Mycobacterium anyangense sp. nov., a rapidly growing species isolated from blood of Korean native cattle, Hanwoo (Bos taurus coreanae)

Byoung-Jun Kim,1,† Jae-Myung Kim,2,† Bo-Ram Kim,1 So-Young Lee,1 GaNa Kim,1 Yun-Ho Jang,2 Soyoon Ryoo,2 Che-Ok Jeon,3 Hyun-Mi Jin,3 Joseph Jeong,4 Seon Ho Lee,4 Ji-Hun Lim,4 Yoon-Hoh Kook1 and Bum-Joon Kim1

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

1Department of Biomedical Sciences, Microbiology and Immunology, Cancer Research Institute, and Institute of Endemic Diseases, Seoul National University Medical Research Center (SNUMRC), Seoul National University College of Medicine, Seoul 110-799, Republic of Korea
2Animal and Plant Health Research Department, Animal and Plant Quarantine Agency, Anyang 430-757, Republic of Korea
3Department of Life Science, Chung-Ang University, Seoul 156-756, Republic of Korea
4Department of Laboratory Medicine, Ulsan University Hospital, University of Ulsan College of Medicine, Ulsan 682-714, Republic of Korea

From the whole blood of Korean native cattle, Hanwoo (Bos taurus coreanae), a previously undescribed, rapidly growing, scotochromogenic isolate of the genus Mycobacterium is reported. Its 16S rRNA gene sequence, and the sequences of three other genes (hsp65, recA and rpoB) were unique and phylogenetic analysis based on 16S rRNA gene sequence (1420 bp) placed the organism into the rapidly growing Mycobacterium group close to Mycobacterium smegmatis (98.5 % sequence similarity). However, phylogenetic analyses based on three different gene sequences (hsp65, recA and rpoB) revealed its location to be distinct from the branch of rapidly growing species. Culture and biochemical characteristics were generally similar to those of Mycobacterium fortuitum. Unique matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS profiles of lipids, unique fatty acid profile, unique mycolic acids profiles and a low DNA–DNA relatedness to M. fortuitum (23.6 %) and M. smegmatis (39.7 %) strongly supported the taxonomic status of this strain as a representative of a novel species of rapidly growing mycobacteria named Mycobacterium anyangense. The type strain is strain QIA-38T (=JCM 30275T=KCTC 29443T).

Non-tuberculous mycobacteria (NTM) are common in the environment and can be the cause of opportunistic infections in humans and in animals (Tortoli, 2009).

†Byoung-Jun Kim and Jae-Myung Kim contributed equally to this study.

Abbreviations: GPL, glycopeptidolipid; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; NTM, non-tuberculous mycobacteria; RGM, rapidly growing mycobacteria

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA, hsp65, recA and rpoB genes of strain QIA-38T are KJ855063, KF910182, KJ855062 and KF910164, respectively. The accession numbers for the partial hsp65 gene sequences of strains Mycobacterium chelonae ATCC 35752T and Mycobacterium smegmatis ATCC 19420T are KP017250 and KP017251, respectively.

Five supplementary tables are available with the online Supplementary Material.

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 NTM (Magee & Ward, 2012). Actually, by applying our combined molecular and conventional taxonomic methods to isolates from Korean patients, several novel NTM species have been successfully identified so far (Kim et al., 2012, 2013a, b, 2014a; Lee et al., 2010a, b; Mun et al., 2007, 2008).

In this study, a strain of NTM designated QIA-38T was isolated from Hanwoo (Bos Taurus coreanae) at the Animal and Plant Quarantine Agency in South Korea (Kim et al., 2014b). From the whole blood of slaughtered cattle, a sample was decontaminated by 10 % oxalic acid solution for 10 min at room temperature (RT) or 1.5 % 1-hexadecylpyridinium chloride for 30 min at RT. After centrifugation at 1000 g for
10 min, the sample was resuspended in PBS and then inoculated on Lowenstein–Jensen slants and Lowenstein–Jensen slants containing 0.1 % glycerol. The isolated strain was subcultured until visible bacterial growth in Middlebrook 7H9 broth supplemented with albumin dextrose catalase (ADC) and on Middlebrook 7H10 agar plates supplemented with oleic albumin dextrose catalase (OADC) for three to five days at 25 °C, 30 °C, 37 °C and 45 °C to analyse characteristics of the strain.

To characterize strain QIA-38T, colony morphology and growth rate were determined. Acid-alcohol-fastness was examined by Ziehl–Neelsen staining. And the enzymic activities of alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase were analysed using an API ZYM kit (bioMérieux) following the manufacturer’s instructions and comparing with other typical rapidly growing mycobacteria (RGM), Mycobacterium abscessus ATCC 19977T, Mycobacterium chelonae ATCC 35752T, Mycobacterium fortuitum ATCC 6841T and Mycobacterium smegmatis ATCC 19420T.

Table 1. Comparison of cultural and biochemical characteristics among strain QIA-38T and related members of the genus Mycobacterium

<table>
<thead>
<tr>
<th>Characteristic</th>
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<td>Growth at:</td>
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<td>30 °C</td>
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<td>Pigmentation</td>
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<td>N</td>
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<td>Enzymic activities (API ZYM kit)</td>
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<td>Alkaline phosphatase</td>
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<td>Acid phosphatase</td>
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<td>Naphthol-AS-BI-phosphohydrolase</td>
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Microscopic analysis showed rod-shaped, acid-fast bacilli with no spores or filaments. When cultured on Middlebrook 7H10 agar plates, mature colonies of strain QIA-38T developed in about three to five days. Colonies grown on Middlebrook 7H10 agar plates were usually smooth and yellow or orange. Strain QIA-38T grew at various temperatures (25 °C, 30 °C, 37 °C and 45 °C), and grew especially well at 37 °C; however, there was no growth at 45 °C. Strain QIA-38T was supposed to be grouped in the RGM due to its growth rate, and enzyme activities of strain QIA-38 were compared with those of other typical RGM strains using the API ZYM kit. Among 19 enzymes, strain QIA-38T showed positive results for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucuronidase, α-glucosidase and β-glucosidase. For the other enzymes, lipase (C14), trypsin, α-chymotrypsin, α-galactosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase, strain QIA-38T showed negative results. In the case of β-galactosidase, strain QIA-38T showed weakly positive results. Compared with other RGM species, the enzymic activities of strain QIA-38T were similar to those of M. fortuitum ATCC 6841T, but not M. abscessus subsp. abscessus ATCC 19977T, M. chelonae ATCC 35752T or M. smegmatis ATCC 19420T. The results of the enzymic activities compared among strain QIA-38T, M. abscessus subsp. abscessus ATCC 19977T, M. chelonae ATCC 35752T, M. fortuitum ATCC 6841T and M. smegmatis ATCC 19420T are shown in Table 1.

For matrix-assisted laser desorption ionization time-of flight (MALDI-TOF) MS analysis, total lipids were extracted in CHCl3/CH3OH (1:1, v/v) from 30 ml 7H9 broth cultures of strain QIA-38T and M. fortuitum ATCC 6841T. The lipid samples extracted were treated with 0.5 μl 2,5-dihydroxybenzoic acid for the matrix in MALDI-TOF MS analysis. MALDI-TOF MS analysis was carried out in duplicate with a Voyager DE-STR MALDI-TOF instrument (Perseptive Biosystems) equipped with a pulsed nitrogen laser emitting at 337 nm as described by Pérez et al. (2004). The MALDI-TOF MS analysis was duplicated for each sample to ensure accuracy.

The MALDI-TOF MS profiles of total lipids of strain QIA-38T and M. fortuitum ATCC 6841T were compared with those of other RGM, M. abscessus subsp. abscessus, M. chelonae and M. smegmatis. In the case of M. smegmatis, diglycosylated glycopeptidolipids (GPLs; m/z 1273) were detected in the lipids; however, M. abscessus subsp. abscessus and M. chelonae produced diglycosylated GPLs (m/z 1273) and triglycosylated GPLs (m/z 1419) in their lipids (Ripoll et al., 2007). Compared with previously reported MALDI-TOF MS profiles of other RGM, M. abscessus subsp. abscessus, M. chelonae and M. smegmatis, strain QIA-38T and M. fortuitum ATCC 6841T showed different MALDI-TOF MS profiles. The two strains showed only one prominent
cluster in their MALDI-TOF MS profiles; however, each cluster range was different. In the case of the MALDI-TOF MS profile of strain QIA-38ᵀ, the prominent cluster ranged from \( m/z \) 1088.1 to \( m/z \) 1214.3 and the cluster range seemed to represent non-O-acetylated GPL. However, the cluster in the MALDI-TOF MS profile of \textit{M. fortuitum} ATCC 6841ᵀ ranged from \( m/z \) 1327.5 to \( m/z \) 1399.5. The cluster seemed to represent the triglycosylated GPLs (Fig. 1). The unique MALDI-TOF MS profiles of lipids from strain QIA-38ᵀ suggested that this strain differed from other RGM, including \textit{M. abscessus} subsp. \textit{abscessus}, \textit{M. chelonae}, \textit{M. fortuitum} and \textit{M. smegmatis}.

Fatty acid methyl esters were obtained from the biomass of strain QIA-38ᵀ as described by Minnikin (1988). Extracted samples were separated by GC (model 6890N; Hewlett Packard) and analysed by using the Sherlock Microbial ID System (MIS; MIDI). The predominant fatty acids of strain QIA-38ᵀ were \( C_{16:0} \) (22.43 %) and \( C_{18:1}\omega9c \) (27.83 %). The fatty acid profile comprised unbranched saturated and unsaturated fatty acid esters such as \( C_{16:1}\omega6c \) (9.22 %), \( C_{14:0} \) (6.10 %), \( C_{16:1}\omega7c \) (3.64 %), \( C_{18:0} \) (1.92 %), \( C_{16:1}\omega9c \) (1.59 %) and \( C_{18:2}\omega6,9c \) (1.04 %) and tuberculostearic acid (10-methyl \( C_{18:0} \); 5.89 %) was also detected. Using the MYCO6 method and MI7H10 3.80 library (MIDI), strain QIA-38ᵀ was identified.

![Fig. 1. MALDI-TOF MS analysis of lipids extracted from strain QIA-38ᵀ (a) and \textit{M. fortuitum} ATCC 6841ᵀ (b).](http://ijs.sgmjournals.org)
as belonging to the Mycobacterium avium–Mycobacterium intracellulare–Mycobacterium scrofulaceum complex (MAIS complex) with low similarity index (0.183 Sim Index).

The mycolic acids of strain QIA-38\(^T\), M. fortuitum ATCC 6841\(^T\) and M. smegmatis ATCC 19420\(^T\) were analysed by HPLC as described by Butler et al. (1992). For peak identification, high-molecular-mass standard (RibiImmunoChem) was added. To identify and compare mycolic acid profiles, an HPLC mycobacterium library (available at http://www.MycobacToscana.it) was used. Mycolic acid profiles of M. fortuitum and M. smegmatis showed two late clusters of peaks. However, in the case of strain QIA-38\(^T\), these showed different profiles, one early prominent cluster of peaks and one late weak cluster of peaks (Fig. 2). HPLC analysis showed that strain QIA-38\(^T\) has a unique mycolic acid profile compared with M. fortuitum and M. smegmatis.

The cell wall peptidoglycan was analysed from whole-cell hydrolysates which were treated with 6 M HCl for 18 h at 100 °C. After that, the sample was spotted onto a TLC cellulose plate and resolved using the solvent system of Rhuland et al. (1955). The result showed that cell-wall peptidoglycan of strain QIA-38\(^T\) contained meso-diaminopimelic acid.

For molecular taxonomic study, genomic DNA of strain QIA-38\(^T\) was extracted by the bead-beater-phenol extraction method as previously reported (Kim et al., 2005). The purified genomic DNA was used as a template for PCR amplifications of four independent gene targets, the 16S rRNA, heat-shock protein 65 (hsp65), recombination (recA) and RNA polymerase β-subunit (rpoB) genes. Three genes, 16S rRNA, partial hsp65 and rpoB genes, were amplified as described by Adékambi et al. (2006), Kim et al. (2005), Roth et al. (1998) and Springer et al. (1996). In the case of the recA gene, the target sequence was amplified by using RecG1 (F: 5′-CTSGATATCGCGACATGCTG-3′) and RecR2 (R: 5′-CTSGATATCGCGACATGCTG-3′) and RecR2 (R: 5′-CTSGATATCGCGACATGCTG-3′) and RecR2 (R: 5′-CTSGATATCGCGACATGCTG-3′).
TTGATCTTTCTTCTCGATCTC-3′) primers (Blackwood et al., 2000). Briefly, template genomic DNA (50 ng) and each of the primer sets (total 20 pmol) were added to PCR premix tubes (AccuPower PCR PreMix; Bioneer) and PCR was conducted by subjecting the mixed 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 (BioRad). All the amplified PCR products were purified using a MEGAquick-spin fragment DNA purification kit (Intron Biotechnology) or a QIAquick Gel Extraction kit (Qiagen) for direct sequencing. Sequencing reactions were performed using a PTC-225 Peltier thermal cycler (MJ Research) and ABI PRISM BigDye Terminator Cycle Sequencing kits with Ampli Taq DNA polymerase (FS enzyme; Applied Biosystems) following the manufacturers’ protocols. Nucleotide sequences obtained were compared with the GenBank database using the BLAST program (http://blast.ncbi.nlm.nih.gov/blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

The 16S rRNA (1420 bp), hsp65 (603 bp), recA (542 bp) and rpoB (711 bp) gene sequences of strain QIA-38T and other reference mycobacterial strains, especially RGM, were aligned by using the multiple alignment algorithm in the MEGAalign software as previously described (Kim et al., 1999, 2005; Turenne et al., 2001). Evolutionary distance matrices were generated according to the Cantor & Jukes model (Cantor & Jukes, 1962). Phylogenetic trees were reconstructed from the four target gene and concatenated sequences using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods in MEGA version 4.0 software (Kumar et al., 2008). The 16S rRNA gene sequence of Tsukamurella paurometabola NCTC 10741, and the hsp65, rpoB and recA gene sequences of Mycobacterium tuberculosis H37Rv were retrieved from GenBank and used as outgroups. Reconstructed trees and their topologies were evaluated by bootstrap analysis based on 1000 resamplings (Felsenstein, 1985).

The BLAST analysis result of the 16S rRNA gene sequence (1420 bp) of strain QIA-38T showed the closest match (1406/1414 bp, 99.9% similarity) with Mycobacterium sp. DFTK7 (GenBank accession no. AB663500), which has aromatic hydrocarbon degrading ability (Kaiya et al., 2012). A phylogenetic tree based on 16S rRNA gene sequences showed that strain QIA-38T was grouped with M. smegmatis ATCC 19420T with a high bootstrap value (99) (Fig. 3a). The 16S rRNA gene sequence similarity was compared among other RGM and the result showed that the most closely related strain was M. smegmatis ATCC 19420T (NR_025311, sequence similarity of 98.5%) with 17 nucleotide differences in 1369 bp (Table S1, available in the online Supplementary Material). Also, the sequence polymorphisms of strain QIA-38T and M. smegmatis ATCC 19420T included six nucleotides in hypervariable region A of the 16S rRNA gene sequence; however, in hypervariable region B, only one nucleotide difference was found among strain QIA-38T, M. fortuitum ATCC 6841T, Mycobacterium peregrinum ATCC 14467T, Mycobacterium senegalense ATCC 35796T and Mycobacterium septicum ATCC 700731T. Compared with M. smegmatis ATCC 19420T, the sequence polymorphisms with strain QIA-38T included five nucleotides in hypervariable region B (Fig. 4).

The phylogenetic tree based on partial hsp65 gene sequences (603 bp) of RGM including strain QIA-38T showed that strain QIA-38T was located near the M. smegmatis and M. fortuitum group, however, forming an independent branch (Fig. 3b). As sequence similarities were compared with other RGM, the most closely related strain was M. smegmatis ATCC 19420T (KP017251, sequence similarity of 95.2%) with 28 nucleotide differences in 603 bp (Table S2).

In the case of the partial recA gene sequence (559 bp) based phylogenetic tree, strain QIA-38T was located on an independent branch near the M. abscessus–M. chelonae group (Fig. 3c). As shown by the 16S rRNA gene and hsp65 gene sequences, when the recA gene sequence was compared with those of other RGM, the most closely related strain was M. smegmatis ATCC 19240T (AY458101, sequence similarity of 93.0%) with 36 nucleotide differences in 559 bp (Table S3).

In the case of the partial rpoB gene sequence (711 bp) based phylogenetic tree, strain QIA-38T was separated from the M. abscessus–M. chelonae and M. fortuitum groups as an independent branch (Fig. 3d). When rpoB sequences were compared with those of other RGM, the most closely related strain was M. senegalense CIP 104941T (AY262738, sequence similarity of 88.7%) with 76 nucleotide differences in 711 bp (Table S4).

A phylogenetic tree based on the concatenation (1873 bp) of the three gene sequences [rpoB (711 bp) + hsp65 (603 bp) + recA (559 bp)] was also reconstructed. As shown in the rpoB gene sequence based phylogenetic tree, the concatenated tree showed that strain QIA-38T was separated as an independent branch (Fig. 3e). The concatenated sequence similarity among the RGM showed the most closely related strain was M. fortuitum (sequence similarity of 91.0%) (Table S5).

DNA–DNA hybridization was carried out to examine the DNA relatedness between strain QIA-38T, M. fortuitum ATCC 6841T and M. smegmatis ATCC 19420T. Briefly, extracted genomic DNA of the three strains were blotted onto Hybond-N+ nylon membranes (Amersham Pharmacia Biotech) in three replicates after denaturation using NaOH solution and heating at 80 °C. Each DNA (4 μg) was digested by HaeIII restriction enzyme and its product was used individually as a labelled DNA probe for cross-hybridization. Random-primed DNA labelling with digoxigenin (DIG)-dUTP and detection of hybrids by enzyme immunoassay on nylon membranes were performed using a DIG High Prime DNA Labelling kit (Roche Applied Science) according to the manufacturer’s instructions.
Fig. 3. Phylogenetic relationships of strain QIA-38T among other species of the genus *Mycobacterium* based on the 16S rRNA gene (a), *hsp65* gene (b), *recA* gene (c), *rpoB* gene (d), and concatenated three-gene (*hsp65*, *recA* and *rpoB*; 1873 bp) (e) sequences. Trees were reconstructed using the neighbour-joining method. Bootstrap values were calculated from 1000 replications. Bootstrap values of <50% are not shown. Solid circles indicate that the corresponding groups were supported in the maximum-parsimony trees. *T. paurometabola* and *M. tuberculosis* were used as an outgroup in the 16S rRNA, *hsp65* and *rpoB* trees, respectively. Bars, number of substitutions per nucleotide position.
M. fortuitum and the levels of DNA relatedness between strain QIA-38T and another RGM support that strain QIA-38T is a representative of mycolic acids, and low DNA–DNA relatedness with M. chelonae, M. abscessus, M. tuberculosis, and M. senegalense (Table 1). The DNA–DNA relatedness was always confirmed by reciprocal hybridization. The DNA–DNA hybridization tests were always confirmed by reciprocal hybridization.

The DNA–DNA relatedness between strain QIA-38T and M. fortuitum ATCC 6841T or strain QIA-38T and M. smegmatis ATCC 19420T. The DNA–DNA hybridization tests were always confirmed by reciprocal hybridization.

Taken together, the unique sequences of four independent genes (16S rRNA, hsp65, recA and rpoB) together with unique MALDI-TOF MS profiles of lipids, HPLC profiles of mycolic acids, and low DNA–DNA relatedness with other RGM support that strain QIA-38T is a representative of a novel RGM.

**Description of Mycobacterium anyangense sp. nov.**

Mycobacterium anyangense (an.yang.en’s N.L. neut. adj. anyangense pertaining to Anyang, Republic of Korea, the geographical location of the agency isolating the type strain).

Generally, rod-shaped and acid-fast bacteria with frequently curved bacilli having no spores or filaments are observed by microscopy. The optimal growth temperature is 37 °C. On Middlebrook 7H10 agar medium, smooth and yellow or orange colonies are formed. Positive enzymic activities for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucuronidase, α-glucosidase and β-glucosidase. Negative enzymic activities for lipase (C14), trypsin, α-chymotrypsin, α-galactosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The MALDI-TOF MS profile of total lipids ranges from ~m/z 1088.1 to ~m/z 1214.3. The predominant fatty acids are C15:0 and C18:1ω9c as in the M. avium–M. intracellulare–M. scrofulaceum complex (MAIS complex). HPLC patterns of mycolic acids show one prominent cluster of peaks and one late weak cluster of peaks. Cell-wall peptidoglycan contains meso-diaminopimelic acid. Genetically, unique sequences for the four independent genes 16S rRNA, hsp65, recA and rpoB are found. The type strain, QIA-38T (=JCM 30275T = KCTC 29443T), was isolated from Hanwoo (Bos taurus coreanae) whole blood in Korea.

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