Brevibacterium ammoniilyticum sp. nov., an ammonia-degrading bacterium isolated from sludge of a wastewater treatment plant

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A Gram-stain-positive, non-motile, chemo-organotrophic, mesophilic, aerobic bacterium, designated A1T, was isolated from sludge of a wastewater treatment plant. Strain A1T showed good ability to degrade ammonia and grew well on media amended with methanol and ammonia. Strain A1T grew with 0–11 % (w/v) NaCl, at 20–42 °C, but not <15 or >45 °C and at pH 6–10 (optimum pH 8.0–9.0). The isolate was catalase-positive and oxidase-negative. The DNA G+C content was 70.7 mol%. A comparative analysis of 16S rRNA gene sequences revealed that strain A1T formed a distinct phyletic lineage in the genus Brevibacterium and showed high sequence similarity with Brevibacterium casei NCDO 2048T (96.9 %), Brevibacterium celere KMM 3637T (96.9 %) and Brevibacterium sanguinis CF63T (96.4 %). DNA–DNA hybridization revealed <43 % DNA–DNA relatedness between the isolate and its closest phylogenetic relatives. The affiliation of strain A1T with the genus Brevibacterium was supported by the chemotaxonomic data: predominant quinone menaquinone MK-7(H2); polar lipid profile containing diphasphatidylglycerol, phosphatidylglycerol and an unidentified glycolipid; characteristic cell-wall diamino acid meso-diaminopimelic acid; whole-cell sugars galactose, xylose and ribose; absence of mycolic acids; and major fatty acids iso-C15 : 0, anteiso-C15 : 0 and anteiso-C17 : 0. The results of physiological and biochemical tests allowed phenotypic differentiation of strain A1T from members of the genus Brevibacterium. On the basis of the results in this study, a novel species, Brevibacterium ammoniilyticum sp. nov., is proposed. The type strain is A1T (=KEMC 41-098T =JCM 17537T =KACC 15558T).

The genus Brevibacterium was first reported by Breed (1953), with Brevibacterium linens as the type species, which was isolated from surface-ripened cheese and described as Gram-positive, non-branching, non-spore-forming, short rods with a high G+C content. Collins (2006) stated that the genus Brevibacterium has meso-diaminopimelic acid and respiratory menaquinones MK-8(H2) in the A1γ-type peptidoglycan. In recent years, in addition to B. linens, the following species in the genus Brevibacterium sensu stricto have been reported: (Ivanova et al., 2004), (Wauters et al., 2004), (Pascual & Collins, 1999), B. marinum (Lee, 2008), B. permense (Gavris et al., 2004), (Bhadra et al., 2008) and (Heyrman et al., 2004). In many cases, members of the genus Brevibacterium have been isolated from dairy products, including cheese (Collins et al., 1983), and from human skin (Collins et al., 1983; Wauters et al., 2004; Roux & Raoult, 2009). In some cases, Brevibacterium strains have been recognized in other environments such as high salinity (Bhadra et al., 2008).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain A1T is JF937067.

A supplementary table and a supplementary figure are available with the online version of this paper.
2008; Lee 2008), poultry (Pascual & Collins 1999), a
damaged mural painting (Heyrman et al., 2004) and a
degraded thallus of a brown alga (Ivanova et al., 2004).
Members of the genus have been reported not only in
relation to cheese production, but also in patents from
biotechnological industry, including those for production
of antibiotics and wastewater treatment.

Strain A1T was isolated from the sludge of a wastewater
treatment plant near a farm area at Gwangju-si, Kyeonggi-
do, South Korea (37° 21' 31.24" N 127° 14' 24.50" E; altitude 49 m). For isolation, 1 % (v/v) sludge was
inoculated into ATCC medium 412 (containing l−1: 5.0 g
NH4Cl, 2.0 g KH2PO4, 0.5 g NaCl, 0.2 g MgSO4, 0.002 g
FeSO4, 0.002 g MnCl2, 0.2 g , yeast extract 0.2 g; pH 7.0;
20 ml methanol added after autoclaving). Enrichment
culture was performed aerobically with shaking (150 r.p.m.)
at 28 °C for 24 h. Single colonies of strain A1T along with
other 119 strains were purified by transferring them onto
new plates and the purified colonies were tentatively
identified by partial 16S rRNA gene sequencing. The isolate
was routinely cultured on ATCC medium 412 at 28 °C and
preserved as a suspension in Luria–Bertani broth (LB; Difco)
with 20 % (w/v) glycerol at −70 °C. Strain A1T was Gram-
istain-positive, non-motile and aerobic and formed white
greyish colonies on ATCC medium 412. Strain A1T was
subjected to a polyphasic taxonomic investigation and
the results indicated it represents a novel Brevibacterium
species.

Strain A1T was able to degrade ammonia nitrogen and
methanol; batch culture experiments showed that 55 mg
ammonia l−1 and 1875 mg methanol l−1 were degraded in
12 h (initial concentrations l−1: 80 mg ammonia and
2500 mg methanol).

Total genomic DNA was extracted by a modification of the
method of Marmur (1961). Cells were lysed with 10 mg
lysozyme ml−1 and 50 U achromopeptidase ml−1 (Ezaki &
Suzuki, 1982) to avoid the difficulty of extraction of DNA
from Gram-positive bacteria. The 16S rRNA gene

Fig. 1. Phylogenetic position of B. ammoniilyticum A1T based on 16S rRNA gene sequence analysis. The tree was
reconstructed using the neighbour-joining method (Saitou & Nei, 1987) in the program MEGA5 (Tamura et al., 2011) with
bootstrap values based on 1000 replications (Felsenstein, 1985). The evolutionary distances were calculated using the Kimura
two-parameter model (Kimura, 1983). Bar, 0.01 substitutions per nucleotide position.
amplified by PCR with primers 27F and 1492R as described by Frank et al. (2008). Sequencing was performed by using a Big Dye Terminator cycle sequencing kit v.3.1 (Applied BioSystems). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system at Macrogen, Seoul, Korea. A nearly complete sequence of the 16S rRNA gene (1432 nt) was obtained and compiled with SeqMan (DNASTAR). 16S rRNA gene sequences of related taxa were obtained from GenBank and edited using BioEdit (Hall, 1999). Multiple alignments were performed with CLUSTAL X (Thompson et al., 1997). The evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood methods in MEGA5 (Tamura et al., 2011) with bootstrap values based on 1000 replications (Felsenstein, 1985).

Neighbour-joining phylogenetic analysis based on 16S rRNA gene sequences revealed that strain A1T formed a distinct lineage in the genus Brevibacterium (Fig. 1). The highest sequence similarities were observed with B. casei NCDO 2048\textsuperscript{T} (96.9 %), KMM 3637\textsuperscript{T} (96.9 %), CF63\textsuperscript{T} (96.4 %) and NCDO 739\textsuperscript{T} (96.4 %). Sequence similarity with other members of the genus Brevibacterium was <96 %. In the neighbour-joining phylogenetic tree (Fig. 1), strain A1T clustered with B. casei NCDO 2048\textsuperscript{T} with a bootstrap value of 83 %, and this cluster joined the cluster comprising species of Brevibacterium along with B. antiquum VKM Ac-2118\textsuperscript{T}. These findings were supported by the maximum-parsimony analysis.

The reference strains B. linens KACC 14346\textsuperscript{T}, B. casei KCTC 3082\textsuperscript{T}, KCTC 3083\textsuperscript{T}, B. epidermidis KCTC 3090\textsuperscript{T}, B. marinus KCTC 19221\textsuperscript{T}, JCM 2590\textsuperscript{T}, JCM 11680\textsuperscript{T}, B. permense JCM 13318\textsuperscript{T}, JCM 13319\textsuperscript{T}, JCM 13386\textsuperscript{T}, JCM 13521\textsuperscript{T} and JCM 21798\textsuperscript{T} were obtained from the relevant culture collections. All experiments on the reference strains and strain A1T were performed with the same methods and conditions. All strains were maintained and cultivated on LB media at pH 7.0 aerobically, unless otherwise mentioned.

The cellular morphology of strain A1T was determined by phase-contrast microscopy and transmission electron microscopy. Gram-stained cells (Cappuccino & Sherman 2010) were examined with a BX 51 phase-contrast microscope (Olympus, Japan) at magnification ×1000. Strain A1T was grown on LB agar for 1, 2 and 3 days and single colonies at each time interval were suspended and stained with 1 % (w/v) phosphotungstic acid (Hayat & Miller 1990; Roux & Raoult 2009). Cell morphology was examined on a JEM 1010 transmission electron microscope (Jeol, Japan) at an operating voltage of 60–80 kV (Fig. 2). Motility testing was performed by stabbing the centre of tube filled with 0.4 % LB agar (Brown, 2008). Growth and colony morphology were examined on complex agars such as trypticase soy agar (TSA; Fgvo), nutrient agar (NA; Difco), LB agar and Columbia blood agar base (Scharlau) with 5 % sheep blood. Growth at pH 3–12 (in increments of one pH unit), at 5, 10, 15, 20, 25, 37 and 42 °C and with 0–15 % (w/v) NaCl (in increments of 1 %) was determined at 28 °C in LB medium (1 % inoculum, OD\textsubscript{600}=1.0) for 72 h aerobically. Results were evaluated as positive if OD\textsubscript{600} increased by more than 0.300. Biochemical characteristics were determined using the API CORYNE, API ZYM, API 50 CH/E, API 20 E and ID 32 GN systems (bioMérieux) as described in the manufacturer’s instructions. Catalase was determined with 3 % H\textsubscript{2}O\textsubscript{2} and oxidase was assayed by applying cells to moistened discs impregnated with dimethyl-p-phenylenediamine (bioMérieux). Hydrolysis of casein, starch and Tween 80 (Atlas, 1993) was also investigated; reactions were read after 5 days. Fermentation and hydrogen sulfide production were examined using TSI slants (Cappuccino & Sherman 2010). The phenotypic
Table 1. Differential characteristics of strain A1\textsuperscript{T} and closely related members of the genus Brevibacterium

Strains: 1, *Brevibacterium ammoniilyticum* sp. nov. A1\textsuperscript{T}; 2, *B. linens* KACC 14346\textsuperscript{T}; 3, *B. casei* KCTC 3082\textsuperscript{T}; 4, KCTC 3083\textsuperscript{T}; 5, *B. epidermidis* KCTC 3090\textsuperscript{T}; 6, *B. marinum* KCTC 19221\textsuperscript{T}; 7, JCM 2590\textsuperscript{T}; 8, *B. avium* JCM 11680\textsuperscript{T}; 9, JCM 13319\textsuperscript{T}; 10, *B. permense* JCM 13318\textsuperscript{T}; 11, JCM 13319\textsuperscript{T}; 12, *B. sanguinis* JCM 13386\textsuperscript{T}; 13, *B. celere* JCM 13521\textsuperscript{T}; 13, *B. oceani* JCM 21798\textsuperscript{T}. All data were taken from this study. All strains are Gram-positive, non-motile, aerobic, non-spore-forming, catalase-positive rods.

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<td>BI</td>
<td>YI</td>
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<td>YO</td>
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<td>Glycogen</td>
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characteristics of strain A1T are given in the species description and those that are differentiated strain A1T from its closest phylogenetic relatives are listed in Table 1.

For cellular fatty acid analysis, strain A1T and the reference strains were grown on TSA for 48 h at 30°C. Two loopfuls of bacterial mass were collected from fourth quadrant of the plates (according to the MIDI technical note) and subjected to saponification, methylation and extraction using the methods of Kuykendall et al. (1988). The fatty acid methyl esters were separated using the Sherlock Microbial Identification System (TSBA version 6.0; MIDI), analysed by GC (model 6890; Hewlett Packard) and identified using the Microbial Identification software package (Sasser, 1990). The fatty acid composition of strain A1T was characterized by predominant amounts of anteiso-C15:0 (46.8%), anteiso-C17:0 (28.1%) and iso-C15:0 (17.5%), which have been also found previously in its close relatives. The fatty acid composition of strain A1T is compared with the reference strains in Table 2. Gruner et al. (1993) proved that anteiso-C15:0 and anteiso-C17:0 were the major fatty acids in B. casei NCDO 2048T, B. linens DSM 20425T and B. epidermidis NCDO 2286T.

For chemotaxonomic characterization, cells were grown in LB medium for 3 days and freeze dried. Polar lipids were extracted and examined by two-dimensional TLC (Minnikin et al., 1977). The polar lipid profile of strain A1T contained predominant amounts of diphosphatidylglycerol, followed by phosphatidylglycerol and a glycolipid, smaller amounts of two aminolipids and minor amounts of three unknown phospholipids (Fig. S1, available in IJSEM Online). Collins (2006) and Jones & Keddie (1986) described diphosphatidylglycerol, phosphatidylglycerol and dimannosyldiacylglycerol as the major polar lipids of the genus Brevibacterium. Kämpfer et al. (2010) proved that diphosphatidylglycerol, phosphatidylglycerol and glycolipid were major polar lipids of B. sandarakinum 01-Je-003T.

Isoprenoid quinones were extracted from freeze-dried cells of strain A1T, B. linens KACC 14346T and B. casei KCTC.
Table 2. Fatty acid compositions of strain A1T and closely related members of the genus Brevibacterium

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<tr>
<th>Fatty acid</th>
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<tr>
<td>C16:0</td>
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<td>tr</td>
<td>1.1</td>
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<td>iso-C14:0</td>
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<tr>
<td>iso-C15:0</td>
<td>17.5</td>
<td>9.2</td>
<td>14.4</td>
<td>8.2</td>
<td>9.7</td>
<td>17.0</td>
<td>8.3</td>
<td>11.0</td>
<td>16.4</td>
<td>8.4</td>
<td>6.1</td>
<td>8.9</td>
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<tr>
<td>iso-C16:0</td>
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<td>4.5</td>
<td>3.6</td>
<td>2.2</td>
<td>3.3</td>
<td>6.0</td>
<td>tr</td>
<td>4.2</td>
<td>3.7</td>
<td>4.9</td>
<td>4.6</td>
<td>5.0</td>
<td>2.5</td>
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<tr>
<td>iso-C17:0</td>
<td>3.1</td>
<td>3.5</td>
<td>5.0</td>
<td>1.7</td>
<td>2.7</td>
<td>6.5</td>
<td>6.3</td>
<td>3.6</td>
<td>4.0</td>
<td>2.9</td>
<td>2.5</td>
<td>3.8</td>
<td>3.4</td>
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<tr>
<td>anteiso-C15:0</td>
<td>46.8</td>
<td>45.3</td>
<td>37.4</td>
<td>57.7</td>
<td>44.3</td>
<td>31.9</td>
<td>37.4</td>
<td>41.0</td>
<td>42.8</td>
<td>40.5</td>
<td>40.5</td>
<td>35.5</td>
<td>43.0</td>
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<tr>
<td>anteiso-C17:0</td>
<td>28.1</td>
<td>36.1</td>
<td>38.7</td>
<td>27.5</td>
<td>38.8</td>
<td>38.1</td>
<td>13.7</td>
<td>39.4</td>
<td>31.0</td>
<td>42.3</td>
<td>44.7</td>
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<td>anteiso-C17:1 A</td>
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3082T with chloroform/methanol (2:1, v/v), evaporated under vacuum and extracted in n-hexane/water (1:1, v/v). The crude n-hexane quinone solution was then purified using silica Sep-Pak Vac cartridges (Waters) and subsequently analysed by HPLC (MD-2015 plus; JASCO, Japan), as described elsewhere (Hiraishi et al., 1996). Strain A1T contained MK-7(H2) as the predominant menaquinone, which is similar to B. linens KACC 14346T and B. casei KCTC 3082T (Collins et al., 1979, 1980, 1983). Strain A1T also contained a minor amount of MK-8(H2), and Gavrish et al. (2004) described that MK-7 and MK-8(H2) are the predominant menaquinones of the genus Brevibacterium.

The cell-wall peptidoglycan was analysed as described by Schleifer & Kandler (1972) using TLC on cellulose sheets instead of paper chromatography. Absence of mycolic acids was demonstrated according to Minnikin et al. (1975) using silica gel TLC. The whole-cell sugars contained galactose, xylose and ribose (Collins, 2006) and the characteristic cell-wall diamino acid was l-glutamic acid, L-alanine and minor amounts of L-aspatic acid.

For analysis of DNA G+C content, genomic DNA was extracted as mentioned above and enzymically degraded into nucleosides. The nucleosides were analysed using HPLC as described elsewhere (Tamaoka & Komagata, 1984; Mesbah et al., 1989). The DNA G+C content of strain A1T was 70.7 mol%.

DNA–DNA hybridization experiments were performed fluorometrically by the method of Ezaki et al. (1989), using photobiotin-labelled DNA probes of strain A1T, KCTC 3082T and JCM 13521T with micro-dilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values were quoted as DNA–DNA relatedness. As a probe, strain A1T showed relatively low DNA–DNA relatedness with B. casei KCTC 3082T(43 ± 4 %), JCM 13521T(42 ± 4 %), B. picturae JCM 13319T (46 ± 3 %), B. sanguinis JCM 13386T (42 ± 6 %), B. aurantiacus JCM 2590T(13 ± 1 %), B. linens KACC 14346T (10 ± 1 %), B. oceani JCM 21798T (8 ± 0 %), KCTC 3083T(8 ± 1 %), B. avium JCM 11680T (8 ± 1 %), B. epidermidis KCTC 3090T (5 ± 0 %), B. marinus KCTC 19221T (3 ± 1 %) and B. permense JCM 13318T (3 ± 0 %). The results of the reciprocal reactions, using the probes of B. casei KCTC 3082T and JCM 13521T, also exhibited low relatedness with the isolate (Table S1). These results indicate that strain A1T is not related at the species level to any recognized member of the genus Brevibacterium (Wayne et al., 1987).

Strain A1T was a Gram-positive, non-motile, chemo-organotrophic, mesophilic, aerobic bacterium. Menaquinone MK-7(H2) was the major quinone. The polar lipid profile contained major amounts of diphosphatidylglycerol, phosphatidylglycerol and a glycolipid. The cell-wall peptidoglycan contained meso-diaminopimelamic acid as the main diamino acid and was of the A1γ type. The major fatty acids iso-C15:0, anteiso-C15:0 and anteiso-C17:0 supported the affiliation of strain A1T with the genus Brevibacterium. Strain A1T could be distinguished from members of the genus by the production of α-galactosidase, α-mannosidase and valine arylamidase and the utilization of mannitol and glycogen. The DNA–DNA relatedness results also support the phenotypic, chemotaxonomic and phylogenetic findings that strain A1T represents a novel species within the genus Brevibacterium, for which the name Brevibacterium ammoniiyticum sp. nov. is proposed.

Description of Brevibacterium ammoniiyticum sp. nov.

Brevibacterium ammoniiyticum [am.mo.ni.i.ly’ti.cum. N.L. n. ammonium ammonia; N.L. neut. adj. lyticum (from Gr.
Brevibacterium ammoniiyticum sp. nov.

neut. adj. latitron) able to loose, able to dissolve; N.L. neut. adj. ammoniiyticum ammonia-degrading, to reflect the activity of the bacterium.

Cells are Gram-stain-positive, non-motile, coryneform, aerobic and non-spore-forming. A rod–coccus cycle was observed: rods (0.45–0.50 μm wide and 0.8–1.2 μm long) in exponential phase (1–2 days); cocci (0.45–0.50 μm wide and 0.55–0.65 μm long) in stationary phase (>3 days). On complex medium, colonies are snowball-like, circular, convex with entire margins and greyish white (0.8–1.2 mm in diameter). On Colombia blood agar, colonies are circular, convex with entire margins and creamy white (0.8–1.2 mm in diameter). Grows with 0–11 % (w/v) NaCl, at 20–42 °C, but <15 or >45 °C, and at pH 6–10 (optimum pH 8.0–9.0). Catalase-positive and oxidase-negative. Grows on ATCC medium 412. With API CORYNE, nitrate is reduced to nitrite, gelatin and aesculin are hydrolysed, alkaline phosphatase, β-galactosidase, α-glucosidase and pyrrolidonyl arylamidase are positive and amygdalin, fructose, galactose, glucose, glycerin, mannitol, mannose, maltose, rhamnose, ribose and sorbitol are utilized, but N-acetyl-β-glucosaminidase, β-glucuronidase, pyrazinamidase and urease are negative and lactose, sucrose and xylose are not utilized. With API ZYM, acid phosphatase, cystine arylamidase, esterase (C4), esterase lipase (C8), α-galactosidase, phosphomimidase and valine arylamidase are positive and β-galactosidase and α-glucosidase are weakly positive, but alkaline phosphatase, chymotrypsin, α-fucosidase, β-glucosidase, leucine arylamidase, lipase (C14), trypsin and N-acetyl-β-glucosaminidase are negative. With ID 32GN, N-acetyl-glucosamine, L-alanine, L-fucose, D-glucose, glycogen, 3-hydroxybutyric acid, lactic acid, L-proline, propionic acid, L-rhamnose, D-ribose, L-serine, sodium acetate, sodium malonate, suberic acid, trisodium citrate, 3-hydroxybenzoic acid and valeric acid were assimilated and L-histidine, 4-hydroxybenzoic acid, trisodium citrate, 3-hydroxybenzoic acid and valeric acid and maltose were weakly assimilated, but L-arabinose, L-fucose, D-glucose, L-mannitol, D-mannose, maltose, D-methyl ribose, salicin, D-sorbitol, potassium 2-ketogluconate and potassium 5-ketogluconate are not assimilated. With API 50 CH/E after 24 h, N-acetylglucosamine, amygdalin, arbutin, cellobiose, aesculin, fructose, glucose, D-glucoside, glycerol, glycerin, inulin, lactose, mannose, mannitol, maltose, α-methyl ribose, salicin, starch, trehalose and D-turanose are positive and after 48 h D-arabitol, D-fucose, galactose, inulin, lactose, melibiose, methyl β-D-xylpyranoside, rhamnose and D-xylene are positive, but adonitol, D-arabinose, D-arabinose, dulcitol, erythritol, gentiobiose, inositol, methyl α-D-mannopyranoside, sorbose, sorbitol, trehalose, xyitol, L-xylitol, D-arabitol, L-fucose, 2-ketogluconate, 5-ketogluconate, D-lyxose, D-tagatose and turanose are negative. With API 20 E, acetoin production (Voges–Proskauer), arginine dihydrolase, citrate utilization and H₂S production are positive, but ortho-nitrophenyl β-D-galactopyranosidase, ornithine decarboxylase, lysine decarboxylase and nitrite and nitrogen production are negative. The major quinone is menaquinone MK-7(H₂) and a minor amount of MK-8(H₂) is also detected. The polar lipid profile consists of major amounts of diphostidglycerol, phosphatidglycerol and glycolipid, moderate amounts of two unknown aminophospholipids and minor amounts of three unknown phospholipids. Mycolic acids are absent. Cell-wall amino acids are anteiso-C₁₅:₀, anteiso-C₁₇:₀ and iso-C₁₅:₀.

The type strain, AT₅ (=KEMC 41-098T =JCM 17537T =KACC 15558T), was isolated from sludge of a wastewater treatment plant near a farm area at Gwangju-si, Kyeonggi-do, South Korea. The DNA G+C content of the type strain is 70.7 mol%.

Acknowledgements

This subject was supported by the Korea Ministry of Educational Science and Technology (2011-0000544) and Korea National Environmental Microorganisms Bank (2010-0007473).

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Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in...


