Tomitella biformata gen. nov., sp. nov., a new member of the suborder Corynebacterineae isolated from a permafrost ice wedge

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Gram-reaction-positive, aerobic, non-spore-forming, irregular rod-shaped bacteria, designated AHU1821T and AHU1820, were isolated from an ice wedge in the Fox permafrost tunnel, Alaska. The strains were psychrophilic, growing at ~5 to 27 °C. Phylogenetic analysis of the 16S rRNA and gyrB gene sequences indicated that the ice-wedge isolates formed a clade distinct from other mycolic-acid-containing bacteria within the suborder Corynebacterineae. The cell wall of strains AHU1821T and AHU1820 contained meso-diaminopimelic acid, arabinose and galactose, indicating chemotype IV. The muramic acids in the peptidoglycan were glycolated. The predominant menaquinone was MK-9(H2). The polar lipids consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides and an unidentified glycolipid. The major fatty acids were hexadecenoic acid (C16 : 1), hexadecanoic acid (C16 : 0), octadecenoic acid (C18 : 1) and tetradecanoic acid (C14 : 0). Tuberculostearic acid was present in relatively small amounts (1 %). Strains AHU1821T and AHU1820 contained mycolic acids with 42–52 carbons. The DNA G+C content of the two strains was 69.3–71.6 mol% (Tm).

16S rRNA, rpoB and recA gene sequences were identical between strains AHU1821T and AHU1820 and those of the gyrB gene showed 99.9 % similarity. Based on phylogenetic and phenotypic evidence, strains AHU1821T and AHU1820 represent a single novel species of a novel genus, for which the name Tomitella biformata gen. nov., sp. nov. is proposed. The type strain of Tomitella biformata is AHU1821T (=DSM 45403T =NBRC 106253T).

At the time of writing, actinomycetes that are characterized by the presence of mycolic acids and cell wall chemotype IV (Lechevalier & Lechevalier, 1970) include the genera Corynebacterium, Dietzia, Gordonia, Millisua, Mycobacterium, Nocardiia, Rhodococcus, Segniliparum, Skermania, Smaragdicoccus, Tsukamurella and Williamsia within the suborder Corynebacterineae (Butler et al., 2005; Goodfellow & Maldonado, 2006; Soddell et al., 2006; Adachi et al., 2007). Members of this suborder are distinguished from those of other suborders by their phylogeny, based on 16S rRNA gene sequence analysis, and their phenotypic properties.

Previously, we reported on the isolation of bacteria that had been preserved within a permafrost ice wedge for ~25 000 years (Katayama et al., 2007). Phylogenetic analysis based on 16S rRNA gene sequences indicated that two of the ice-wedge isolates, namely strains AHU1821T and AHU1820, were distinct from genera within the

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, gyrB, rpoB and recA gene sequences of strains AHU1821T and AHU1820 are AB491283 and AB491294 (16S rRNA), AB491285 and AB491286 (gyrB), AB491287 and AB491288 (rpoB) and AB491289 and AB491290 (recA), respectively.

A scanning electron micrograph of cells of strain AHU1821T, a neighbour-joining tree showing the relationship between strains AHU1821T and AHU1820 and other mycolic-acid-containing bacteria and images of AHU1821T and AHU1820 colony morphology are available as supplementary material with the online version of this paper.
suborder Corynebacterineae. In this paper, we describe these two strains further and suggest that they represent a novel genus and species in the suborder Corynebacterineae.

Strains AHU1821T and AHU1820 were isolated from an ice wedge in the Fox permafrost tunnel, Alaska, USA (64.952°N 147.617°W), which is preserved at about −3°C by the US Army’s Cold Regions Research and Engineering Laboratory. Sample collection and laboratory isolation methods have been described previously (Katayama et al., 2007). Strains AHU1821T and AHU1820 were originally isolated on agar plates containing Hickey–Tresner revised medium with antibiotics. The strains grew well aerobically at 20°C in tryptic soy broth (TSB) supplemented with 2% (w/v) D-fructose or 2% (v/v) ethanol. Cell cultures used for all experiments were prepared in TSB with 2% D-fructose at 20°C unless indicated otherwise.

The 16S rRNA, gyrB, rpoB and recA gene sequences of strains AHU1821T and AHU1820 were determined. The lengths of the gene sequences were 1471 bp for the 16S rRNA gene (Escherichia coli positions 28–1522), 1506 bp for gyrB (Nocardia farcinica IFM 10152 (GenBank accession no. NC_006361) positions 172–1671; 72.0% of the total length), 2892 bp for rpoB (N. farcinica IFM 10152 positions 436–3321; 82.9%) and 902 bp for recA (N. farcinica IFM 10152 positions 76–977; 86.4%). Phylogenetic analyses and physical and chemotaxonomic characterizations were performed according to methods described previously (Katayama et al., 2009).

Cell morphology was observed under a scanning electron microscope (JOEL, JSM-6301F) (Supplementary Fig. S1, available in IJSEM Online). Growth at −5, 15, 20, 23 and 25°C was determined based on increase in OD600. The upper temperature limit for growth was determined from colony formation at 25, 27, 30 and 37°C on tryptic soy agar (TSA) supplemented with 2% D-fructose. Growth at pH 4.0–11.5, at intervals of 0.5 pH units, was examined by culturing cells on TSA supplemented with 0.5% D-fructose. Enzyme activities were determined using the commercial API ZYM system (bioMérieux). Analysis of whole-cell sugars and the detection of diaminopimelic acid were performed according to the method of Staneck & Roberts (1974). Polar lipids were extracted and identified by two-dimensional TLC (Minnikin et al., 1984). Muramic acids were extracted and analysed using GC-MS (QP2010; Shimadzu) as described by Yano et al. (1972).

The 16S rRNA, rpoB and recA gene sequences of strains AHU1821T and AHU1820 were identical and the gyrB gene sequences showed 99.9% similarity (1 nt difference). The two strains showed less than 97% 16S rRNA gene sequence similarity with the members of the suborder Corynebacterineae. They were also related to Rhodococcus coprophilus (95.6% similarity to the type strain) and other members of the genus Rhodococcus (95.4%–92.3%) and to members of the genera Nocardia (94.9% and lower) and Tsukamurella (94.8% and lower). Based on gyrB gene sequences, the ice-wedge isolates were related to Rhodococcus equi (82.7% gyrB gene sequence similarity to the type strain), Nocardia neocaledoniensis (82.6%) and Nocardia thailandica (82.5%). The neighbour-joining trees based on 16S rRNA and gyrB gene sequences indicated that the ice-wedge isolates formed a monophyletic branch distinct from members of the suborder Corynebacterineae (Fig. 1 and Supplementary Fig. S2). The DNA G+C contents of strains AHU1821T and AHU1820 were 69.3 and 71.6 mol%, respectively.

Physiological and morphological characteristics common to strains AHU1821T and AHU1820 are given in the genus and species descriptions below. No differences were evident between the two strains except for their colony morphology. When grown on TSA supplemented with 2% D-fructose, the colonies of strain AHU1821T were circular with entire margins that were smooth, convex and beige, whereas the colonies of strain AHU1820 were circular with an undulating edge, dry, flat and beige (Supplementary Fig. S3).

The predominant menaquinone in both AHU1821T and AHU1820 was MK-9(H2), which is different from that of the related genera Rhodococcus, Nocardia and Tsukamurella (Table 1). Minor amounts of MK-10(H2) (3% of total menaquinones) and MK-8(H2) (1%) were also present. Muramic acid residues in the peptidoglycans of the strains AHU1821T and AHU1820 were glycolated. Whole-cell hydrolysates of strains AHU1821T and AHU1820 included the diamino acid meso-diaminopimelic acid and the sugars arabinose, galactose, glucose and ribose, indicating wall chemotype IV. The polar lipids detected in strains AHU1821T and AHU1820 included diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides and an unidentified glycolipid. Cells of strains AHU1821T and AHU1820 contained mycolic acids with between 42 and 52 carbons. Fatty acids found in cells of strain AHU1821T cultured at 15°C were C16:1 (39.8%), C16:0 (23.0%), C18:1 (20.1%) and C14:0 (9.9%). Tuberculostearic acid (10-methyl C18:0), which is a major fatty acid component in related genera Rhodococcus, Nocardia and Tsukamurella (Goodfellow & Maldonado, 2006), was present in relatively small amounts (1% and lower) in strain AHU1821T. Small proportions or the absence of methyl- branched fatty acids were seen in the distantly related genera Corynebacterium, Millisia and Smaragdococcus (Soddell et al., 2006; Adachi et al., 2007). In GC-MS analysis, two peaks of C16:1 A and B (Supplementary Table S1), were obtained. The size of both peaks was strongly dependent on growth temperature. The proportion of C16:1 A increased with decreasing temperature, while that of C16:1 B decreased with decreasing temperature. In general, to maintain membrane fluidity at low temperatures, bacteria alter their fatty acid profiles by increasing the cellular proportion of fatty acids that have a lower melting point (Gounot & Russell, 1999). cis Fatty acids have a lower phase transition temperature than their corresponding trans isomers (Keweloh & Heipieper, 1996), suggesting that C16:1 A and B might be in the cis and trans configurations.
respectively. The fatty acid profile and its temperature
dependence were almost identical between strains
AHU1821T and AHU1820.

The 16S rRNA and gyrB gene trees and the phenotypic
characteristics indicated that the strains AHU1821T and
AHU1820 are clearly distinct from the genera within the
suborder Corynebacterineae. To evaluate the genomic
relatedness between strains AHU1821T and AHU1820, we
compared the sequences of gyrB, rpoB and recA genes as
well as those of the 16S rRNA gene, as described above. In a
recent study by Adékambi et al. (2008), the intraspecific

Fig. 1. Neighbour-joining tree showing the relationship between strains AHU1821T and AHU1820 and other mycolic acid-
containing bacteria. Bootstrap values ≥50% are shown at nodes. Dots indicate branches that were also found in the maximum-
likelihood tree. Bar, 0.01 substitutions per nucleotide position.
rpoB sequence similarity was estimated to be between 98.2 and 100 % by correlation with DNA–DNA relatedness analysis. These data strongly support the genomic consistency between the two strains. Accordingly, the strains AHU1821T and AHU1820 represent a single novel genus and species, for which the name Tomitella biformata gen. nov., sp. nov. is proposed.

On the basis of 16S rRNA signature nucleotide patterns, which were updated by Zhi et al. (2009), Tomitella biformata strains were affiliated with the family Tsukamurellaceae in the suborder Corynebacterineae. However, the 16S rRNA gene tree indicates that the two Tomitella strains form a clade distinct from members of the family Tsukamurellaceae. Further comparative taxonomic studies on additional Tomitella strains are needed to determine the family assignment.

**Description of Tomitella biformata gen. nov.**

Tomitella (To.mi.tel’la. N.L. fem. n. Tomitella named in honour of Emeritus Professor Fusao Tomita, a celebrated Japanese microbiologist).

Cells are aerobic, Gram reaction-positive, non-spore-forming, irregular rods. The predominant quinone is a dihydrogenated menaquinone with nine isoprene units. Whole-cell hydrolysates are rich in meso-diaminopimelic acid, arabinose and galactose. The muramic acid residues are glycolated. The polar lipids detected include diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides and an unidentified glycolipid. Mycolic acids are present with between 42 and 52 carbon atoms. C$_{14:0}$ are the major fatty acids. 10-methyl-C$_{18:0}$ is present in relatively small proportions. The DNA G+C content of the type strain of the type species is 69.3 mol% ($T_m$). The type species is Tomitella biformata.

**Description of Tomitella biformata sp. nov.**

Tomitella biformata (bi.for.ma’ta. L. fem. adj. biformata two-shaped).

Has the following characteristics in addition to those given for the genus. Cells exhibit snapping division and produce V-forms. Cells turn into short coccoid rods after prolonged culture. On TSA supplemented with 2 % D-fructose colonies are circular with smooth entire margins, convex and beige, or colonies are circular with an undulating edge, dry, flat and beige. Grows at $-$5 to 27 $^\circ$C and at pH 5–10. The optimal temperature for growth is 20 $^\circ$C. Catalase-positive and oxidase-negative. Acid is produced from D-fructose, glycerol and ethanol and is produced weakly from D-mannose, but not from D- or L-arabinose, D-galactose, D-glucose, L-rhamnose, D-xyllose, D-ribose, cellulbiose, maltose, sucrose, turanose, D- or L-fucose, D- or L-xyllose, trehalose, raffinose, D-sorbitol, D-mannitol, xylitol, starch or inulin. Positive for alkaline phosphatase, acid phosphatase, esterase lipase (C$_9$), leucine arylamidase, trypsin and naphthol-AS-BI-phosphohydrolase.

The type strain is AHU1821$^T$ (=DSM 45403$^T$ =NBRC 106253$^T$), which was isolated from an ice wedge in the Fox permafrost tunnel, Alaska. Strain AHU1820 (=NBRC 106252) is a second strain of the species, isolated from the same source.

### Table 1. Chemical properties of strains AHU1821$^T$ and AHU1820 and other mycolic acid-containing genera

<table>
<thead>
<tr>
<th>Strain or genus</th>
<th>Predominant menaquinone(s)</th>
<th>Acyl type</th>
<th>PE</th>
<th>Major fatty acids</th>
<th>Mycolic acids (no. of carbons)</th>
<th>DNA G+C content (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHU1821$^T$ and AHU1820</td>
<td>MK-9(H$_2$)</td>
<td>G</td>
<td>+</td>
<td>S, U</td>
<td>42–52</td>
<td>69.3–71.6</td>
</tr>
<tr>
<td><strong>Corynebacterium</strong></td>
<td>MK-8(H$_2$), -9(H$_2$)</td>
<td>A</td>
<td>–</td>
<td>S, U</td>
<td>22–38</td>
<td>51–63</td>
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<tr>
<td><strong>Dietzia</strong></td>
<td>MK-8(H$_2$)</td>
<td>A</td>
<td>–</td>
<td>S, U, T</td>
<td>34–38</td>
<td>73</td>
</tr>
<tr>
<td><strong>Tsukamurella</strong></td>
<td>MK-9</td>
<td>G</td>
<td>+</td>
<td>S, U, T</td>
<td>64–78</td>
<td>67–78</td>
</tr>
<tr>
<td><strong>Mycobacterium</strong></td>
<td>MK-9(H$_2$)</td>
<td>G</td>
<td>+</td>
<td>S, U, T</td>
<td>60–90</td>
<td>62–70</td>
</tr>
<tr>
<td><strong>Rhodococcus</strong></td>
<td>MK-8(H$_2$)</td>
<td>G</td>
<td>+</td>
<td>S, U, T</td>
<td>30–54</td>
<td>63–73</td>
</tr>
<tr>
<td><strong>Smaragdicoccus</strong></td>
<td>MK-8(H$_4$, o-cycl.)</td>
<td>G</td>
<td>+</td>
<td>S, U, T</td>
<td>48–60</td>
<td>64–72</td>
</tr>
<tr>
<td><strong>Segniliparus</strong></td>
<td>SQA-8(H$_4$, o-cycl.), SQB-8(H$_4$, dicycl.)</td>
<td>G</td>
<td>+</td>
<td>S, U</td>
<td>43–49</td>
<td>63.7</td>
</tr>
<tr>
<td>Skermania</td>
<td>MK-8(H$_4$, o-cycl.)</td>
<td>G</td>
<td>+</td>
<td>S, U, T</td>
<td>58–64</td>
<td>67.5</td>
</tr>
<tr>
<td>Millisla</td>
<td>MK-8(H$_2$)</td>
<td>G</td>
<td>+</td>
<td>S, U</td>
<td>44–52</td>
<td>64.7</td>
</tr>
<tr>
<td>Gordonia</td>
<td>MK-9(H$_2$)</td>
<td>G</td>
<td>+</td>
<td>S, U, T</td>
<td>46–66</td>
<td>60–66</td>
</tr>
<tr>
<td>Williamsia</td>
<td>MK-9(H$_2$)</td>
<td>G</td>
<td>+</td>
<td>S, U, T</td>
<td>50–56</td>
<td>64–65</td>
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<tr>
<td>Skermania</td>
<td>MK-8(H$_4$, o-cycl.)</td>
<td>G</td>
<td>+</td>
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<td>+</td>
<td>S, U</td>
<td>44–52</td>
<td>64.7</td>
</tr>
</tbody>
</table>

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**Data for established genera were taken from the following studies:** Segniliparus, Butler et al. (2005); Smaragdicoccus, Adachi et al. (2007); Millisla, Soddell et al. (2006); other genera, Goodfellow & Maldonado (2006). SQA, Smaragdiquinone A; SQB, smaragdiquinone B; G, glycolated; A, acetylated; PE, phosphatidylethanolamine; S, saturated fatty acid; U, unsaturated fatty acid; T, tuberculostearic acid; ND, no data.
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References


