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. Kolganova³ and I. V. Kublanov¹

Correspondence

O. A. Podosokorskaya
podosokorskaya@gmail.com

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

²Gubkin Russian State University of Oil and Gas, Leninskiy Prospekt 65, 117485 Moscow, Russia

³Bioengineering 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 H₂ 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

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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 $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (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 ($58^\circ 50' 05.3'' \text{ N } 81^\circ 30' 07.5'' \text{ E}$). 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 $0.3\text{--}0.7 \mu\text{m}$; the length of a single cell was indefinite, whereas the length of straight or curved multicellular filaments ranged from $15\text{--}200 \mu\text{m}$ (Fig. 1a). Occasionally, filaments of up to $400 \mu\text{m}$ 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 $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ was considerably weaker than that in reduced medium, and ceased completely under microaerophilic conditions (2% oxygen,

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, $10 \mu\text{m}$ (a) and $1 \mu\text{m}$ (b).

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 $20\text{--}50^\circ\text{C}$ (optimum between 42 and 45°C , with no growth at temperatures of ≤ 15 or $\geq 52^\circ\text{C}$), at pH $6.5\text{--}9.0$ (optimum between pH 7.5 and pH 8.0, with no growth at pH values of ≤ 5.7 or ≥ 9.5) and with $0\text{--}0.5\%$ (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.

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 H₂/CO₂ (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 H₂, 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* KOMI-1^T (Yamada *et al.*, 2007); 6, *Bellilinea caldifistulae* GOMI-1^T (Yamada *et al.*, 2007); 7, '*Thermanaerotherix daxensis*' GNS-1 (Grégoire *et al.*, 2011). All strains form multicellular filaments, show no respiration of O₂, ferment yeast extract and sucrose, do not utilize lactate, acetate, ethanol, glycerol, formate plus acetate or H₂/CO₂ plus acetate, and produce hydrogen as a product of fermentation. As far as is known (the relevant data for '*Thermanaerotherix 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 glycerophospholipid, 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 chemotrophically in the presence of yeast extract, producing H₂ 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, H₂/CO₂ 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.

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