Ornatilinea apprima gen. nov., sp. nov., a cellulolytic representative of the class *Anaerolineae*

O. A. Podosokorskaya,[1] E. A. Bonch-Osmolovskaya,[1] A. A. Novikov,[2] T. V. Kolganova[3] and I. V. Kublanov[1]

Correspondence
O. A. Podosokorskaya
podosokorskaya@gmail.com

[1]Winogradsky Institute of Microbiology, Russian Academy of Sciences, Prospekt 60-Letiya Oktyabrya 7/2, 117312 Moscow, Russia

[2]Gubkin Russian State University of Oil and Gas, Leninskiy Prospect 65, 117485 Moscow, Russia

[3]Bioengineering Center, Russian Academy of Sciences, Prospekt 60-Letiya Oktyabrya 7/1, 117312 Moscow, Russia

A novel obligately anaerobic, mesophilic, organotrophic bacterium, strain P3M-1^T, was isolated from a microbial mat formed in a wooden bath filled with hot water emerging from a 2775 m-deep well in the Tomsk region of western Siberia, Russia. Cells of strain P3M-1^T were rod-shaped, 0.3–0.7 μm in width and formed multicellular filaments that reached up to 400 μm in length. Strain P3M-1^T grew optimally at 42–45 °C, pH 7.5–8.0, and with 0.1% (w/v) NaCl. Under optimal conditions, the doubling time was 6 h. The isolate was able to ferment a variety of proteinaceous substrates and sugars, including microcrystalline cellulose. Acetate, ethanol and H_2 were the main products of glucose fermentation. The genomic DNA G+C content was 55 mol%. 16S rRNA gene sequence-based phylogenetic analyses showed that strain P3M-1^T was a member of the class *Anaerolinea*, with 92.8 % sequence similarity to *Levilinea saccharolytica* KIBI-1^T. Based on phylogenetic analysis and physiological properties, strain P3M-1^T represents a novel species in a new genus, for which the name *Ornatilinea apprima* gen. nov., sp. nov. is proposed; the type strain of *O. apprima* is P3M-1^T (=DSM 23815^T=VKM B-2669^T).

The phylum *Chloroflexi*, a deep-branching bacterial lineage, has attracted the interest of many researchers and this has resulted in many novel taxa being described within this phylum over the last decade (Sekiguchi *et al.*, 2003; Cavaletti *et al.*, 2006; Moe *et al.*, 2009). At the time of writing, the phylum consisted of six classes of micro-organisms with diverse morphology and physiology: from cocci to multicellular filaments, from psychrophiles to thermophiles, and from phototrophs to chemo-organoheterotrophs (Hanada *et al.*, 2002; McKeown *et al.*, 2009). Moreover, 16S rRNA gene sequence similarity levels between representatives of the different classes is often found to be <85 %, and thus corresponds to the level that is commonly used as a cut-off for distinguishing new phyla (Hugenholtz *et al.*, 1998; Rappé & Giovannoni, 2003; Geissinger *et al.*, 2009).

One recently discovered group in the phylum *Chloroflexi* is the class *Anaerolineae*, which was described in 2006 and, at the time of writing, comprises six genera (Sekiguchi *et al.*, 2003; Yamada *et al.*, 2006, 2007; Grégoire *et al.*, 2011). Sources of isolation of cultured members of the class *Anaerolineae*, as well as DNA of uncultured clones, are extremely diverse and include sediments, subsurface habitats, anaerobic dechlorinating consortia, hot springs, deep hot aquifers and anaerobic wastewater sludges. Nevertheless, members of the class *Anaerolineae* form a surprisingly uniform bacterial group. All cultured members of the class are Gram-negative, non-motile and non-spore-forming micro-organisms that form multicellular filaments and have relatively long doubling times (45–100 h) in culture. They are neutrophilic, strictly anaerobic chemo-organotrophs that are able to utilize sugars and polysaccharides such as starch, pectin and xylan. Growth of members of the class *Anaerolineae* on cellulose and its derivatives does not appear to have been investigated previously. Here, we propose a novel species of a new genus that appears to be the first representative of the class *Anaerolineae* that has been found able to grow on microcrystalline cellulose (Avicel) as sole energy and carbon source.

Isolate P3M-1^T was obtained using modified Widdel freshwater medium (Widdel & Bak, 1992) that had reduced

The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence of strain P3M-1^T is JQ292916.

Two supplementary figures are available with the online version of this paper.

concentrations of NaCl (1 g l^{-1}) and $NaHCO_3$ (to 0.1 g l^{-1}) and excluded selenite solution. The medium was supplemented with trace element solution (1 ml l^{-1}; Kevbrin & Zavarzin, 1992); vitamin solution (1 ml l^{-1}; Wolin *et al.*, 1963) and yeast extract (Helicon; 0.1 g l^{-1}), as the source of growth factors. Microcrystalline cellulose (Avicel; Fluka) was added to the medium as the sole energy and carbon source up to a final concentration of 2 g l^{-1}. The medium was prepared anaerobically under an atmosphere of 100 % N_2 and reduced by the addition of $Na_2S.9H_2O$ (0.3 g l^{-1}). Resazurin (1 mg l^{-1}) was used as a redox indicator and the pH of the medium, measured at 20 °C using a pH meter calibrated at the same temperature, was adjusted to pH 7.5 using anoxic 2 M NaOH. The medium was then sterilized at 121 °C for 40 min. Hungate tubes (18 ml), each containing 10 ml medium, were prepared, inoculated with a sample of the microbial mat collected from the surface of a wooden bath filled with hot water (47 °C, pH 7.5) emerging from a 2775 m-deep well in the Tomsk region of western Siberia, Russia (58° 50′ 05.3″ N 81° 30′ 07.5″ E). Visible growth was obtained after 5 days of incubation at 47 °C. The dominant micro-organism was isolated by serial 10-fold dilutions of the primary enrichment. The isolate obtained from the eighth dilution, designated strain P3M-1^T, was characterized using the same medium as used for the isolation. Purity of the strain was verified by transfer on organic-rich medium at various pH values and temperatures and by 16S rRNA gene sequence analysis. Rods of two different morphological types were observed in lower dilutions of the same enrichment culture; cells were successfully isolated after replacement of the microcrystalline cellulose with carboxymethyl cellulose (Sigma). Both of these isolates, designated strains P3M-2 and P3M-3, also proved to be cellulolytic and they have been tentatively assigned to the proposed new phylum '*Ignavibacteriae*' (O. A. Podosokorskaya and others, unpublished) and to the genus *Clostridium*, respectively.

Morphologically, the cells of strain P3M-1^T were rods that gathered into long multicellular filaments. The width of each cell was 0.3–0.7 μm; the length of a single cell was indefinite, whereas the length of straight or curved multicellular filaments ranged from 15–200 μm (Fig. 1a). Occasionally, filaments of up to 400 μm were observed. After growth on certain organic substrates (e.g. glucose, yeast extract or beef extract), many multicellular filaments became interwoven (Fig. S1, available in IJSEM Online), forming flock-like aggregates and biofilms (Fig. S2). Gliding motility and spores were not observed under any cultivation conditions or in any growth phase. Electron microscopy of thin sections (Bonch-Osmolovskaya *et al.*, 1990) revealed two electron-dense layers, one electron-transparent layer and septa (Fig. 1b).

Strain P3M-1^T was strictly anaerobic: growth on medium without prereduction with $Na_2S.9H_2O$ was considerably weaker than that in reduced medium, and ceased completely under microaerophilic conditions (2 % oxygen, v/v, in the gas phase). The novel strain was negative for catalase activity when tested by the method of Smibert & Krieg (1994). To determine the optimal and range of temperature, pH and NaCl concentrations for growth, modified Widdel medium containing Avicel (2 g l^{-1}) as a substrate, as described above, was used. Strain P3M-1^T grew at 20–50 °C (optimum between 42 and 45 °C, with no growth at temperatures of $\leqslant$15 or $\geqslant$52 °C), at pH 6.5–9.0 (optimum between pH 7.5 and pH 8.0, with no growth at pH values of $\leqslant$5.7 or $\geqslant$9.5) and with 0–0.5 % (w/v) NaCl (optimum 0.1 %). In medium containing 20 g NaCl l^{-1}, cells did not divide but survived and subsequently grew successfully when transferred to medium of suitable salinity. Under optimal conditions and with glucose (2 g l^{-1}) as the growth substrate, the doubling time of strain P3M-1^T was 6 h.

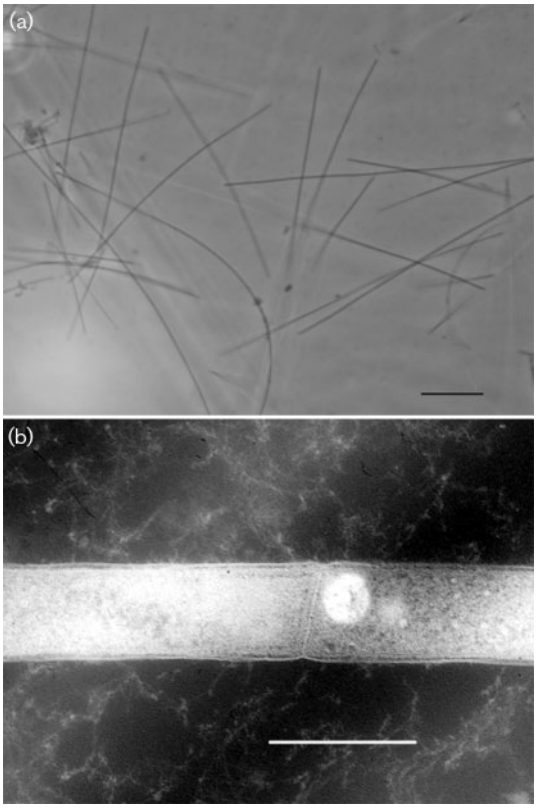

Fig. 1. Phase-contrast micrograph of strain P3M-1^T showing straight and curved filaments (a) and a transmission electron micrograph of a thin section of the novel strain, showing a distinct septum (b). Bars, 10 μm (a) and 1 μm (b).

With H_2 in high concentrations (>20 %, v/v) in the gas phase, the growth of strain P3M-1^T was inhibited and the strain's filaments became significantly longer. However, the growth of strain P3M-1^T was not completely inhibited when pure H_2 formed the gas phase, and the presence of a relatively low concentration of H_2 in the gas phase (5 %)

had no apparent effect on cell yield. As co-cultivation with hydrogenotrophic methanogens is known to stimulate the growth of some established members of the class *Anaerolineae*, strain P3M-1^T was cultivated with *Methanobacterium bryantii* VKM B-1629^T, but no significant increase in the growth of strain P3M-1^T was observed.

Strain P3M-1^T did not grow on medium without yeast extract; the minimal concentration of yeast extract required to support growth of the novel strain and the optimal concentration required (as substrate) were 0.025 and 1 g l^{-1}, respectively. In the presence of 2 g yeast extract l^{-1}, visible stagnation of growth occurred. Increasing the yeast extract concentration to 10 g l^{-1} led to complete inhibition of growth of strain P3M-1^T.

Utilization of growth substrates was studied, over three subcultures under anaerobic conditions, in modified Widdel medium with yeast extract at 0.05 g l^{-1} and the test compound at 2 g l^{-1} or 20 mM. Under these conditions, strain P3M-1^T was able to grow on yeast extract, beef extract, casein hydrolysate, glucose, xylose, sucrose, maltose, cellobiose and microcrystalline cellulose but not on peptone, starch, pectin, xylan, chitin, galactose, fructose, arabinose, pyruvate (2 g l^{-1}), acetate, lactate, ethanol or glycerol (20 mM). Also, the novel strain could not utilize formate (20 mM) plus acetate (20 mM) or H_2/CO_2 (80 : 20, v/v; 1 atm) plus acetate (20 mM). The products of glucose fermentation, which were investigated by the method of Miroshnichenko *et al.* (2008), were predominantly acetate, ethanol and H_2, with traces of lactate and formate also detected.

The presence of elemental sulfur (5 g l^{-1}), sodium sulphate (10 mM), sodium thiosulphate (10 mM), sodium nitrate (10 mM) or crystalline iron (III) oxide (90 mM) did not influence the growth of the novel isolate in the Avicel-containing medium, while sulphite (10 mM) inhibited growth. Sulphide formation (Trüper & Schlegel, 1964) was not detected when any reduced sulphur compounds were added to the medium.

For the analysis of cellular fatty acid methyl esters, cells of strain P3M-1^T were harvested from a culture grown under optimal conditions on modified Widdel medium containing glucose (1 g l^{-1}) and yeast extract (0.1 g l^{-1}), in the early stationary phase of growth. The analyses, performed by the methods of Sasser (1990), revealed that strain P3M-1^T contained iso-$C_{15:0}$ (39.3 %) and anteiso-$C_{15:0}$ (24.3 %) as its major cellular fatty acids. Isoprenoid quinones were extracted according to the method of Collins (1985) and analysed using an LCQ Advantage Max tandem-type mass spectrometer and a Mat 8430 ionization mass spectrometer (Finnigan); no quinones were detected in cells of strain P3M-1^T that had been grown under anaerobic conditions.

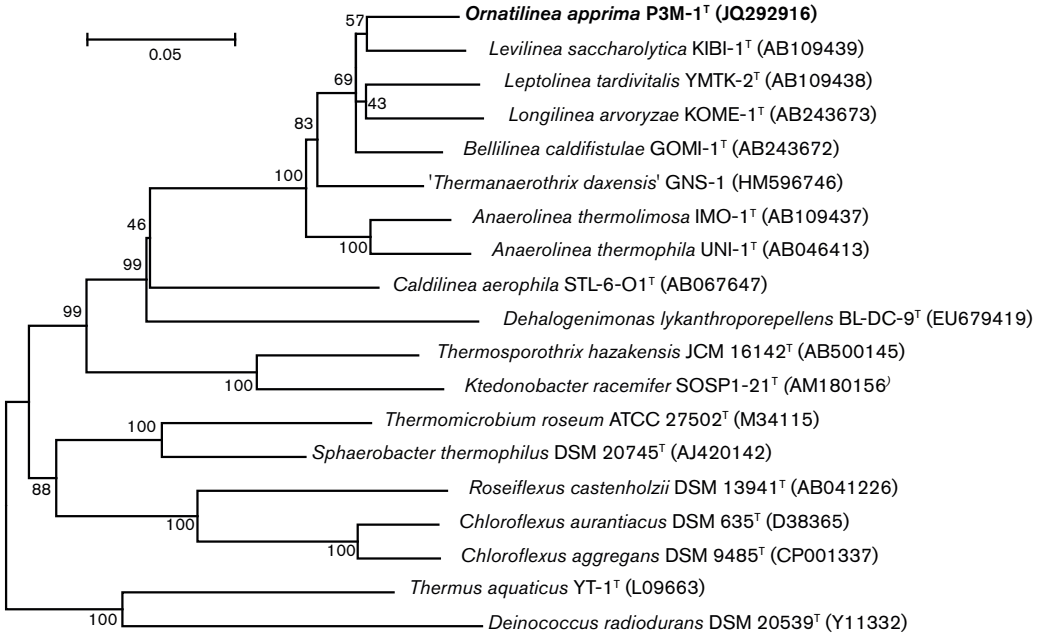

Fig. 2. A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain P3M-1^T and established members of the class *Anaerolineae* and other representatives of the phylum *Chloroflexi*. Percentage bootstrap values (based on 1000 replications) are shown at branch nodes. *Deinococcus radiodurans* DSM 20539^T and *Thermus aquaticus* YT-1^T were used as outgroups. Bar, 0.05 substitutions per nucleotide position.

Table 1. Characteristics of strain P3M-1^T and type strains of the type species of members of the class *Anaerolineae*

Strains: 1, P3M-1^T (data from this study); 2, *Levilinea saccharolytica* KIBI-1^T (Yamada *et al.*, 2006); 3, *Leptolinea tardivitalis* YMTK-2^T (Yamada *et al.*, 2006); 4, *Anaerolinea thermophila* UNI-1^T (Sekiguchi *et al.*, 2003); 5, *Longilinea arvoryzae* KOME-1^T (Yamada *et al.*, 2007); 6, *Bellilinea caldifistulae* GOMI-1^T (Yamada *et al.*, 2007); 7, '*Thermanaerothrix daxensis*' GNS-1 (Grégoire *et al.*, 2011). All strains form multicellular filaments, show no respiration of O_2, ferment yeast extract and sucrose, do not utilize lactate, acetate, ethanol, glycerol, formate plus acetate or H_2/CO_2 plus acetate, and produce hydrogen as a product of fermentation. As far as is known (the relevant data for '*Thermanaerothrix daxensis*' GNS-1 are not available), all strains also have no detectable quinones and do not use electron acceptors. +, Positive; −, negative; W, weakly positive; ND, no data available; SM, small amounts.

Characteristic	1	2	3	4	5	6	7
Origin	Mesophilic anaerobic mat	Mesophilic anaerobic sludge	Mesophilic anaerobic sludge	Thermophilic anaerobic sludge	Rice paddy soil	Thermophilic anaerobic sludge	Deep hot aquifer
Cell diameter (μm)	0.3–0.7	0.4–0.5	0.15–0.2	0.2–0.3	0.4–0.6	0.2–0.4	0.2–0.3
Min./optimum/max. temp. for growth (°C)	20/42–45/50	25/37/50	25/37/50	50/55/60	30/37/40	45/55/65	50/65/73
Min./optimum/max. pH for growth	6.5/7.5–8.0/9.0	6.0/7.0/7.2	6.0/7.0/7.2	6.0/7.0/8.0	5.0/7.0/8.5	6.0/7.0/7.5	5.8/7.0/8.5
NaCl tolerance (%, w/v)	<2	⩽3	<1.5	<1	<1.5	⩽3	⩽10
Doubling time (h)*	6 (6)	56 (56)	50 (50)	72 (48)	92 (38)	45 (29)	100
Substrates for growth:							
Glucose	+	+	+	+	−	+	+
Galactose	−	W	W	+	−	+	W
Fructose	−	+	+	+	W	+	+
Arabinose	−	−	W	W	−	+	W
Xylose	+	+	+	W	+	W	W
Xylan	−	W	+	W	+	W	+
Starch	−	−	W	+	−	−	−
Pectin	−	+	+	W	+	+	ND
Avicel	+	ND	ND	ND	ND	ND	ND
Peptone	−	W	W	ND	+	W	−
Pyruvate	−	+	W	W	−	+	+
Growth products:							
Acetate	+	+	+	+	+	+	+
Lactate	SM	SM	+	SM	+	+	+
Succinate	−	−	SM	SM	−	SM	−
Pyruvate	−	−	+	−	−	SM	−
Formate	SM	+	SM	SM	−	+	−
Ethanol	+	−	−	−	−	−	−
Major fatty acids	i-$C_{15:0}$, ai-$C_{15:0}$	Branched-$C_{14:0}$, i-$C_{15:0}$, $C_{16:0}$, branched-$C_{17:0}$	Branched-$C_{17:0}$, $C_{16:0}$, $C_{14:0}$, $C_{17:0}$	$C_{16:0}$, $C_{15:0}$, $C_{14:0}$, $C_{18:0}$	i-$C_{15:0}$, ai-$C_{15:0}$, $C_{14:0}$	$C_{16:0}$, $C_{14:0}$, i-$C_{15:0}$	$C_{16:0}$, $C_{18:0}$, i-$C_{17:0}$, $C_{20:0}$
DNA G+C content (mol%)	55	59.5	48.2	54.5	57.6	54.7	57.6

*Values in parentheses are the doubling times recorded when the strains were co-cultured with hydrogenotrophic methanogens.

Polar lipids were extracted and analysed by two-dimensional TLC, as described by Tindall (1990), on high-performance 100 × 100 mm plates (Sorbfil). Specific spray reagents for lipid phosphate (Vaskovsky & Kostetsky, 1968), free amino groups (ninhydrin), quaternary nitrogen compounds (Dragendorff's reagent) and sugars (α-naphthol, with subsequent charring) were used. The polar lipid profile of strain P3M-1^T was found to be dominated by two unidentified aminophosphoglycolipids and an unidentified glycophospholipid, with an unidentified phospholipid and an unidentified glycolipid also detected.

For the determination G+C content and the phylogenetic analyses, genomic DNA was extracted from the cells of strain P3M-1^T by the method of Park (2007). The genomic DNA G+C content of strain P3M-1^T was 55 mol%, as calculated from the complete genome sequence of the type strain. The almost-complete 16S rRNA gene sequence of strain P3M-1^T (1462 nt) was determined as described previously (Sokolova *et al.*, 2002). Comparison of this sequence with the 16S rRNA gene sequences of established species, using version 2.1 of the EzTaxon server (Kim *et al.*, 2012), indicated that the novel strain's closest relatives were members of the class *Anaerolineae* in the phylum *Chloroflexi*. In pairwise comparisons, strain P3M-1^T showed 16S rRNA gene sequence similarities of 89.6–92.8 % with type strains of established species in the class *Anaerolineae*. A phylogenetic tree based on the 16S rRNA gene sequences was constructed, by the neighbour-joining method (Saitou & Nei, 1987), within version 5.05 of the MEGA software package (Tamura *et al.*, 2011). Evolutionary distances were computed using the maximum-composite-likelihood method (Tamura *et al.*, 2004) and bootstrap values were determined (Felsenstein, 1985), with 1000 replications. In the tree (Fig. 2), the novel strain clustered with *Levilinea saccharolytica* KIBI-1^T but with an evolutionary distance between the two strains that indicated they should be considered to belong to different genera. A tree produced from the same sequences but by the maximum-likelihood method (Tamura & Nei, 1993) showed similar clustering (data not shown).

At the time of writing, the class *Anaerolineae* comprised seven species in six genera. Strain P3M-1^T has several features that are widespread among the members of this class: it is a non-spore-forming, non-motile, uncoloured micro-organism that forms multicellular filaments, has a Gram-reaction-negative cell wall, is strictly anaerobic, has no detectable quinones, and is able to grow chemo-organotrophically in the presence of yeast extract, producing H_2 and acetate (Table 1). There are, however, many features that differentiate strain P3M-1^T from established members of the class *Anaerolineae*: it is able to form biofilms; it is the first member of the class that has a slightly alkaline pH optimum; and it has a doubling time that is significantly shorter than that of other members of the class (i.e. 6 h for strain P3M-1^T vs 45 h for *Bellilinea caldifistulae* GOMI-1^T). Furthermore, strain P3M-1^T does not grow on fructose, xylan or pectin but is able to grow on various proteinaceous substrates and microcrystalline cellulose. Finally, unlike all established members of the class *Anaerolineae*, strain P3M-1^T produces ethanol during fermentation of glucose. Some phenotypic characteristics, such as DNA G+C content, the composition and ratio of fatty acids and the substrate utilization pattern (with strain P3M-1^T unable to grow on peptone, pyruvate or galactose) allow strain P3M-1^T to be distinguished from its closest relative, *L. saccharolytica* KIBI-1^T. The morphological, physiological and phylogenetic data allow strain P3M-1^T to be distinguished clearly from established members of the class *Anaerolineae*. Strain P3M-1^T represents a novel species of a new genus, for which the name *Ornatilinea apprima* gen. nov., sp. nov. is proposed.

Description of *Ornatilinea* gen. nov.

Ornatilinea (Or.na.ti.li′ne.a. L. adj. *ornatus* adorned, handsome; L. fem. n. *linea* line; N.L. fem. n. *Ornatilinea* handsome, line-shaped organism).

Cells are non-motile, Gram-reaction-negative and form multicellular filaments. Spores are not formed. Obligately anaerobic. The main fatty acids are iso-$C_{15:0}$ and anteiso-$C_{15:0}$. The genus lies in the family *Anaerolineaceae*. The type species is *Ornatilinea apprima*.

Description of *Ornatilinea apprima* sp. nov.

Ornatilinea apprima (ap.pri′ma. L. adj. *apprimus*, *-a*, *-um* the very first; L. fem. adj. *apprima* the very first representative of the genus).

Forms multicellular filaments that are longer than 200 μm and 0.3–0.7 μm wide. Growth occurs at 20–50 °C (optimum between 42 and 45 °C), at pH 6.5–9.0 (optimum between pH 7.5 and pH 8.0), and with 0–2.0 % (w/v) NaCl (optimum 0.1 %). Doubling time in culture under optimal conditions is 6 h. Yeast extract is required for growth. In the presence of yeast extract at 0.05 g l^{-1}, grows on beef extract, casein hydrolysate, glucose, xylose, sucrose, maltose, cellobiose and microcrystalline cellulose but not peptone, starch, pectin, xylan, chitin, galactose, fructose, arabinose, pyruvate, acetate, lactate, ethanol, glycerol, H_2/CO_2 plus acetate or formate plus acetate. Does not use sulphate, sulphite, thiosulphate, elemental sulphur, nitrate or crystalline iron (III) oxide as electron acceptors.

The type strain, P3M-1^T (=DSM 23815^T=VKM B-2669^T), was isolated from a microbial mat that had formed in a wooden bath filled with hot water from a 2775 m-deep well in the Tomsk region (Siberia, Russia). The genomic DNA G+C content of the type strain is 55 mol%, as calculated from the complete genome sequence of the type strain.

Acknowledgements

This work was supported by the Russian Federal Ministry of Education and Science (via grants #16.512.11.2152, #11.519.11.2029 and П646)

and by the 'Molecular and cell biology' and 'The origin and evolution of the biosphere' programmes of the Russian Academy of Sciences. We thank Andrew Mardanov (Bioengineering Center, Russian Academy of Sciences, Moscow) for his assistance with the determination of DNA G+C contents, Victoria Shcherbakova (All-Russian Collection of Microorganisms, Pushchino) for her help with the acquisition of *M. bryantii* VKM B-1629^T, Nikolai Chernyh (Winogradsky Institute of Microbiology, Russian Academy of Sciences, Moscow) for his help with the confocal laser scanning microscopy, and Olga Karnachuk (Tomsk State University, Tomsk) and Sergey Gavrilov (Winogradsky Institute of Microbiology, RAS, Moscow) for their help with sampling.

References

Bonch-Osmolovskaya, E. A., Sokolova, T. G., Kostrikina, N. A. & Zavarzin, G. A. (1990). *Desulfurella acetovorans* gen. nov., sp. nov. – a new thermophilic sulfur-reducing eubacterium. *Arch Microbiol* **153**, 151–155.

Cavaletti, L., Monciardini, P., Bamonte, R., Schumann, P., Rohde, M., Sosio, M. & Donadio, S. (2006). New lineage of filamentous, spore-forming, Gram-positive bacteria from soil. *Appl Environ Microbiol* **72**, 4360–4369.

Collins, M. D. (1985). Analysis of isoprenoid quinones. *Methods Microbiol* **18**, 329–366.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Geissinger, O., Herlemann, D. P. R., Mörschel, E., Maier, U. G. & Brune, A. (2009). The ultramicrobacterium "*Elusimicrobium minutum*" gen. nov., sp. nov., the first cultivated representative of the termite group 1 phylum. *Appl Environ Microbiol* **75**, 2831–2840.

Grégoire, P., Fardeau, M.-L., Joseph, M., Guasco, S., Hamaide, F., Biasutti, S., Michotey, V., Bonin, P. & Ollivier, B. (2011). Isolation and characterization of *Thermanaerothrix daxensis* gen. nov., sp. nov., a thermophilic anaerobic bacterium pertaining to the phylum "*Chloroflexi*", isolated from a deep hot aquifer in the Aquitaine Basin. *Syst Appl Microbiol* **34**, 494–497.

Hanada, S., Takaichi, S., Matsuura, K. & Nakamura, K. (2002). *Roseiflexus castenholzii* gen. nov., sp. nov., a thermophilic, filamentous, photosynthetic bacterium that lacks chlorosomes. *Int J Syst Evol Microbiol* **52**, 187–193.

Hugenholtz, P., Pitulle, C., Hershberger, K. L. & Pace, N. R. (1998). Novel division level bacterial diversity in a Yellowstone hot spring. *J Bacteriol* **180**, 366–376.

Kevbrin, V. V. & Zavarzin, G. A. (1992). The effect of sulfur compounds on growth of halophilic the homoacetic bacterium *Acetohalobium arabaticum*. *Microbiology (English translation of Microbiologiia)* **61**, 563–567.

Kim, O.-S., Cho, Y.-J., Lee, K., Yoon, S.-H., Kim, M., Na, H., Park, S.-C., Jeon, Y. S., Lee, J.-H., Yi, H., Won, S. & Chun, J. (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* **62**, 716–721.

McKeown, R. M., Scully, C., Enright, A.-M., Chinalia, F. A., Lee, C., Mahony, T., Collins, G. & O'Flaherty, V. (2009). Psychrophilic methanogenic community development during long-term cultivation of anaerobic granular biofilms. *ISME J* **3**, 1231–1242.

Miroshnichenko, M. L., Kublanov, I. V., Kostrikina, N. A., Tourova, T. P., Kolganova, T. V., Birkeland, N. K. & Bonch-Osmolovskaya, E. A. (2008). *Caldicellulosiruptor kronotskyensis* sp. nov. and *Caldicellulosiruptor hydrothermalis* sp. nov., two extremely thermophilic, cellulolytic, anaerobic bacteria from Kamchatka thermal springs. *Int J Syst Evol Microbiol* **58**, 1492–1496.

Moe, W. M., Yan, J., Nobre, M. F., da Costa, M. S. & Rainey, F. A. (2009). *Dehalogenimonas lykanthroporepellens* gen. nov., sp. nov., a reductively dehalogenating bacterium isolated from chlorinated solvent-contaminated groundwater. *Int J Syst Evol Microbiol* **59**, 2692–2697.

Park, D. (2007). Genomic DNA isolation from different biological materials. In *Methods in Molecular Biology, Version 353: Protocols for Nucleic Acid Analysis by Nonradioactive Probes*, 2nd edn, pp. 3–13. Edited by E. Hilario & J. Mackay. Totowa, NJ: Humana Press.

Rappé, M. S. & Giovannoni, S. J. (2003). The uncultured microbial majority. *Annu Rev Microbiol* **57**, 369–394.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Sasser, M. (1990). *Identification of bacteria by gas chromatography of cellular fatty acids*, MIDI Technical Note 101. Newark, DE: MIDI Inc.

Sekiguchi, Y., Yamada, T., Hanada, S., Ohashi, A., Harada, H. & Kamagata, Y. (2003). *Anaerolinea thermophila* gen. nov., sp. nov. and *Caldilinea aerophila* gen. nov., sp. nov., novel filamentous thermophiles that represent a previously uncultured lineage of the domain *Bacteria* at the subphylum level. *Int J Syst Evol Microbiol* **53**, 1843–1851.

Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–651. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.

Sokolova, T. G., Kostrikina, N. A., Chernyh, N. A., Tourova, T. P., Kolganova, T. V. & Bonch-Osmolovskaya, E. A. (2002). *Carboxydocella thermautotrophica* gen. nov., sp. nov., a novel anaerobic, CO-utilizing thermophile from a Kamchatkan hot spring. *Int J Syst Evol Microbiol* **52**, 1961–1967.

Tamura, K. & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* **10**, 512–526.

Tamura, K., Nei, M. & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc Natl Acad Sci U S A* **101**, 11030–11035.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731–2739.

Tindall, B. J. (1990). Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* **66**, 199–202.

Trüper, H. G. & Schlegel, H. G. (1964). Sulphur metabolism in *Thiorhodaceae*. I. Quantitative measurements on growing cells of *Chromatium okenii*. *Antonie van Leeuwenhoek* **30**, 225–228.

Vaskovsky, V. E. & Kostetsky, E. Y. (1968). Modified spray for the detection of phospholipids on thin-layer chromatograms. *J Lipid Res* **9**, 396.

Widdel, F. & Bak, F. (1992). Gram-negative mesophilic sulfate reducing bacteria. In *The Prokaryotes*, 2nd edn, vol. 4, pp. 3352–3378. Edited by A. Balows, H. G. Truper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.

Wolin, E. A., Wolin, M. J. & Wolfe, R. S. (1963). Formation of methane by bacterial extracts. *J Biol Chem* **238**, 2882–2886.

Yamada, T., Sekiguchi, Y., Hanada, S., Imachi, H., Ohashi, A., Harada, H. & Kamagata, Y. (2006). *Anaerolinea thermolimosa* sp. nov., *Levilinea saccharolytica* gen. nov., sp. nov. and *Leptolinea tardivitalis* gen. nov., sp. nov., novel filamentous anaerobes, and description of the new classes *Anaerolineae* classis nov. and

Caldilineae classis nov. in the bacterial phylum *Chloroflexi*. *Int J Syst Evol Microbiol* **56**, 1331–1340.

Yamada, T., Imachi, H., Ohashi, A., Harada, H., Hanada, S., Kamagata, Y. & Sekiguchi, Y. (2007). *Bellilinea caldifistulae* gen. nov., sp. nov. and *Longilinea arvoryzae* gen. nov., sp. nov., strictly anaerobic, filamentous bacteria of the phylum *Chloroflexi* isolated from methanogenic propionate-degrading consortia. *Int J Syst Evol Microbiol* **57**, 2299–2306.