mycobacterium* sp. IWGM 90160. In hypervariable region 'B', a unique 1 bp deletion distinguished strain 03-19 $^T$ from these closely related strains (Fig. 2). Perhaps these polymorphisms in the 16S rRNA gene could be used to detect this strain in the future.

A neighbour-joining tree based on the aligned 16S rRNA gene sequences of strain 03-19 $^T$ and 42 other *Mycobacterium* strains indicated a close relationship between strain 03-19 $^T$ and *M. nebraskense, Mycobacterium scrofulaceum* and *Mycobacterium* sp. IWGM 90160 within the SGM. The high bootstrapping values and topology of the maximum-parsimony tree strongly supported the grouping of these species (Fig. 3).

Trees based on *hsp65* and *rpoB* gene sequences showed different groupings of strain 03-19 $^T$, compared with the 16S rRNA gene sequences. In the *hsp65* neighbour-joining tree (Supplementary Fig. S1 available in IJSEM Online), strain 03-19 $^T$ was closely related to *Mycobacterium interjectum*, rather than to *M. nebraskense* or *M. scrofulaceum*, and this was strongly supported by high bootstrap values and the recovery of this grouping in the maximum-parsimony tree. In the *rpoB* neighbour-joining tree (Supplementary Fig. S2), strain 03-19 $^T$ was closely related to *Mycobacterium xenopi*. In spite of low bootstrap values, the same grouping was observed in the maximum-parsimony tree. Overall, phylogenetic analysis based on the three different gene sequences showed slightly different results for the relationship of strain 03-19 $^T$ to known species. The distinct grouping of 03-19 $^T$ among different trees confirmed the taxonomic status of this strain as a member of a novel species. It also strongly supported the notion that the description of the novel species could not have been achieved with results from a single gene. Sequence alignments of partial *hsp65* and *rpoB* genes for strain 03-19 $^T$ and closely related strains are available as Supplementary Fig. S3(a, b) in IJSEM Online.

The distinct sequences of these three gene targets together with the uniqueness of its mycolic acid profile and phenetic traits confirm the taxonomic status of strain 03-19 $^T$ as a member of a novel mycobacterial species rather than a variant of a previously described species. Moreover, the successive isolations of this strain from sputum samples of a patient at different stages in the absence of other mycobacteria strongly support the possibility that it may be a causative agent of pulmonary disease.

**Description of *Mycobacterium seoulense* sp. nov.**

*Mycobacterium seoulense* (seo.ul'en'se. N.L. neut. adj. *seoulense* pertaining to Seoul, Republic of Korea, the geographical origin of the type strain).

The bacillus stains acid–alcohol-fast. Cells are generally rod-shaped and frequently bent. Occasional coccid forms are noted. Spores and filaments are not present. The optimal

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<td><em>M. scrofulaceum</em> (A2536034)</td>
<td>G.C G.G C.G G.A A.G A.G</td>
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<tr>
<td><em>M. nebraskense</em> (AY368456)</td>
<td>A.A</td>
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<tr>
<td>03-19 $^T$ (DQ536432)</td>
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<td>03-19 $^T$ (DQ536432)</td>
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**Fig. 2.** Alignment of hypervariable regions A and B of the 16S rRNA gene from strain 03-19 $^T$ and genotypically similar reference strains. Nucleotide positions are indicated according to the *Escherichia coli* sequence. Species are represented by the corresponding type strains. The sequence of 03-19 $^T$ was determined in the present study; other sequences were obtained from GenBank.
The type strain is 03-19T (= DSM 44998T = KCTC 19146T), isolated from human sputum samples in Seoul, Republic of Korea.

Acknowledgements

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References


