**Mycobacterium paragordonae** sp. nov., a slowly growing, scotochromogenic species closely related to *Mycobacterium gordonae*

Byoung-Jun Kim,† Seok-Hyun Hong,† Yoon-Hoh Kook and Bum-Joon Kim

A previously undescribed, slowly growing, scotochromogenic mycobacterial strain (49061<sup>T</sup>) was isolated from a patient with pulmonary infections during the *hsp65*-sequence-based identification of Korean clinical isolates. Its 16S rRNA gene sequence was unique and the phylogenetic analysis based on 16S rRNA gene sequence (1393 bp) placed the organism into the slow-growing *Mycobacterium* group close to *Mycobacterium gordonae* (99.0% sequence similarity). Growth characteristics and acid-fastness also supported the placement of this species into the genus *Mycobacterium*. Phenotypically, this strain was generally similar to *Mycobacterium gordonae*; however, of particular interest, the optimal growth temperature of strain 49061<sup>T</sup> was 25–30 °C, and it was not able to grow at 37 °C on 7H10 agar slants. Unique MALDI-TOF MS profiles of lipids, phylogenetic analysis based on another two gene sequences (*hsp65* and *rpoB*) and a low DNA–DNA relatedness (46.52 ± 0.7) strongly supported the taxonomic status of this strain as a representative of a distinct species from *M. gordonae*. It was concluded that the strain represents a novel species for which the name *Mycobacterium paragordonae* is proposed with the type strain 49061<sup>T</sup> (=JCM 18565<sup>T</sup>=KCTC 29126<sup>S</sup>).

Non-tuberculous mycobacteria (NTM) are common in the environment and can be opportunistic pathogens. Recently, the application of molecular techniques to the taxonomy and identification of isolates from environmental sources and clinical specimens have led to an increased awareness of the diversity within NTM (Magee & Ward, 2012). Since our combined molecular and conventional taxonomic methods were applied to Korean patients, several novel NTM species have been successfully identified so far (Kim et al., 2012, 2013a, b; Lee et al., 2010a, b; Mun et al., 2007, 2008).

*Mycobacterium gordonae* exists ubiquitously in the environment and is generally considered to be saprophytic and non-pathogenic to humans. Genetic heterogeneity within *M. gordonae* has been shown by other reports (Itoh et al., 2003). However, to date, descriptions of novel species taxonomically similar to *M. gordonae* have not been published. In the present study, we introduce a novel species phenotypically and genetically related to *M. gordonae*, but distinct with respect to some phenotypic traits, particularly low optimal growth temperature (25–30 °C) and no growth at 37 °C.

The strain used in this study, 49061<sup>T</sup>, was a clinical isolate from a sputum sample of a patient with a symptomatic pulmonary infection. The strain was isolated as described in a previous report (Mun et al., 2008). It was isolated in 2010 from the Asan medical centre and submitted as a frozen stock of the strain. The strain was cultured on Middlebrook 7H9 broth (Becton Dickinson) supplemented with albumin dextrose catalase (ADC) and Middlebrook 7H10 agar (Becton Dickinson) plates supplemented with oleic albumin dextrose catalase (OADC) for a week at 30 °C and 37 °C to analyse its characteristics.

The phenotypic characteristics of strain 49061<sup>T</sup> and the type strains of the related species, *Mycobacterium asiaticum* ATCC 25276<sup>T</sup> and *M. gordonae* ATCC 14470<sup>T</sup> were analysed by biochemical study and then compared (Table 1). The biochemical characteristics were analysed as previously reported (Kent & Kubica, 1985; Pfaffer, 2007). Colony morphology, pigmentation in the dark, photo-induction and growth at various temperatures (25, 37 and 45 °C) were tested during 6 weeks of incubation on

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**Abbreviations:** NTM, nontuberculous mycobacteria; PNB, p-nitrobenzoate; TCH, thiophene-2-carboxylic acid hydrazide.

The GenBank/EMBL/DDJB accession numbers for the 16S rRNA, *hsp65* and *rpoB* gene sequences of strain 49061<sup>T</sup> are KC525204, KC525205 and KC525206, respectively.

A supplementary figure and four supplementary tables are available with the online version of this paper.
Middlebrook 7H10 agar plates supplemented with OADC. Acid–alcohol fastness was examined by Ziehl–Neelsen and auramine O staining.

Biochemical characteristics such as niacin accumulation, nitrate reductase, Tween 80 hydrolysis, urease and pyrazinamidase, were tested (Kent & Kubica, 1985; Pfyffer, 2007). Inhibition tests, including tolerance to thiophene-2-carboxylic acid hydrazide (TCH), p-nitrobenzoate (PNB), 5 % sodium chloride, ethambutol and picric acid, were carried out, and the ability to grow on MacConkey agar without crystal violet was determined (Pfyffer, 2007).

Generally, rod-shaped and acid-fast bacteria with frequently curved bacilli having no spores or filaments were observed by microscopy. Interestingly, strain 49061T showed no growth at 37 °C, which is generally an optimal temperature for growth of bacteria, including mycobacterial strains (Vincent & Gutierrez, 2007). Growth of smooth and orange colonies was observed on 7H10 agar plates at 25 °C (Table 1). Also when cultured at 30 °C, strain 49061T showed good growth (data not shown). This strain showed tolerance to 10 mg TCH ml⁻¹, but no growth on media containing 500 mg PNB ml⁻¹, 5 % NaCl, picric acid or ethambutol or on MacConkey agar plates without crystal violet. Negative responses were observed for niacin accumulation, nitrate reductase, arylsulfatase (after 3 days) and Tween 80 hydrolysis (within 5 days). In addition, the activities of catalase, Tween 80 hydrolysis (within 10 days), arylsulfatase (after 14 days), urease, tellurite reductase and pyrazinamidase showed positive responses. Furthermore, strain 49061T grew at lower growth temperatures (25–30 °C) and could not grow at 37 °C on 7H10 agar medium. Details from a comparison of the biochemical profiles and cultural characteristics of 49061T, M. asiaticum ATCC 25276T and M. gordonae ATCC 14470T are shown in Table 1.

Table 1. Cultural and biochemical characteristics that differentiate strain 49061T from M. asiaticum ATCC 25276T and M. gordonae ATCC 14470T

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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</thead>
<tbody>
<tr>
<td>Growth at:</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>25 °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37 °C</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth detectable after:</td>
<td>−</td>
<td>−</td>
<td>±</td>
</tr>
<tr>
<td>&lt;7 days</td>
<td></td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>&gt;7 days</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Colour*</td>
<td>O</td>
<td>W/Y</td>
<td>Y</td>
</tr>
<tr>
<td>Pigmentation†</td>
<td>S</td>
<td>P</td>
<td>S</td>
</tr>
<tr>
<td>Arylsulfatase (14 days)</td>
<td>+</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Tellurite reductase</td>
<td>+</td>
<td>−</td>
<td>±</td>
</tr>
<tr>
<td>Tween hydrolysis (&lt;5 days)</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Growth with:</td>
<td>±</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>10 mg TCH ml⁻¹</td>
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<tr>
<td>500 mg PNB ml⁻¹</td>
<td>−</td>
<td>±</td>
<td>±</td>
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<tr>
<td>Growth on picric acid</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>*Y, yellow; W, white; O, orange.</td>
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<td>†S, scotochromogenic; P, photochromogenic.</td>
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</tbody>
</table>

DNA–DNA hybridization was carried out to examine the DNA relatedness between strain 49061T and M. gordonae ATCC 14470T. Briefly, extracted genomic DNA of the two strains was blotted onto Hybond-N+ nylon membranes (Amersham Pharmacia Biotech) in three replicates after denaturation using NaOH solution and heating at 80 °C. Each DNA sample (4 μg) was digested with HaeIII restriction enzyme and its product was used individually as a labelled DNA probe for cross hybridization. Randomly primed DNA labelling with digoxigenin (DIG)-dUTP and detection of hybrids by enzyme immunoassay on nylon membrane were performed using a DIG High Prime DNA Labelling kit (Roche Applied Science) according to the manufacturer’s instructions and standard procedures (Sambrook & Russell, 2001). The hybridization signals were detected using a scanner (Scanjet 3770; Hewlett Packard) and analysed using the program Adobe Photoshop (version 7.0). The signal produced by hybridization of the probe to the homologous target DNA was taken to be 100 % and signal intensities by the self-hybridization of the series of dilutions were used for the calculation of the levels of DNA relatedness between strain 49061T and M. gordonae ATCC 14470T. The DNA–DNA hybridization tests were confirmed by cross hybridization.

The DNA–DNA relatedness between strain 49061T and M. gordonae ATCC 14470T was 46.52 ± 0.7 %, below the 70 % DNA–DNA relatedness which is generally used as a standard for species delineation (Wayne et al., 1987). For the MALDI-TOF mass spectrometry analysis, lipids were extracted with CHCl₃/CH₃OH (1:1, v/v) from 30 ml Middlebrook 7H9 broth cultures (supplemented with ADC) of strain 49061T and M. gordonae ATCC 14470T. A 0.5 μl volume of 2,5-dihydroxybenzoic acid was added to 10 μl of the purified lipids. MALDI-TOF mass spectrometry was performed on the extracted samples with a Voyager DE-STR MALDI-TOF instrument (Perseptive Biosystems) equipped with a pulse nitrogen laser emitting at 337 nm as previously described (Pérez et al., 2004) and was duplicated independently.

The MALDI-TOF MS profiles from the total lipids of strain 49061T and M. gordonae ATCC 14470T showed similar patterns. Each mass spectrum exhibited a cluster of peaks.
ranging from ~m/z 1361.9 to ~m/z 2747.8, with two major peaks at m/z 1415.6 and m/z 2600.7 (strain 49061\textsuperscript{T}) and m/z 1459.6 and m/z 2596.9 (\textit{M. gordonae}). However, there were minor peak clusters at m/z 2729.9 in MALDI-TOF MS profiles of \textit{M. gordonae} ATCC 14470\textsuperscript{T} but not in those of strain 49061\textsuperscript{T} (Fig. S1, available in IJSEM Online). The similar, but, distinct MALDI-TOF MS profiles of strain 49061\textsuperscript{T} and \textit{M. gordonae} ATCC 14470\textsuperscript{T} indicated differences in their lipids.

Genomic DNA of strain 49061\textsuperscript{T} was extracted by the bead beater–phenol extraction method for molecular taxonomic study as previously reported (Kim et al., 2005). As a negative control and test for the PCR amplification process, distilled water and purified genomic DNA were used as templates for PCR amplifications of three independent gene targets, the 16S rRNA, heat-shock protein 65 (\textit{hsp65}) and RNA polymerase β-subunit (\textit{rpoB}) genes. An almost complete 16S rRNA gene, the partial \textit{hsp65} gene and the partial \textit{rpoB} gene sequences were amplified as described previously (Kim et al., 1999, 2005; Roth et al., 1998; Springer et al., 1996). Briefly, template DNA (50 ng) and each primer set (20 pmol) were added to PCR tubes (containing AccuPower PCR PreMix; Bioneer) and PCR was conducted by subjecting the samples to 5 min at 95°C, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 45 s; and a final extension at 72°C for 5 min, which was performed in a MyCycler thermal cycler (Bio-Rad). The PCR products were detected and then purified using a Megaquick-spin fragment DNA purification kit (iNtRON) for direct sequencing. In the case of the 16S rRNA gene, the PCR product was cloned into pCR 2.1-TOPO vector (Invitrogen) and transformed into TOP10 \textit{Escherichia coli} (Invitrogen) for more precise sequencing results. Sequencing reactions were performed using a PTC-225 Peltier thermal cycler (MJ Research) and ABI PRISM BigDye Terminator Cycle Sequencing kits with Ampli \textit{Tag} DNA polymerase (FS enzyme; Applied Biosystems) following the manufacturers’ protocols. Obtained sequences were compared with the GenBank database using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The 16S rRNA (1404 bp), \textit{hsp65} (603 bp) and \textit{rpoB} (306 bp) gene sequences of strain 49061\textsuperscript{T} and other reference mycobacterial strains, which include a wide range of both slowly and rapidly growing mycobacteria, were aligned by using the multiple alignment algorithm in the MEGA software package as previously described (Kim et al., 1999, 2005; Turenne et al., 2001). Evolutionary distance matrices were generated according to the Jukes & Cantor model (Jukes & Cantor, 1969). Phylogenetic trees were inferred from the three target gene sequences using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods in the MEGA version 4.0 software (Kumar et al., 2008). The 16S rRNA gene sequence and \textit{hsp65} gene sequence of \textit{Tsukamurella paurometabola} NCTC 10741\textsuperscript{T} and KCTC 9821\textsuperscript{T} and the \textit{rpoB} gene sequence of \textit{Rhodococcus equi} ATCC 10146 were retrieved from GenBank and used as outgroups. In addition, concatenated trees based on 16S rRNA, \textit{hsp65} and \textit{rpoB} gene sequences were reconstructed. The reconstructed trees and their topologies were evaluated by bootstrap analysis based on 1000 replications (Felsenstein, 1985).

The BLAST analysis results for the 16S rRNA gene sequence (1393 bp) of strain 49061\textsuperscript{T} showed the closest match (99%) with \textit{M. gordonae} strain ASCr-1.42 (GenBank accession number JX575114), which was recently isolated from farmed sturgeons in China. A phylogenetic tree based on the 16S rRNA gene sequences of species of the genus \textit{Mycobacterium} showed the closest relationship between strain 49061\textsuperscript{T} and \textit{M. gordonae} ATCC 14470\textsuperscript{T} with a high bootstrap value (100) (Fig. 1a). In a comparison of the 16S rRNA gene sequences with \textit{M. asiaticum} ATCC 25276\textsuperscript{T} and \textit{M. gordonae} ATCC 14470\textsuperscript{T}, those of strain 49061\textsuperscript{T} showed sequence similarities of 98.1% and 99.0%, respectively (Table S1). The sequence polymorphisms between strain 49061\textsuperscript{T} and \textit{M. asiaticum} ATCC 25276\textsuperscript{T} and \textit{M. gordonae} ATCC 14470\textsuperscript{T} were found to involve ten and two nucleotides in hypervariable region A, respectively and one and three nucleotides in hypervariable region B, respectively (Fig. 2). These results supported the hypothesis that the closest relationship for the 16S rRNA gene sequence is that between strain 49061\textsuperscript{T} and \textit{M. gordonae}.

The phylogenetic analysis based on the partial \textit{hsp65} gene sequences (603 bp) also supported the grouping of strain 49061\textsuperscript{T} and \textit{M. gordonae} ATCC 14470\textsuperscript{T} (with a bootstrap value of 88), as shown in the 16S rRNA gene-based tree (Fig. 1b). Sequence similarities for \textit{hsp65} between strain 49061\textsuperscript{T} and \textit{M. asiaticum} ATCC 25276\textsuperscript{T} and \textit{M. gordonae} ATCC 14470\textsuperscript{T} were 95.7% and 95.9%, respectively (Table S2).

In the case of the \textit{rpoB}-sequence-based phylogenetic analysis, strain 49061\textsuperscript{T} was clustered with \textit{M. asiaticum} ATCC 25276\textsuperscript{T}, not with \textit{M. gordonae} ATCC 14470\textsuperscript{T} as in the 16S rRNA- and \textit{hsp65}-sequence-based trees. However, the bootstrap value of the group (strain 49061\textsuperscript{T} and \textit{M. asiaticum} ATCC 14470\textsuperscript{T}) was below 50 (Fig. 1c). Sequence similarities for \textit{rpoB} between strain 49061\textsuperscript{T} and \textit{M. asiaticum} ATCC 25276\textsuperscript{T} and \textit{M. gordonae} ATCC 14470\textsuperscript{T} were 95.4% and 95.1%, respectively (Table S3).

Also, a phylogenetic tree based on the concatenation (2302 bp) of the three gene sequences [16S rRNA (1393 bp) + \textit{hsp65} (603 bp) + \textit{rpoB} (306 bp)] was reconstructed. The tree showed that strain 49061\textsuperscript{T} was clustered together with \textit{M. gordonae} ATCC 14470\textsuperscript{T} and the topology was strongly supported by high bootstrap value (100) and maximum-parsimony analysis (Fig. 1d). Concatenated gene sequence similarities among strain 49061\textsuperscript{T}, \textit{M. asiaticum} ATCC 25276\textsuperscript{T} and \textit{M. gordonae} ATCC 14470\textsuperscript{T} showed that the closest phylogenetic relationship was between strain 49061\textsuperscript{T} and \textit{M. gordonae} ATCC 14470\textsuperscript{T} (97.7% sequence similarity) (Table S4). Collectively, strain 49061\textsuperscript{T} was genetically close to \textit{M. gordonae} ATCC 14470\textsuperscript{T}; however, there were unique gene sequences that could differentiate the two strains.
Fig. 1. Phylogenetic relationships of strain 49061T with other species of the genus *Mycobacterium* based on the 16S rRNA gene (a), *hsp65* gene (b), *rpoB* gene (c) and concatenated three-gene (16S rRNA, *hsp65* and *rpoB*; 2289–2305 bp) (d) sequences. These trees were reconstructed using the neighbour-joining method. The bootstrap values were calculated from 1000 replications. Bootstrap values of <95% are not shown. Filled circles indicate that the corresponding groups were supported in the maximum-parsimony trees. *Tsukamurella paurometabola* DSM 20162T and KCTC 9821T and *Rhodococcus equi* ATCC 10146 were used as outgroups in the 16S rRNA-, *hsp65*- and *rpoB*-based trees, respectively. Bars indicate numbers of substitutions per nucleotide position.
Toward defining course of evolution: minimum approach using the bootstrap. 


