Mycobacterium aquiterrae sp. nov., a rapidly growing bacterium isolated from groundwater

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Abstract

A strain representing a rapidly growing, Gram-stain-positive, aerobic, rod-shaped, non-motile, non-sporulating and non-pigmented species of the genus Mycobacterium, designated strain S-I-6⁷, was isolated from groundwater at Daejeon in Korea. The strain grew at temperatures between 10 and 37 °C (optimal growth at 25 °C), between pH 4.0 and 9.0 (optimal growth at pH 7.0) and at salinities of 0–5% (w/v) NaCl, growing optimally with 2% (w/v) NaCl. Phylogenetic analyses based on multilocus sequence analysis of the 16S rRNA gene, hsp65, rpoB and the 16S–23S internal transcribed spacer indicated that strain S-I-6⁷ belonged to the rapidly growing mycobacteria, being most closely related to Mycobacterium sphagni. On the basis of polyphasic taxonomic analysis, the bacterial strain was distinguished from its phylogenetic neighbours by chemotaxonomic properties and other biochemical characteristics. DNA–DNA relatedness among strain S-I-6⁷ and the closest phylogenetic neighbour strongly support the proposal that this strain represents a novel species within the genus Mycobacterium, for which the name Mycobacterium aquiterrae sp. nov. is proposed. The type strain is S-I-6⁷ (=KACC 17600T =NBRC 109805T =NCAIM B 02535T).

The genus Mycobacterium of the family Mycobacteriaceae, belonging to the suborder Corynebacterineae, was first described by Lehmann and Neumann in 1896 and proposed by Skerman et al. [1] with Mycobacterium tuberculosis as the type species and listed in the Approved Lists of Bacterial Names [1]. At the time of writing, the genus Mycobacterium comprises 185 recognized species and 13 subspecies [2], and they are divided into major groups based on their rate of growth and appearance: slowly growing and rapidly growing, and pigmented and non-pigmented species [3]. Species of the genus Mycobacterium are aerobic, non-motile, mycolic-acid-containing bacteria and they are characterized as acid-fast organisms and contain DNA with a G+C content of 62–72 mol%. Mycobacteria are widespread organisms distributed as free-living and non-pathogenic strains in environments such as water, while many members of the genus have been isolated from clinical specimens and might be pathogenic to humans and animals [4–6]. Strain S-I-6⁷ was isolated from groundwater in Korea, and a polyphasic taxonomic approach was used including multilocus sequence analysis (MLSA) to determine its taxonomic position representing a novel species of the genus Mycobacterium.

Strain S-I-6⁷ was isolated from groundwater at Daejeon in Korea during studies focused on the isolation of oligotrophic bacteria. The strain was isolated by diluting a water sample in sterile distilled water and plating on R2A (Difco) agar, followed by aerobic incubation at 28 °C for 5 days. The pH of the medium was adjusted to pH 7.0 with 1 M NaOH. The strain was subsequently purified three times by plating on R2A medium at 28 °C for 3 days and maintained on the same medium. The strain was stored at −80 °C in this medium without agar and supplemented with 20% (v/v) glycerol solution. In order to characterize strain S-I-6⁷ phenotypically, the isolate was routinely grown aerobically on R2A agar for 3 days at 25 °C and pH 7.0, except where indicated otherwise.

The morphology of the isolate was observed by Gram staining and transmission electron microscopy (CM20; Philips), and motility was observed by phase-contrast microscopy (Eclipse 80i; Nikon), using cells from exponentially growing cultures. Gram staining was performed by the Burke method [7]. The presence of endospores was determined by a specific endospore-staining test, using malachite green (Shaeffer and Fulton endospore stain kit; Sigma). Catalase

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Abbreviations: ITS, 16S-23S internal transcribed spacer; MLSA, multilocus sequence analysis.

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene, hsp65, rpoB and 165–235 ITS sequences of strain S-I-6⁷ are FJ796421, LC126333, LC126334 and LC126335, respectively.

Two supplementary tables and four supplementary figures are available with the online Supplementary Material.
and oxidase activities, nitrate reduction, and hydrolysis of aesculin, casein, DNA, gelatin, hypoxanthine, l-tyrosine, starch, Tween 80, xanthine and urea were tested as recommended by Cowan and Steel [8], Lánya [9] and Smibert and Krieg [10] with substrate concentrations of 0.01 % (w/v). Substrate utilization profiles and acid production from carbohydrates were determined as described by Leifson [11] in minimal medium supplemented with carbon sources corresponding to API50CH (bioMérieux) gallery ingredients. All compounds were sterilized by filtration and were added to the medium. Cell suspension was performed in sterile distilled water. Additional biochemical characteristics were determined using API 20NE (bioMérieux) at 25°C. The remaining biochemical and nutritional tests were conducted using traditional methods and followed the recommended minimal standards for describing novel taxa of aerobic, endospore-forming bacteria [12]. Enzyme activities were tested using the API ZYM kit system according to the instructions of the manufacturer (bioMérieux).

To determine the optimal temperature and pH for growth of strain S-I-6T, broth cultures in R2A broth were incubated at 4, 10, 15, 20, 22, 25, 28, 30, 35, 37, 45, 50 and 55°C and at pH 3–11 (at intervals of 0.5 pH units). Media at pH <6, 6–9 and >9 were obtained by using sodium acetate/acetate acid, Tris/HCl and glycine/NaOH buffers, respectively. Growth in the presence of 0, 0.1, 0.5, 1.0, 2.0, 3.0, 5.0, 7.0 and 10.0 % NaCl (w/v) was tested in 10–2 nutrient broth (NB; BD) medium at pH 7.0. Growth was monitored by turbidity at OD600 by using a spectroscopic method (model UV-1650PC; Shimadzu). Anaerobic growth was determined by incubation in the BBL GasPak Anaerobic System (BD) for 3 days at 25°C on R2A.

Antibiotic susceptibility testing was performed on cation-supplemented Mueller–Hinton agar by the disc diffusion method, according to the recommendations of the Clinical and Laboratory Standards Institute for rapidly growing mycobacteria [13], seeding with a bacterial suspension containing 1.5x106 c.f.u. ml–1 and using discs (Advantec) impregnated with various antibiotics. Susceptibility to antibiotics was tested on R2A plates using antibiotic discs containing the following (µg per disc unless otherwise stated): teicoplanin (30), amikacin (30), ampicillin (20), lincomycin (15), nalidixic acid (30), kanamycin (30), gentamicin (10), streptomycin (10), neomycin (30), penicillin (10 U), vancomycin (30), erythromycin (15), oleandomycin (15), amoxicillin (10), spiramycin (100), rifampicin (10), polymixin B (100 U), nystatin (50), bacitracin (10 U), tetracycline (30), cycloheximide (30), roxithromycin (15), spingomyelin (30), apramycin (30), salinomycin (30), hygromycin (30), capreomycin (30), sisomycin (10), amphotericin (2), gramicidin S (30), phosphomycin (30) and chloramphenicol (30).

Strain S-I-6T was susceptible to teicoplanin, amikacin, ampicillin, gentamicin, kanamycin, neomycin, vancomycin, erythromycin, spiramycin, rifampicin, tetracycline, apramycin, hygromycin, sisomycin and chloramphenicol, but resistant to lincomycin, nalidixic acid, streptomycin, polymixin B, penicillin, oleandomycin, amoxicillin, nystatin, bacitracin, cycloheximide, roxithromycin, spingomyelin, salinomycin, capreomycin, amphotericin, gramicidin S and phosphomycin.

Genomic DNA from strain S-I-6T was extracted using a commercial genomic DNA extraction kit (Solgent). The 16S rRNA gene was amplified by PCR with the forward primer 27F and the reverse primer 1492R [14]. Amplification of the hsp65 and rpoB genes and the ITS region (16S–23S internal transcribed spacer) was performed using methods described by Devulder et al. [15]. Direct sequence determination of the PCR-amplified DNA was carried out using an automated DNA sequencer (ABI 3730XL; Applied Biosystems). The full sequences of the genes were compiled using SeqMan software (DNASTAR). The 16S rRNA, hsp65 and rpoB gene sequences were aligned with published sequences of closely related bacteria with CLUSTAL W 2.0 software [16]. Gaps at the 5' and 3' ends of the alignment were omitted in further analyses.

To determine the phylogenetic position of strain S-I-6T among the closely related mycobacteria, MLSA of a concatenated gene sequence (2251 bp) using 16S rRNA (1429 bp), hsp65 (424 bp) and rpoB (398 bp) gene sequences was performed as described by Devulder et al. [15]. The ITS gene sequence was not included in the analysis as the reference sequences available were too few to reconstruct reliable trees. Phylogenetic trees were reconstructed by using three different methods: the neighbour-joining [17], maximum-likelihood [18] and maximum-parsimony [19] algorithms within the MEGA6 program [20]. Evolutionary distance matrices for the neighbour-joining method were calculated using the algorithm of Kimura’s two-parameter model [21]. To evaluate the stability of the phylogenetic tree, a bootstrap analysis (1000 replications) was performed [22]. The 16S rRNA, hsp65 and rpoB gene sequences used for phylogenetic
comparisons were obtained from the GenBank database and their strain designations and GenBank accession numbers are shown in Figs 1, 2, S2 and S3. To determine genomic relatedness, DNA–DNA hybridization was performed using the modified method of Ezaki et al. [23]. Probe labelling for DNA–DNA hybridization was conducted by using the non-radioactive DIG-High Prime system (Roche); hybridized DNA was visualized using the DIG luminescent detection kit (Roche), and the level of DNA–DNA relatedness was quantified by using a densitometer (Bio-Rad). Isolation of DNA [24] and determination of the DNA G+C content were performed by HPLC (SPD-10AV; Shimadzu), as described by Mesbah et al. [25].

The almost-complete 16S rRNA gene sequence (1438 bp), partial hsp65 (444 bp), partial rpoB (441 bp) and entire ITS sequence (575 bp) of strain S-I-6T were determined and used for initial BLAST searches of the GenBank database and for phylogenetic analysis. The 16S rRNA gene, hsp65, rpoB and ITS sequences of related taxa were obtained from the GenBank database and EzBioCloud server, and the identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene, hsp65, rpoB and ITS sequence similarity were achieved by using the EzBioCloud server (http://www.ezbiocloud.net) [26]. According to pairwise 16S rRNA, hsp65 and rpoB gene sequence comparisons, strain S-I-6T was shown to have the closest sequence similarity to Mycobacterium sphagni DSM 44076T (98.4, 95.3 and 97.5 %, respectively), with sequence similarity ranging from 98.1 to 95.7, 94.6 to 89.9 and 97.4 to 93.5 %, respectively, with members of the other species of the genus Mycobacterium. The ITS sequence similarity was below 90 %. Forty-nine species in the genus Mycobacterium showed high sequence similarities (>97 %) in 16S rRNA
gene sequences, and sequence similarity of closely related members of the genus ranged from 98.4 to 97.5% (Fig. 2).

Phylogenetic analysis of the hsp65 gene sequence showed that strain S-I-6T was located in a clade with *M. sphagni* DSM 44076T [27] with 87% bootstrap value (Fig. S2), while in the rpoB tree S-I-6T was located in a clade adjacent to *Mycobacterium aromaticivorans* JCM 16368T [28] and *Mycobacterium rhodesiae* CIP 106806T [29] with a very low bootstrap value (<50%) being within the cluster containing *M. sphagni* DSM 44076T (Fig. S3). But in the phylogenetic tree based on 16S rRNA gene sequences, strain S-I-6T was not located in the same clade containing *M. sphagni* DSM 44076T (Fig. 2). However, the concatenated phylogenetic tree using three genes confirmed that strain S-I-6T was located in a clade with *M. sphagni* DSM 44076T with 84% bootstrap value, and located close to *M. aromaticivorans* JCM 16368T, *M. rhodesiae* CIP 106806T, *Mycobacterium aichiense* 49005T and *Mycobacterium crocinum* czh-42T, showing similar tree topologies based on hsp65 and rpoB gene sequences (Fig. 1). Following the determination of a reliable phylogenetic position of strain S-I-6T, the following strains were subjected to DNA–DNA hybridization resulting in DNA–DNA relatedness values between strain S-I-6T and *M. sphagni* DSM 44076T, *M. aromaticivorans* JCM 16368T, *M. rhodesiae* CIP 106806T, *M. aichiense* 49005T and *M. crocinum* JCM 16369T of 43±4, 37±2, 35±7, 32±7 and 31±8%, respectively (Table S1), and these values were significantly lower than 70%, the threshold value recommended for the assignment of genomic species [30]. The G+C content of the DNA of strain S-I-6T was 64.7 mol%, which is in the range (62.0–67.0 mol%) for members of the genus *Mycobacterium*.

For analysis of fatty acids, strain S-I-6T was cultured on TSA at 25° C for 3 days and cells were obtained at the late-exponential growth phase, similarly for reference strains on their growth media. Cellular fatty acids were extracted and analysed by gas chromatography (6890 N; Agilent Technologies) according to the standard protocol of the Sherlock Microbial Identification System (version 4.5; database TSBA40 4.10; MIDI). For the analysis of cell-wall mycolic acids, quinones and polar lipids, cells were harvested in the late-exponential phase and freeze-dried. For the
identification of the mycolic acids in the cell wall, one-dimensional TLC was carried out following the standard procedure described by Minnikin et al. [31]. Isoprenoid quinones were extracted and purified as described by Collins [32]. Extracts were analysed by HPLC (SPD-10AV; Shimadzu) using an Cosmosil C18-AR column (150×4.6 mm, internal diameter 5 µm) at 270 nm. The mobile phase was methanol/isopropanol (2:1, v/v), and the flow rate was 1.0 ml min⁻¹ and the run time was 25 min. The injection volume was 5 µl, and the chromatographic column was controlled at 30 °C. For polar lipid analysis, the cellular lipids were extracted, washed and hydrolysed as described by Minnikin et al. [33]. The total lipids were separated on silica-gel plates by two-dimensional TLC with a solvent system composed of chloroform/methanol/water (65:25:4, by vol.) in the first direction and chloroform/methanol/acetic acid/water (80:15:12:4, by vol.) in the second direction. To detect spots and their colour reaction, 5 % ethanolic molybdatophosphoric acid, Dittmer’s reagent, ninhydrin solution, Dragendorff’s reagent and α-naphthol reagent were used for all lipids, phospholipids, aminolipids, choline lipids and glycolipids, respectively.

Fig. 2. Rooted neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain S-I-6T and related bacteria in the genus Mycobacterium. Bootstrap values, expressed as a percentage of 1000 replications, are given at branching points when >50 %. Filled circles, open circles and open squares at nodes indicate generic branches that were also recovered using the maximum-likelihood and maximum-parsimony algorithms, the maximum-likelihood algorithm, and the maximum-parsimony algorithm, respectively. Nocardia abscessus IMMIB D-1592T was used as an outgroup. Bar, 0.005 substitutions per nucleotide position.
Major fatty acids in strain S-I-6T were C17:1ω7c (22.8 %), C16:0 (20.1 %), summed feature 3 (15.4 %; C16:1ω7c and/or iso-C15:0 2-OH) and C18:1ω9c (11.5 %). Moderate amounts of C14:0 (9.6 %), 10-methyl C18:0 and 10-methyl C16:0 were also present (Table S2). These are common characteristic features of members of the genus Mycobacterium. The presence of 10-methyl C16:0, 10-methyl C18:0 and C20:4 ω6,9,12,15c, and the absence of 10-methyl C19:0 and C16:1ω9c distinguished strain S-I-6T from closely related type strains of species of the genus Mycobacterium (Table S2). The major menaquinone of isolate S-I-6T was MK-9(H2). Polar lipids of S-I-6T comprised diphasphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside, an aminoglycolipid, two unknown aminolipids and two unknown lipids (Fig. S4).

Strain S-I-6T had distinct sequence characteristics of three independent genes (16S rRNA, hsp65, rpoB) and the 16S–23S ITS region belonging to a rapidly growing mycobacterial species within the M. sphagni subclade, and shared similar chemotaxonomic characteristics within the members of the genus Mycobacterium in terms of DNA G+C content, mycolic acid, polar lipids and major fatty acids. However, strain S-I-6T could be distinguished from other closely related species by some chemotaxonomic and phenotypic features, such as assimilation of D-sorbitol, no acid production from D-mannose, production of the enzyme naphthol-AS-BI-phosphohydrolase, and the presence of 10-methyl C16:0, 10-methyl C18:0 and C20:4 ω6,9,12,15c, and the absence of 10-methyl C19:0 and C16:1ω9c (Tables 1 and S2). Therefore, on the basis of the results of this polyphasic taxonomic study, we propose that the newly isolated strain represents a novel species of the genus Mycobacterium, for which the name Mycobacterium aquiterrae sp. nov. is proposed, with the type strain S-I-6T.

**DESCRIPTION OF MYCOBACTERIUM AQUITERRAE SP. NOV.**


Cells are Gram-stain-positive, aerobic, non-motile, non-spore-forming rods and 0.4–0.5 × 1.0–1.2 μm in size. Colonies are circular, convex, entire, light-yellow in colour and 0.1–0.5 mm in diameter on R2A agar after 3 days of incubation at 25 °C. Growth also occurs on NA, LB, TSA, ISP2, ISP4, PDA, Bennet’s agar, Mueller–Hinton agar and Middlebrook 7H10. Growth at 10–37 °C (optimally at 25 °C), pH 4.0–9.0 (optimally at pH 7.0) and with 0–5 % (w/v) NaCl, with optimal growth at 2 % (w/v) NaCl. Oxidase-positive and catalase-negative. Nitrate is reduced to nitrite, but not to nitrogen. Aesculin and starch are hydrolysed but gelatin, urea, DNA, xanthine, hypoxanthine and l-tyrosine are not. Utilizes glycerol, D-fructose, D-mannose, D-rhamnose, dulcitol, inositol, D-mannitol, D-ribose, D-sorbitol, arbutin, aesculin, salicin, cellobiose, melibiose, sucrose, trehalose, raffinose, starch, glycogen, turanose, D-fucose and gluconate, but does not utilize erythritol, D-arabinose, L-arabinose, D-xylene, L-xylene, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-glucose, L-sorbose, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, N-acetylgalactosamine, amygdalin, maltose, lactose, inulin, melezitose, xyitol, gentiobiose, D-lyxose, D-tagatose, L-fucose, D-arabitol, L-arabitol, 2-ketogluconate, 5-ketogluconate, citrate or succinate. Acid is produced from erythritol, D-ribose, D-glucose, D-fructose, N-acetylgalactosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, sucrose, trehalose, starch, glycogen, gentiobiose and turanose, but not from glycerol, D-arabinose, L-arabinose, D-xylene, L-xylene, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, melibiose, inulin, melezitose, raffinose, xyitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, glucosate, 2-ketogluconate or 5-ketogluconate. Enzyme activity is observed for esterase (C4), esterase lipase (C8), leucine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase and N-acetyl-β-glucosaminidase, but not for alkaline phosphatase, lipase (C14), valine arylamidase, α-chymotrypsin, β-galactosidase, β-glucuronidase, α- or β-glucosidase, α-mannosidase or α-fucosidase (API ZYM). Possesses mycolic acid, and the major fatty acids are C17:1ω7c, C16:0 summed feature 3 (C16:0ω7c and/or iso-C15:0 2-OH) and C18:1ω9c. The predominant isoprenoid quinone is MK-9 (H2). Polar lipids are diphasphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannoside, an aminoglycolipid, two unknown aminolipids and two unknown lipids. Phylogenetic analyses show that it belongs to the rapidly growing mycobacteria and is closely related to *M. sphagni* DSM 44076T.

The type strain, S-I-6T (=KACC 17600T=NBRC 109805T =NCAIM B 02355T), was isolated from groundwater at Daejeon in Korea. The DNA G+C content of the type strain is 64.7 mol%.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**