

## *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 AHU1821<sup>T</sup> 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 AHU1821<sup>T</sup> 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(H<sub>2</sub>). The polar lipids consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides and an unidentified glycolipid. The major fatty acids were hexadecenoic acid (C<sub>16:1</sub>), hexadecanoic acid (C<sub>16:0</sub>), octadecenoic acid (C<sub>18:1</sub>) and tetradecanoic acid (C<sub>14:0</sub>). Tuberculostearic acid was present in relatively small amounts (1%). Strains AHU1821<sup>T</sup> and AHU1820 contained mycolic acids with 42–52 carbons. The DNA G + C content of the two strains was 69.3–71.6 mol% (*T<sub>m</sub>*). 16S rRNA, *rpoB* and *recA* gene sequences were identical between strains AHU1821<sup>T</sup> and AHU1820 and those of the *gyrB* gene showed 99.9% similarity. Based on phylogenetic and phenotypic evidence, strains AHU1821<sup>T</sup> 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 AHU1821<sup>T</sup> (=DSM 45403<sup>T</sup> =NBRC 106253<sup>T</sup>).

At the time of writing, actinomycetes that are characterized by the presence of mycolic acids and cell wall chemotype IV

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *gyrB*, *rpoB* and *recA* gene sequences of strains AHU1821<sup>T</sup> and AHU1820 are AB491283 and AB491284 (16S rRNA), AB491285 and AB491286 (*gyrB*), AB491287 and AB491288 (*rpoB*) and AB491289 and AB491290 (*recA*), respectively.

A scanning electron micrograph of cells of strain AHU1821<sup>T</sup>, a neighbour-joining tree showing the relationship between strains AHU1821<sup>T</sup> and AHU1820 and other mycolic-acid-containing bacteria and images of AHU1821<sup>T</sup> and AHU1820 colony morphology are available as supplementary material with the online version of this paper.

(Lechevalier & Lechevalier, 1970) include the genera *Corynebacterium*, *Dietzia*, *Gordonia*, *Millisia*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Segniliparus*, *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 AHU1821<sup>T</sup> and AHU1820, were distinct from genera within the

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 AHU1821<sup>T</sup> 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 AHU1821<sup>T</sup> 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 AHU1821<sup>T</sup> 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).

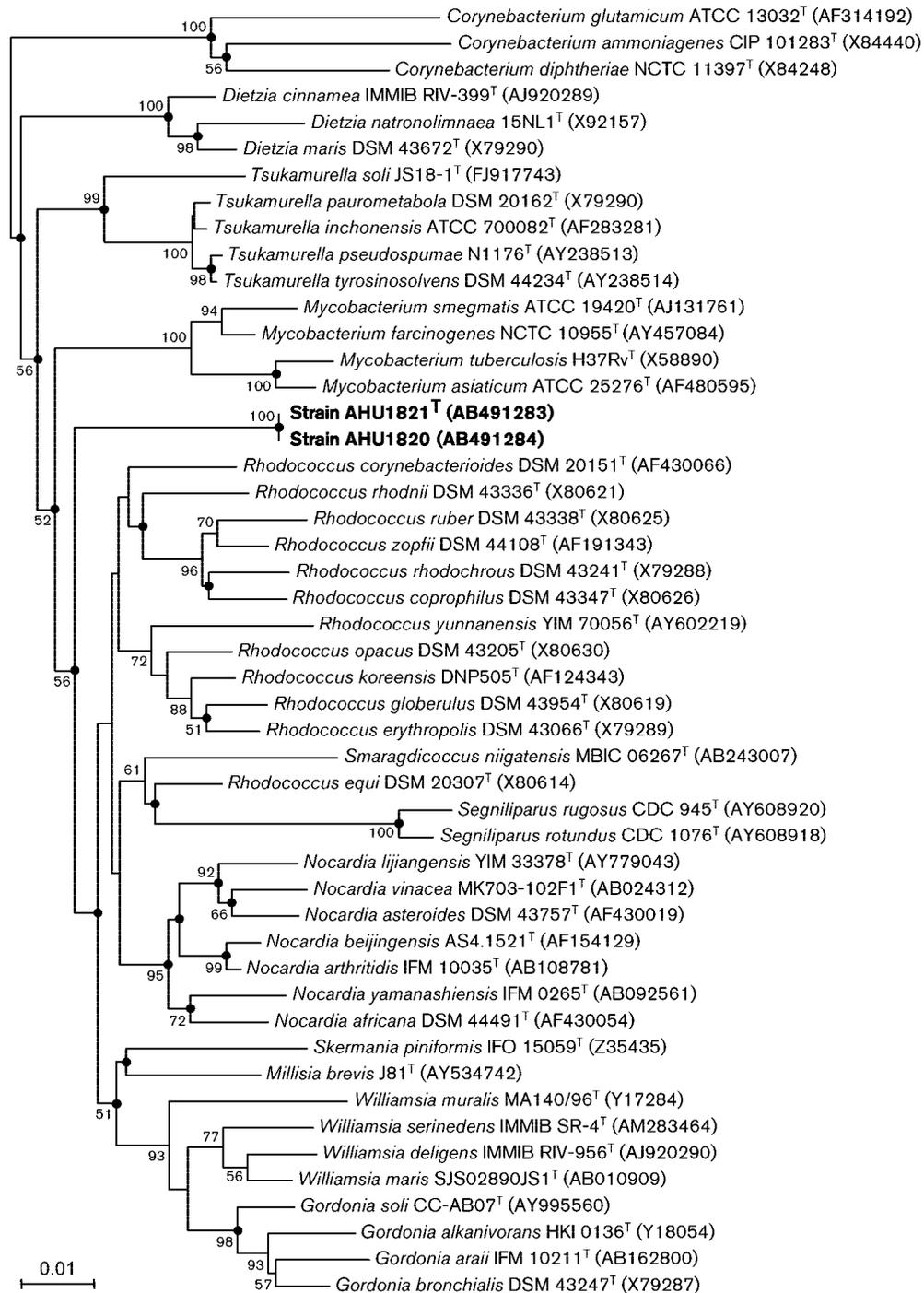
Cell morphology was observed under a scanning electron microscope (JOEL, JSM-6301F) (Supplementary Fig. S1, available in IJSEM Online). Growth at -5, 15, 20, 23 and 25 °C was determined based on increase in OD<sub>600</sub>. The upper temperature limit for growth was determined from colony formation 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 Stanek & Roberts (1974). Polar lipids were extracted and identified by two-dimensional TLC (Minnikin *et al.*, 1984). Mycolic 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 AHU1821<sup>T</sup> 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 AHU1821<sup>T</sup> and AHU1820 were 69.3 and 71.6 mol%, respectively.

Physiological and morphological characteristics common to strains AHU1821<sup>T</sup> 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 AHU1821<sup>T</sup> 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 AHU1821<sup>T</sup> and AHU1820 was MK-9(H<sub>2</sub>), which is different from that of the related genera *Rhodococcus*, *Nocardia* and *Tsukamurella* (Table 1). Minor amounts of MK-10(H<sub>2</sub>) (3% of total menaquinones) and MK-8(H<sub>2</sub>) (1%) were also present. Muramic acid residues in the peptidoglycans of the strains AHU1821<sup>T</sup> and AHU1820 were glycolated. Whole-cell hydrolysates of strains AHU1821<sup>T</sup> 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 AHU1821<sup>T</sup> and AHU1820 included diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides and an unidentified glycolipid. Cells of strains AHU1821<sup>T</sup> and AHU1820 contained mycolic acids with between 42 and 52 carbons. Fatty acids found in cells of strain AHU1821<sup>T</sup> cultured at 15 °C were C<sub>16:1</sub> (39.8%), C<sub>16:0</sub> (23.0%), C<sub>18:1</sub> (20.1%) and C<sub>14:0</sub> (9.9%). Tuberculostearic acid (10-methyl C<sub>18:0</sub>), 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 AHU1821<sup>T</sup>. Small proportions or the absence of methyl-branched fatty acids were seen in the distantly related genera *Corynebacterium*, *Millisia* and *Smaragdicoscus* (Soddell *et al.*, 2006; Adachi *et al.*, 2007). In GC-MS analysis, two peaks of C<sub>16:1</sub>, A and B (Supplementary Table S1), were obtained. The size of both peaks was strongly dependent on growth temperature. The proportion of C<sub>16:1</sub> A increased with decreasing temperature, while that of C<sub>16:1</sub> 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 C<sub>16:1</sub> A and B might be in the *cis* and *trans* configurations,



**Fig. 1.** Neighbour-joining tree showing the relationship between strains AHU1821<sup>T</sup> 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.

respectively. The fatty acid profile and its temperature dependence were almost identical between strains AHU1821<sup>T</sup> and AHU1820.

The 16S rRNA and *gyrB* gene trees and the phenotypic characteristics indicated that the strains AHU1821<sup>T</sup> and

AHU1820 are clearly distinct from the genera within the suborder *Corynebacterineae*. To evaluate the genomic relatedness between strains AHU1821<sup>T</sup> 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

**Table 1.** Chemical properties of strains AHU1821<sup>T</sup> and AHU1820 and other mycolic acid-containing genera

Data for established genera were taken from the following studies: *Segniliparus*, Butler *et al.* (2005); *Smaragdicoccus*, Adachi *et al.* (2007); *Millisia*, 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.

Strain or genus	Predominant menaquinone(s)	Acyl type	PE	Major fatty acids	Mycolic acids (no. of carbons)	DNA G+C content (mol%)
Strains AHU1821 <sup>T</sup> and AHU1820	MK-9(H <sub>2</sub> )	G	+	S, U	42–52	69.3–71.6
<i>Corynebacterium</i>	MK-8(H <sub>2</sub> ), -9(H <sub>2</sub> )	A	–	S, U	22–38	51–63
<i>Dietzia</i>	MK-8(H <sub>2</sub> )	A	–	S, U, T	34–38	73
<i>Tsukamurella</i>	MK-9	G	+	S, U, T	64–78	67–78
<i>Mycobacterium</i>	MK-9(H <sub>2</sub> )	G	+	S, U, T	60–90	62–70
<i>Rhodococcus</i>	MK-8(H <sub>2</sub> )	G	+	S, U, T	30–54	63–73
<i>Nocardia</i>	MK-8(H <sub>4</sub> , ω-cycl.)	G	+	S, U, T	48–60	64–72
<i>Smaragdicoccus</i>	SQA-8(H <sub>4</sub> , ω-cycl.), SQB-8(H <sub>4</sub> , dicycl.)	G	+	S, U	43–49	63.7
<i>Segniliparus</i>	ND	ND	ND	S, U, T	ND	68–72
<i>Gordonia</i>	MK-9(H <sub>2</sub> )	G	+	S, U, T	46–66	60–66
<i>Williamsia</i>	MK-9(H <sub>2</sub> )	G	+	S, U, T	50–56	64–65
<i>Skermania</i>	MK-8(H <sub>4</sub> , ω-cycl.)	G	+	S, U, T	58–64	67.5
<i>Millisia</i>	MK-8(H <sub>2</sub> )	G	+	S, U	44–52	64.7

*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 AHU1821<sup>T</sup> 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* 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<sub>16:1</sub>, C<sub>16:0</sub>, C<sub>18:1</sub> and

C<sub>14:0</sub> are the major fatty acids. 10-methyl-C<sub>18:0</sub> is present in relatively small proportions. The DNA G+C content of the type strain of the type species is 69.3 mol% (*T<sub>m</sub>*). 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 °C and at pH 5–10. The optimal temperature for growth is 20 °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-xylose, D-ribose, cellobiose, maltose, sucrose, turanose, D- or L-fucose, D- or L-xylose, trehalose, raffinose, D-sorbitol, D-mannitol, xylitol, starch or inulin. Positive for alkaline phosphatase, acid phosphatase, esterase lipase (C<sub>8</sub>), leucine arylamidase, trypsin and naphthol-AS-BI-phosphohydrolase.

The type strain is AHU1821<sup>T</sup> (=DSM 45403<sup>T</sup> =NBRC 106253<sup>T</sup>), 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.

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