Methylobacter tundripaludum sp. nov., a methane-oxidizing bacterium from Arctic wetland soil on the Svalbard islands, Norway (78° N)

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A Gram-negative, rod-shaped, non-motile, non-spore forming bacterium (SV96^T) was isolated from wetland soil near Ny-Ålesund, Svalbard. On the basis of 16S rRNA gene sequence similarity, strain SV96^T was shown to belong to the *Gammaproteobacteria*, related to *Methylobacter psychrophilus* Z-0021^T (99.1%), *Methylobacter luteus* ATCC 49878^T (97.3%), *Methylobacter marinus* A45^T (97.0%) and *Methylobacter whittenburyi* ATCC 51738^T (95.8%); the closest related species within the genus *Methylomicrobium* with a validly published name was *Methylomicrobium album* ATCC 33003^T (95.0%). Chemotaxonomic data (including the major fatty acids: 16:1 ω 8, 16:1 ω 7 and 16:1 ω 5t) supported the affiliation of strain SV96^T to the genus *Methylobacter*. The results of DNA–DNA hybridization, physiological and biochemical tests allowed genotypic and phenotypic differentiation of strain SV96^T from the four *Methylobacter* species mentioned above. Strain SV96^T therefore represents a novel species, for which the name *Methylobacter tundripaludum* sp. nov. is proposed (type strain SV96^T = DSM 17260^T = ATCC BAA-1195^T).

The genus *Methylobacter* was formed following the emendation of the description of the genus *Methylococcus* (Bowman *et al.*, 1993), and *Methylobacter* species are equivalent to the similarly named group first coined by Whittenbury *et al.* (1970). The genus was further revised when three members of the genus *Methylobacter* were renamed as a new taxon *Methylomicrobium* (Bowman *et al.*, 1995). At present the genus comprises the four species *Methylobacter luteus* (Bowman *et al.*, 1993; Romanovskaya *et al.*, 1978), *Methylobacter whittenburyi* (Bowman *et al.*, 1993; Romanovskaya *et al.*, 1978; Whittenbury *et al.*, 1970), *Methylobacter marinus* (Bowman *et al.*, 1993; Lidstrom, 1988) and *Methylobacter psychrophilus* (Omelchenko *et al.*, 1996; Tourova *et al.*, 1999).

Strain SV96^T was isolated from a soil core collected from a wetland near the settlement Ny-Ålesund (78° 56' N 11° 53' E), Svalbard, in July 1996. The soil emitted methane (Høj

et al., 2005) and had a pH of 6.4. The temperature of the soil was 10 °C at the surface and 5 °C at 10 cm below the surface; the permafrost level was at 25 cm. After the fresh vegetation layer was removed, the upper 10 cm of the soil core was mixed, 2 g soil was taken and added to 10 ml nitrate mineral salt medium (NMS) (Whittenbury *et al.*, 1970) at pH 6.8, and shaken for 10 min at 200 r.p.m. After 10 min of sedimentation, 1 ml supernatant was mixed with 9 ml NMS in a 120 ml serum bottle (Alltech). The bottle was sealed with a rubber stopper and crimp cap. Twenty millilitres of air was replaced with 20 ml CH₄/CO₂ (95:5) mixture (both gases were 99.95% purity). The bottle was incubated at 20 °C and subcultured every 2–3 weeks (1 ml culture to 9 ml fresh NMS medium). After four or five subculturing steps in liquid media, bacteria were plated on NMS medium containing 1.5% noble agar (bacteriological agar type E; Biokar diagnostics). The plates were incubated at 20 °C in sealed chambers containing approximately 35% methane in air. Colonies were picked and restreaked. Heterotrophic contamination was checked by streaking colonies on agar plates with rich medium containing 0.5% tryptone, 0.25% yeast extract, 0.1% glucose and 2.0% agar. These plates were incubated at room temperature without additional methane. The cultures were considered to be pure when only one cell type was observed under light microscopy and no growth on nutrient rich medium was observed. Exospore

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Abbreviations: PLFA, phospholipid fatty acid; RuMP, ribulose monophosphate; sMMO, soluble methane monooxygenase.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, *pmoA* and *nifH* sequences of *Methylobacter tundripaludum* SV96^T are AJ414655, AJ414658 and AY937260, respectively.

The growth of SV96^T was measured at temperatures ranging from 5.0 to 40.0 °C, using a temperature gradient apparatus with the opportunity to grow bacteria simultaneously at 10 different chosen temperatures. The temperature gradient was achieved using a metal cylinder with a flat top (14 cm in diameter and 50 cm in length), where one end was attached to a cooling water bath and the other to a thermostat-regulated heat block. The top of the cylinder had 10 holes, 5 cm apart in two parallel rows, which fitted a 27 ml serum bottle (Alltech). The temperatures were measured at every second hole, where a small hole was drilled between the two parallel rows. To achieve temperatures from 5 to 40 °C, two different experiments with partly overlapping temperatures were run. The temperature was first set from 5 to 12 °C (a gradient of 0.14 °C cm⁻¹) and then from 11 to 40 °C (a gradient of 0.58 °C cm⁻¹). Cultures of SV96^T were prepared by adding 5 ml starting culture to 27 ml serum bottles. The bottles were sealed with rubber stoppers and crimp caps before 5 ml air was replaced with 5 ml CH₄/CO₂ (95:5) mixture and the cultures were grown for 5 days. The OD₆₀₀ was measured for the starting and 5-day-old cultures using a Spectramax 250 microplate spectrophotometer system (Molecular Devices). The maximum OD₆₀₀ of SV96^T was 0.72, and was measured after 15 days at 20 °C. The experiment was repeated in three replicates at each temperature setting and the net growth calculated by subtracting the OD₆₀₀ of the starting culture from the OD₆₀₀ values of the 5-day-old cultures. Mean values for net growth and standard deviations were calculated.

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closest matches with cultured bacteria were *Methylobacter marinus* A45^T (84 %) and *Methylobacter luteus* NCIMB 11914^T (=ATCC 49878^T) (84 %). The DNA base composition was determined by thermal denaturation using a Cary 4E Varian spectrophotometer at a heating rate of 0.5 °C min⁻¹. The G + C content (mol%) of the DNA was calculated by the equation of Mandel *et al.* (1970). DNA–DNA hybridization was performed with *Methylobacter luteus* ATCC 49878^T according to the method described by Ezaki *et al.* (1989). It was not performed with *Methylobacter psychrophilus* Z-0021^T or *Methylobacter marinus* A45^T because they were, to our knowledge, both unavailable from any culture collection.

The most closely related bacterium, *Methylobacter psychrophilus* Z-0021^T (Tourova *et al.*, 1999), sharing 99.1 % 16S rRNA gene similarity, was isolated from a moss-vegetated area on the tundra in the polar Ural (Omelchenko *et al.*, 1993). Despite the high 16S rRNA gene sequence similarity and the similar origin, the bacterial strains were different in several important morphological traits (Table 1). SV96^T was non-motile, 0.8–1.3 µm wide and 1.9–2.5 µm in length. Cells often appeared in pairs or in long chains. Colonies were pale-pink pigmented, but lost the pigmentation upon methane starvation. Cells of *Methylobacter psychrophilus* Z-0021^T were reported to be cocci or diplococci, 1.0–1.7 µm in diameter, and colonies were opaque cream (Omelchenko *et al.*, 1996). The cells of SV96^T lysed in 2 % SDS. The strain did not grow when NaCl was added to

solid NMS medium and poor to no growth was observed with methanol as a carbon source. SV96^T grew at all pH values tested except for pH 9.0 and no exospores or cysts were revealed using the described methods. A type I membrane structure, with bundles of disc-shaped membranes, was confirmed using transmission electron microscopy (Fig. 3). The major phospholipid fatty acids (PLFAs) for SV96^T were 16:1ω8 (34.9 %), 16:1ω7 (23.4 %) and 16:1ω5t (26.3 %). Isolate SV96^T had a G + C content of 47 mol% (±1 mol%), while *Methylobacter psychrophilus* Z-0021^T had a G + C content of 45.6 mol% (Omelchenko *et al.*, 1996). SV96^T was positive for the *nifH* gene by PCR (Poly *et al.*, 2001). It was negative for the *mmoX* gene in PCR assays (Fuse *et al.*, 1998; Miguez *et al.*, 1997) and no soluble methane monooxygenase (sMMO) was detected by colorimetric assay performed as described by Brusseau *et al.* (1990), with the modifications of Graham *et al.* (1992). SV96^T had an optimum growth temperature of 23 °C, but grew well at temperatures from 10 to 27 °C (Fig. 4) and was clearly not psychrophilic. *Methylobacter psychrophilus* Z-0021^T was reported to have a growth optimum between 3.5 and 10 °C with no growth at temperatures above 20 °C (Omelchenko *et al.*, 1996). To confirm that SV96^T and *Methylobacter psychrophilus* Z-0021^T represent different species, a DNA–DNA hybridization should be performed; however, *Methylobacter psychrophilus* Z-0021^T is not available from any culture collection (P. Kämpfer, personal communication). This is also the situation for *Methylobacter marinus* A45^T. The 16S rRNA gene sequence similarity

Table 1. Characteristics that differentiate species belonging to the genus *Methylobacter*

Strains/species: 1, *Methylobacter tundripaludum* SV96^T; 2, *Methylobacter psychrophilus* Z-0021^T (data from Omelchenko *et al.*, 1996); 3, *Methylobacter luteus* (four strains); 4, *Methylobacter marinus* (three strains); 5, *Methylobacter whittenburyi* (six strains) (data in columns 3–5 from Bowman *et al.*, 1993, 1995). +, 91–100 % of strains are positive; –, 0–10 % of strains are positive; d, 21–81 % of strains are positive; (?), uncertain (strain is lost and result cannot be confirmed); ND, not determined. All strains have type I membranes and utilize the RuMP pathway.

Characteristic	1	2	3	4	5
Cell morphology					
Cocci	–	+	+	+	+
Ellipsoidal	–	–	d	+	+
Rods	+	–	–	–	–
Coccobacilli	–	–	–	+	d
Cyst formation	–	– (?)	+	+	+
Motility	–	–	–	+	+
Pigmentation*	P	W/B	Y	DB	DB
Optimum growth temperature (°C)	23	3.5–10	30	37	30
NaCl requirement	–	–	–	+	–
Oxidase	+	ND	+	+	+
Urease	–	ND	+	–	–
<i>nifH</i> gene	+	ND	+	+	–
G + C content (mol%)	47	45.6	49–51	54–55	49–54
Major PLFA(s)	16:1ω8, 16:1ω7	16:1	16:1ω7c	16:1ω7c	16:1ω7c

*DB, Diffusible brown; P, pink; W/B, white or buff; Y, yellow.

Fig. 3. Transmission electron micrograph of a cell of *Methylobacter tundripaludum* SV96^T. Bar, 200 nm.

between SV96^T and the second most closely related bacterium, *Methylobacter luteus* ATCC 49878^T, was 97 %. DNA–DNA hybridization demonstrated a relatedness value of 10 % between SV96^T and *Methylobacter luteus* ATCC 49878^T. The differences between *Methylobacter luteus* ATCC 49878^T, *Methylobacter psychrophilus* Z-0021^T, *Methylobacter marinus* A45^T, *Methylobacter whittenburyi* ATCC 51738^T and SV96^T are described in Table 1. Because of the genotypic and phenotypic differences between SV96^T and the

Fig. 4. Growth of *Methylobacter tundripaludum* SV96^T at temperatures ranging from 5 to 40 °C. Data points represent mean values for net growth, calculated by subtracting the OD₆₀₀ of the starting culture from the OD₆₀₀ of the 5-day-old cultures. Error bars represent standard deviations of three replicates at each temperature.

other *Methylobacter* species (including 16S rRNA gene sequence and DNA–DNA hybridization), we propose the name *Methylobacter tundripaludum* sp. nov. for strain SV96^T.

Description of *Methylobacter tundripaludum* sp. nov.

Methylobacter tundripaludum (tun.dri.pa'lu.dum. N.L. n. *tundra* treeless polar region with permanently frozen sub-soil; L. n. *palus* -udis marsh; N.L. gen. pl. n. *tundripaludum* of the tundra marshes).

Gram-negative, straight, fat, rod-shaped cells. Cells are 0.8–1.3 µm in diameter and 1.9–2.5 µm long. Cells are pale-pink pigmented, occur singly, in pairs or in long chains, and are non-motile. Grows on methane as sole carbon and energy source; shows poor to no growth on methanol. Possesses a type I intracytoplasmic membrane system. Assimilates carbon via the ribulose monophosphate (RuMP) pathway and does not possess sMMO. Cells are catalase- and oxidase-positive. Possesses a *nifH* gene. Optimal growth occurs at 23 °C, but grows well down to 10 °C; no growth occurs above 30 °C. Does not require NaCl for growth and cells are lysed by 2 % SDS. Grows well from pH 5.5 to 7.9. Major PLFAs are 16:1ω8 (34.9 %), 16:1ω7 (23.4 %) and 16:1ω5t (26.3 %). The DNA G+C content is 47 mol%. *Methylobacter tundripaludum* strain SV96^T shows 10 % DNA–DNA hybridization with *Methylobacter luteus* ATCC 49878^T.

The type strain is SV96^T (=ATCC BAA-1195^T=DSM 17260^T), isolated from an Arctic wetland soil near Ny-Ålesund, Svalbard.

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