**Mycobacterium koreense** sp. nov., a slowly growing non-chromogenic species closely related to **Mycobacterium triviale**

Byoung-Jun Kim,1 Joseph Jeong,2 Seon Ho Lee,2 Sung-Ryul Kim,2 Hee-Kyung Yu,3 Young-Gil Park,3 Ki-Jeong Kim,4 Yoon-Hoh Kook1 and Bum-Joon Kim1

Correspondence
Bum-Joon Kim
kbumjoon@snu.ac.kr

1Department of Microbiology and Immunology, Cancer Research Institute and Liver Research Institute, College of Medicine, Seoul National University, Seoul 110-799, Republic of Korea

2Department of Laboratory Medicine, Ulsan University Hospital, Ulsan, Republic of Korea

3The Korean Institute of Tuberculosis, The Korean National Tuberculosis Association, Seoul 137-140, Republic of Korea

4Department of Microbiology, School of Medicine, Joong-Ang University, Seoul, Republic of Korea

A novel slow-growing, non-chromogenic mycobacterium (strain 01-305T) was isolated from a patient with pulmonary dysfunction. Growth characteristics, acid-fastness and the results of 16S rRNA gene sequencing supported the placement of this strain within the genus *Mycobacterium*. Phenotypically, strain 01-305T was generally similar to *Mycobacterium triviale* ATCC 23292T, but some unique biochemical characteristics were observed. The 16S rRNA gene sequence of strain 01-305T was similar to those of *M. triviale* ATCC 23290 (GenBank accession no. AY734996, 99.9 % similarity) and *M. triviale* ATCC 23291 (AY734995, 99.9 %); however, it differed substantially from that of *M. triviale* ATCC 23292T (X88924, 98.2 %). Phylogenetic analysis based on 16S rRNA gene sequences placed strain 01-305T in the slow-growing *Mycobacterium* group close to *M. triviale* ATCC 23290 and *M. triviale* ATCC 23291, but not *M. triviale* ATCC 23292T. Unique mycolic acid profiles and phylogenetic analysis based on two different chronometer molecules, and the *hsp65* and *rpoB* genes, strongly supported the taxonomic status of this strain as representing a distinct species. These data support the conclusion that strain 01-305T represents a novel mycobacterial species, for which the name *Mycobacterium koreense* sp. nov. is proposed. The type strain is 01-305T (=DSM 45576T =KCTC 19819T).

Mycobacteria are widely distributed in the environment, and some are pathogenic to humans and animals, some being saprophytic. In addition to strict pathogens, including the *Mycobacterium tuberculosis* complex and *Mycobacterium leprae*, non-tuberculous mycobacteria (NTM) are able to cause opportunistic infections (Tortoli, 2009). As they generally show species-specific resistance to antibacterial agents, there is an increasing need for precise identification of clinically isolated NTM. In an effort to differentiate and identify NTM, approximately 150 mycobacterial species have been described to date. Recently, the combination of conventional methods and molecular analyses, especially PCR-mediated sequencing methods, has been successfully applied to describe novel species in the genus *Mycobacterium* (Lee et al., 2010; Mun et al., 2007, 2008).

The *Mycobacterium terrae* complex comprises several different species that are phenotypically very similar but genetically distinct, classically including *M. terrae*, *Mycobacterium nonchromogenicum* and *Mycobacterium triviale* (Lee et al., 2004; Tortoli, 2003). However, it is now clear that *M. triviale* is separate from this complex. Therefore, taxonomic separation between these species based on conventional biochemical tests is almost impossible, but they can be differentiated into distinct species based on genetic methods. Despite phenotypic similarity between

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

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA, *hsp65* and *rpoB* gene sequences of strain 01-305T are JF271826, JF271827 and JF271828, respectively. Those for the partial *hsp65* and *rpoB* gene sequences of *Mycobacterium triviale* ATCC 23290 are JN037843 and JN037841, respectively. Those for the partial *hsp65* and *rpoB* gene sequences of *Mycobacterium triviale* ATCC 23291 are JN037844 and JN037842, respectively.

Three supplementary figures and five supplementary tables are available with the online version of this paper.
species belonging to the M. terrae complex, M. triviale is genetically distantly related to two other members, M. terrae and M. nonchromogenicum (Tortoli, 2003). Phylogenetic analysis based on 16S rRNA gene sequences supports the genetic disparity of M. triviale from other members of the complex. Recently, polyphasic approaches have broadened the taxonomic diversity of the M. terrae complex, with several novel species added, including Mycobacterium arupense, Mycobacterium hiberniae, Mycobacterium kumamotonense and Mycobacterium senuense (Cloud et al., 2006; Kazda et al., 1993; Lee et al., 2010; Masaki et al., 2006; Mun et al., 2008). However, all recently described members of the complex are related to M. terrae or M. nonchromogenicum rather than to M. triviale. To the best of our knowledge, no novel species phylogenetically related to M. triviale has been described to date.

In the present study, we describe a novel clinical isolate closely related to M. triviale based on data from a polyphasic taxonomic approach via a combination of biochemical tests and molecular analyses targeting 16S rRNA (Rogall et al., 1990; Springer et al., 1996), hsp65 (Kim et al., 2005, 2006) and rpoB (Kim et al., 1999) gene sequences.

The strain used in the study (01-305T) was one of several ‘difficult-to-identify’ isolates submitted to the Korean Institute of Tuberculosis by mycobacteriology laboratories in Korea during 2001. Decontamination of the sputa sample and cultivation were as described previously (Lee et al., 2010; Mun et al., 2008).

The phenotypic (biochemical) characteristics of strain 01-305T and the type strain of M. triviale were analysed and compared (Table 1). Colony morphology, pigmentation in the dark, photo-induction and growth at 25, 37 and 45 °C were tested during 6 weeks of incubation on Middlebrook 7H10 agar plates supplemented with OADC (BD Bioscience). Acid–alcohol-fastness was examined by Ziehl–Neelsen and auramine O staining. We also tested for biochemical characteristics such as niacin accumulation, nitrate reductase, arylsulfatase after 3 and 14 days, heat-stable catalase (pH 7, 68 °C), tellurite reductase, Tween 80 hydrolysis, urease and pyrazinamidase (Kent & Kubica, 1985). 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 examined. Antimicrobial susceptibility was determined by the agar proportion method on Middlebrook 7H10 medium (Kent & Kubica, 1985).

For fatty acid and quinone analyses, strain 01-305T and M. triviale ATCC 23292T were cultured in Middlebrook 7H9 broth supplemented with ADC for 2 weeks at 37 °C under aerobic conditions. For HPLC, strain 01-305T and three strains of M. triviale (ATCC 23290, ATCC 23291 and ATCC 23292T) were cultured on Middlebrook 7H10 agar plates supplemented with OADC for 2 weeks at 37 °C under aerobic conditions.

Cells of strain 01-305T were acid-fast and generally rod-shaped bacilli that were frequently curved and had no spores or filaments. The strain grew at 25 °C but not at 45 °C; the optimal growth temperature was 37 °C. On Middlebrook 7H10 agar plates, mature colonies were detected after about 2 weeks. Microcolonies developed after about 1 week. The growth rate of strain 01-305T was similar to that of M. triviale. On Middlebrook 7H10 agar plates under both dark and photo-induction conditions, colonies of strain 01-305T were rough or smooth without pigment. This strain showed tolerance to 10 mg TCH ml⁻¹, 500 mg PNB ml⁻¹ and 5 % NaCl. It showed negative responses to tests for niacin accumulation, arylsulfatase (at 3 days), tellurite reductase, urease activity and nitrate reductase, but positive responses to tests for Tween 80 hydrolysis, heat-stable catalase and pyrazinamidase. The biochemical characteristics of strain 01-305T were very similar to those of M. triviale. However, two distinct characteristics of strain 01-305T, a negative response for nitrate reductase and the ability to grow on MacConkey agar, differentiated it from M. triviale ATCC 23292T. Differential biochemical and cultural characteristics between strain 01-305T and the type strain of M. triviale are shown in Table 1.

Antimicrobial susceptibility (minimum inhibitory concentration) tests showed that strain 01-305T was more susceptible to cefoxitin (32 μg ml⁻¹), ciprofloxacin (1 μg ml⁻¹), clarithromycin (8 μg ml⁻¹) and doxycycline (16 μg ml⁻¹) than M. triviale. These differences might be used for clinical diagnosis. The detailed results of antibiotic susceptibility tests are given in Table S1, available in IJSEM online.

Fatty acid methyl esters were obtained from biomass of strain 01-305T and M. triviale ATCC 23292T as described by Minnikin (1988). Extracted samples were separated by GC (model 5898A; Hewlett Packard) and analysed by using the Sherlock Microbial ID System. The predominant fatty acids of strain 01-305T were C₁₆:₀ (35.95 %) and C₁₈:₁ω9c.

### Table 1. Differential cultural and biochemical characteristics between strain 01-305T and M. triviale ATCC 23292T

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain 01-305T</th>
<th>M. triviale ATCC 23292T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth after &lt;7 days</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Colony details*</td>
<td>IWY</td>
<td>RWY</td>
</tr>
<tr>
<td>Nitrate reductase</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Growth with 5 % NaCl</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth on MacConkey agar</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

*R, rough; I, intermediate; Y, yellow; W, white.
(31.71 %). The fatty acid profile comprised unbranched saturated and unsaturated fatty acid esters with chain lengths of 14, 15, 16, 17, 18 and 20 carbon atoms, and tuberculostearic acid (10-methyl C18:0; 8.12 %) was also detected. Strain 01-305T could be differentiated from M. triviale ATCC 23292T, based on the presence of C17:1ω7c/C18-OH (2.77 %). The detailed fatty acid profiles of strain 01-305T and M. triviale ATCC 23292T are given in Table S2.

Isoprenoid quinones were extracted from 300 mg freeze-dried pellets and purified by using the small-scale integrated procedure described by Minnikin et al. (1984). The dried menaquinone (MK) was dissolved in 200 μl 2-propanol and separated by HPLC as described by Kroppenstedt (1982, 1985). MK-9(H2) (96.1 %) was the predominant menaquinone and a small amount of MK-8(H2) (3.9 %) was also detected. This result is typical of the genus Mycobacterium, and the menaquinone pattern is similar to that of M. triviale ATCC 23292T. However, the ratio of MK-8(H2) to MK-9(H2) in M. triviale ATCC 23292T was higher than in strain 01-305T. The results of isoprenoid quinone analyses are shown in Fig. S1.

The mycolic acids of strains 01-305T, M. triviale ATCC 23290, M. triviale ATCC 23291 and M. triviale ATCC 23292T were analysed by HPLC as described by Butler et al. (1992). Low- and high-molecular-mass standards (Ribi ImmunoChem) were added for peak identification. The Microbial Identification system (MIDI Inc.) and HPLC mycobacterium library (available at http://www.MycobacteriaToscana.it) were used to identify and compare mycolic acid patterns. The HPLC profile of strain 01-305T showed a double-cluster peak pattern and was similar to those of M. triviale ATCC 23290 and M. triviale ATCC 23291. However, M. triviale ATCC 23292T showed only one late cluster peak pattern (Fig. 1). HPLC analysis indicated that strain 01-305T was related more closely to M. triviale ATCC 23290 and M. triviale ATCC 23291 than to M. triviale ATCC 23292T.

Genomic DNA of strain 01-305T was extracted by the bead-beater phenol extraction method (Kim et al., 2005). Three gene targets, 16S rRNA (1473 bp), heat-shock protein 65 (hsp65; 644 bp) and RNA polymerase β-subunit (rpoB, 352 bp), were amplified by PCR with purified genomic DNA as template (Kim et al., 1999, 2005, 2006; Springer et al., 1996). PCR products of the three gene targets were cloned directly using TOPO TA cloning kits (Invitrogen) to obtain precise sequence information. Three independent colonies were chosen and used for sequencing (Kim et al., 2005). The 16S rRNA gene sequence of strain 01-305T...
Fig. 2. Alignments of hypervariable regions A and B of the 16S rRNA gene sequences of strain 01-305\(^T\) and M. terrae-related reference strains. Nucleotide positions are according to the 16S rRNA gene sequence of Escherichia coli. Only base pairs that differ from M. tuberculosis are shown. Dashes indicate deletions. Reference sequences were retrieved from the GenBank database.

---

Fig. 3. Phylogenetic relationship between strain 01-305\(^T\) and members of recognized species of the genus Mycobacterium based on 16S rRNA (a), rpoB (b) and hsp65 (c) gene sequences. Trees were reconstructed by using the neighbour-joining method. Numbers at nodes are bootstrap values (percentages of 1000 replications); only values >50% are shown. Solid circles indicate that the corresponding groups were supported in the maximum-parsimony trees. Tsukamarella paurometabola DSM 20162\(^T\) and KCTC 9821\(^T\) and Rhodococcus equi ATCC 10146 were used as an outgroup in the 16S rRNA, hsp65 and rpoB gene trees, respectively. Bars, substitutions per nucleotide position.
determined in this study was compared against those in the GenBank database by using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/).

The three target gene sequences of strain 01-305^T were used for multiple alignments with other mycobacterial reference strains by using the multiple-alignment algorithm in MEALIGN as previously described (Kim et al., 1999, 2005). Phylogenetic trees based on the three independent gene sequences were reconstructed via the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods with 16S rRNA and hsp65 gene sequences of Tsukamurella paurometabola DSM 20162^T and KCTC 9821^T, respectively, or the partial rpoB gene sequence of Rhodococcus equi ATCC 10146^T as outgroups. Evolutionary distance matrices were generated according to the Jukes–Cantor model (Jukes & Cantor, 1969). All phylogenetic analyses were carried out by using the neighbour-joining model in the program MEGA 4 (Kumar et al., 2008), and the trees reconstructed were evaluated by bootstrap analysis based on 1000 replicates (Felsenstein, 1985).

Based on 16S rRNA gene sequence analysis, strain 01-305^T showed the closest match (99.8% similarity) to Mycobacterium sp. NLA001000736 (GenBank accession no. HM627011), recently isolated in the Netherlands. The 16S rRNA gene sequence of strain 01-305^T differed by only 3 bp substitutions among 1473 bp compared with Mycobacterium sp. NLA001000736, and by 2 bp substitutions among 1395 and 1394 bp compared with M. triviale ATCC 23290 (AY734996) and M. triviale ATCC 23291 (AY734995), respectively.

Levels of 16S rRNA gene sequence similarity among strain 01-305^T and other species of the M. terrae complex ranged from 94.5% (M. senenuense DSM 44999^T; DQ536408) to 99.9% (M. triviale ATCC 23290 and M. triviale ATCC 23291; AY734996 and AY734995) (Table S3). Strain 01-305^T showed no differences in hypervariable regions of the 16S rRNA gene sequence compared with M. triviale ATCC 23290 and M. triviale ATCC 23291, but different sequences of 7 bp (four in hypervariable region A and three in hypervariable region B) were found between strain 01-305^T and M. triviale ATCC 23290 or M. triviale ATCC 23291 (Fig. 2). The neighbour-joining tree based on 16S rDNA gene sequences of species of the genus Mycobacterium indicated that strain 01-305^T, M. triviale ATCC 23290 and M. triviale ATCC 23291 belong to the same cluster, separate from M. triviale ATCC 23292^T, strongly suggesting that strain 01-305^T, M. triviale ATCC 23290 and M. triviale ATCC 23291 may be different members of the same species, and distinct from M. triviale. Recovery of this
topology in the maximum-parsimony tree with high bootstrap support also supported this conclusion (Fig. 3a).

Phylogenetic trees based on \(rpoB\) and \(hsp65\) gene sequences also supported a close relationship between strain 01-305\(^T\), \(M.\) \(triviale\) ATCC 23290 and \(M.\) \(triviale\) ATCC 23291, but a more distant relationship to \(M.\) \(triviale\) ATCC 23292\(^T\) (Fig. 3b, c). Collectively, the combination of phylogenetic analyses based on three independent genes and HPLC analysis of mycolic acids suggests that two strains previously classified as members of \(M.\) \(triviale\), ATCC 23290 and ATCC 23291, should be transferred to a novel species of the genus, along with strain 01-305\(^T\).

Multiple alignments of the partial \(hsp65\) and \(rpoB\) gene sequences of strain 01-305\(^T\) and of members of the \(M.\) \(terrae\) complex, including \(M.\) \(triviale\) ATCC 23290, \(M.\) \(triviale\) ATCC 23291 and \(M.\) \(triviale\) ATCC 23292\(^T\), are shown in Figs S2 and S3. Sequence similarity values of the partial \(hsp65\) and \(rpoB\) genes between strain 01-305\(^T\) and the three \(M.\) \(triviale\) strains (ATCC 23290, ATCC 23291 and ATCC 23292\(^T\)) also indicated a close phylogenetic relationship among strain 01-305\(^T\), \(M.\) \(triviale\) ATCC 23290 and \(M.\) \(triviale\) ATCC 23291, but not \(M.\) \(triviale\) ATCC 23292\(^T\) (Tables S4 and S5).

Taken together, the distinct sequence characteristics of the three independent genes together with unique HPLC profiles of mycolic acids and phenetic traits strongly support the conclusion that strain 01-305\(^T\), \(M.\) \(triviale\) ATCC 23290 and \(M.\) \(triviale\) ATCC 23291 belong to a distinct mycobacterial species, rather than being variants of the previously described species \(M.\) \(triviale\), for which the name Mycobacterium koreense sp. nov. is proposed.

**Description of Mycobacterium koreense sp. nov.**

Mycobacterium koreense (ko.re.en’s.e. N.L. neut. adj. koreense of or pertaining to the Republic of Korea, the geographical origin of the type strain).

Cells are generally rod-shaped, and frequently curved. Acid–alcohol-fast. Spores and filaments are not present. The optimal growth temperature is 37 °C; grows at 25 °C but not at 45 °C. On Middlebrook 7H10 agar medium, mature colonies develop in about 2 weeks. Microcolonies develop in about 1 week. Colonies grown on Middlebrook 7H10 agar medium are rough or smooth and have no pigmentation under both dark and photo-induced conditions. Tolerant to 10 mg TCH ml\(^{-1}\), 500 mg PNB ml\(^{-1}\) and 5% NaCl. Negative for niacin accumulation, arylsulfatase (at 3 days), tellurite reductase, urease activity and nitrate reductase. Positive for Tween 80 hydrolysis, heat-stable catalase, pyrazinamidase and growth on MacConkey agar. The predominant fatty acids are C\(_{16:0}\) and C\(_{18:1\(\Delta9\)}\).
10-methyl C₁₈:₀ is also detected. MK-9(H₂) is the predominant menaquinone; a small amount of MK-8(H₂) is also found. HPLC analysis shows a unique profile of mycolic acids. Genetically, 16S rRNA, hsp65 and rpoB gene sequences are almost the same as those of M. triviale ATCC 23290 and M. triviale ATCC 23291, but different from those of M. triviale ATCC 23292T. Phylogenetic analyses show that the type strain is closely related to M. triviale ATCC 23290 and M. triviale ATCC 23291 within the slowly growing mycobacteria cluster.

The type strain, 01-305T (=DSM 45576T =KCTC 19819T), was isolated from human sputum in Seoul, Korea.

Acknowledgements

This study was supported by the Korean Healthcare Technology R&D project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (grant A101205). The first and second authors of this paper contributed equally to this study and should be considered as co-first authors.

This study was supported by the Korean Healthcare Technology R&D project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (grant A101205). The first and second authors of this paper contributed equally to this study and should be considered as co-first authors.

References


