# *Bryobacter aggregatus* gen. nov., sp. nov., a peat-inhabiting, aerobic chemo-organotroph from subdivision 3 of the *Acidobacteria*

Irina S. Kulichevskaya,[1] Natalia E. Suzina,[2] Werner Liesack[3] and Svetlana N. Dedysh[1]

Correspondence
Svetlana N. Dedysh
dedysh@mail.ru

[1]S. N. Winogradsky Institute of Microbiology, Russian Academy of Sciences, Moscow 117312, Russia

[2]G. K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow region, 142292, Russia

[3]Max-Planck-Institut für terrestrische Mikrobiologie, D-35043 Marburg, Germany

*Bryobacter aggregatus* gen. nov., sp. nov. is proposed to accommodate three strains of slowly growing, chemo-organotrophic bacteria isolated from acidic *Sphagnum* peat bogs. These bacteria were strictly aerobic, Gram-negative, colourless, non-motile coccoids or short rods that multiplied by normal cell division and formed irregularly shaped cell aggregates. Strains MPL3$^T$, MPL1011 and MOB76 were acidotolerant, mesophilic organisms capable of growth at pH 4.5–7.2 and between 4 and 33 °C (optimum growth at pH 5.5–6.5 and 22–28 °C). The preferred growth substrates were sugars, some heteropolysaccharides and galacturonic and glucuronic acids, which are released during decomposition of *Sphagnum* moss. The major fatty acids were iso-$C_{15:0}$, $C_{16:0}$ and summed feature 3 (iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$); the major quinones were MK-9 and MK-10. The DNA G+C content was 55.5–56.5 mol%. Strains MPL3$^T$, MPL1011 and MOB76 possessed nearly identical 16S rRNA gene sequences and belonged to the phylum *Acidobacteria*. They represent the first taxonomically characterized members of acidobacterial subdivision 3 and display only 81.7–86.7 % 16S rRNA gene sequence similarity to other members of the *Acidobacteria* with validly published names. Therefore, strains MPL3$^T$, MPL1011 and MOB76 are classified as representatives of a novel species in a new genus, for which the name *Bryobacter aggregatus* gen. nov., sp. nov. is proposed; strain MPL3$^T$ (=ATCC BAA-1390$^T$ =DSM 18758$^T$) is the type strain of *Bryobacter aggregatus*.

The *Acidobacteria* is one of the globally distributed but little understood phylogenetic groups of the domain *Bacteria* (Ludwig *et al.*, 1997; Hugenholtz *et al.*, 1998; Barns *et al.*, 1999; Janssen, 2006). The taxonomically described diversity within this phylum remains limited. So far, only subdivisions 1 and 8 of the *Acidobacteria* include taxonomically characterized representatives. These are *Acidobacterium capsulatum* (Kishimoto *et al.*, 1991), *Terriglobus roseus* (Eichorst *et al.*, 2007), *Edaphobacter modestus* and *Edaphobacter aggregans* (Koch *et al.*, 2008) in subdivision 1 and *Holophaga foetida* (Liesack *et al.*, 1994), *Geothrix fermentans* (Coates *et al.*, 1999) and *Acanthopleuribacter pedis* (Fukunaga *et al.*, 2008) in subdivision 8. A variety of acidobacterial strains from subdivisions 1, 2, 3 and 4 have been isolated from different soils by using newly developed media and extended incubation times (Janssen *et al.*, 2002; Sait *et al.*, 2002; Joseph *et al.*, 2003; Davis *et al.*, 2005; Stott *et al.*, 2008). Two of these strains, Ellin345 from subdivision 1 of the *Acidobacteria* and Ellin6076 from subdivision 3, have been tentatively named '*Koribacter versatilis*' and '*Solibacter usitatus*', respectively, and subjected to genome sequencing (Ward *et al.*, 2009), but they have not yet been described taxonomically.

Recently, we showed that members of the *Acidobacteria* are a characteristic component of the microbial community in *Sphagnum*-dominated acidic wetlands (Dedysh *et al.*, 2006; Pankratov *et al.*, 2008). Using low-nutrient media and fluorescent *in situ* hybridization-mediated monitoring of the isolation procedure, several peat-inhabiting acidobacteria were obtained in pure culture. Three of these strains, MPL3$^T$, MPL1011 and MOB76, belonged to subdivision 3 of the *Acidobacteria*, for which no representatives with validly published names are known. Here, the characteristics of our novel isolates are described and a novel species in a new genus is proposed to accommodate these bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Bryobacter aggregatus* strains MPL3$^T$, MPL1011 and MOB76 are AM162405, AM887761 and AM887762, respectively.

Two strains, MPL3$^T$and MPL1011, were isolated from a peat soil (pH 4.0) sampled at a depth of 10–20 cm of the *Sphagnum* peat bog Bakchar, Tomsk region, West Siberia (56° 51′ N 82° 50′ E), in July 2004. The third strain, MOB76, was obtained from a peat sample (pH 4.2) collected from the upper (0–10 cm) oxic layer of the bog Obukhovskoe, Yaroslavl region, European North Russia (58° 14′ N 38° 12′ E). The biofilm-mediated enrichment approach used for isolation of these bacteria was described by Dedysh *et al.* (2006). All isolates were obtained and maintained on tenfold-diluted R2A medium (Difco), pH 5.8.

Morphological observations and cell size measurements were made with a Zeiss Axioplan 2 microscope and Axiovision 4.2 software (Zeiss). For preparation of ultrathin sections, cells of exponentially growing cultures were collected by centrifugation and pre-fixed with 1.5 % (w/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 6.5) for 1 h at 4 °C and then fixed in 1 % (w/v) $OsO_4$ in the same buffer for 4 h at 20 °C. After dehydration in an ethanol series, samples were embedded in Spurr epoxy resin. Thin sections were cut on an LKB-4800 microtome and stained with 3 % (w/v) uranyl acetate in 70 % (v/v) ethanol. Specimen samples were examined with a JEM-100C transmission electron microscope.

Physiological tests were performed in batch cultures grown in liquid MM medium in tightly closed 120 ml serum bottles containing 10 ml medium. MM medium (pH 5.8) contained 1 ml metal salt solution '44' (Staley *et al.*, 1992) and (g per litre distilled water): $KH_2PO_4$, 0.1; $(NH_4)_2SO_4$, 0.1; $MgSO_4 . 7H_2O$, 0.1; glucose, 0.5; and yeast extract, 0.1. Growth of strains MPL3$^T$, MPL1011 and MOB76 could not be monitored by using nephelometry as they form cell aggregates in liquid culture. Therefore, their growth was followed by measuring the rate of $CO_2$ production in liquid cultures incubated for 14 days under a variety of conditions, including temperatures of 4–37 °C and at pH 3.0–8.0. $CO_2$ was measured by GC with a thermal conductivity detector. With the exception of the temperature experiment, flasks were incubated on a rotary shaker at 120 r.p.m. and 24 °C. Variations in pH were achieved by mixing 0.1 M solutions of $H_2SO_4$ and KOH. Carbon source utilization and the ability of isolates to degrade different biopolymers were determined using liquid MM medium in which glucose was replaced with one of the various carbon sources or polymer substrates (0.05 %, w/v). Control incubations that lacked substrate were run in parallel under the same conditions. Oxidative and fermentative utilization of carbohydrates was determined by using the API 20NE kit (bioMérieux). Enzyme activities were examined by using the API ZYM kit (bioMérieux). Nitrogen sources were tested using liquid MM medium with 0.05 % glucose in which $(NH_4)_2SO_4$ was replaced with one of the following compounds at a concentration of 0.01 % (w/v): $KNO_3$, $KNO_2$, urea or one of the amino acids listed in the species description. Cultures were tested for growth under anaerobic conditions in anaerobic jars by using AnaeroGen anaerobic system envelopes (Oxoid) and also in liquid cultures using MM medium prepared under a nitrogen atmosphere. Nitrate respiration was examined in the same medium by replacing $(NH_4)_2SO_4$ with $KNO_3$. Susceptibility to antibiotics was determined on MM agar plates using discs containing the following antibiotics (Oxoid): ampicillin (10 μg), gentamicin (10 μg), kanamycin (30 μg), neomycin (10 μg), novobiocin (30 μg), streptomycin (10 μg), chloramphenicol (30 μg) and lincomycin (10 μg).

Cell biomass for cellular fatty acid and isoprenoid quinone analyses and DNA extraction was obtained from batch cultures grown in liquid MM medium at 24 °C for 1 month. Fatty acid profiles were analysed by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) as described by Kämpfer & Kroppenstedt (1996). Isoprenoid quinones were extracted according to Collins (1985) and analysed using tandem-type MS LCQ Advantage Max and ionization MS Finnigan MAT 8430. The DNA G+C contents of the strains were determined by thermal denaturation using a Unicam SP1800 spectrophotometer at a heating rate of 0.5 °C $min^{-1}$; G+C contents were calculated according to Owen *et al.* (1969). The 16S rRNA gene sequences of strains MPL3$^T$, MPL1011 and MOB76 were determined previously (Dedysh *et al.*, 2006; Pankratov *et al.*, 2008). Phylogenetic analysis was carried out using the ARB program package (Ludwig *et al.*, 2004). Trees were constructed using distance-based (neighbour-joining), maximum-likelihood (DNAML) and maximum-parsimony methods. The significance levels of interior branch points obtained in the neighbour-joining analysis were determined by bootstrap analysis (1000 data resamplings) using PHYLIP (Felsenstein, 1989).

On solid agar medium, strains MPL3$^T$, MPL1011 and MOB76 formed very small (<0.5 mm in diameter), raised, smooth, colourless, circular colonies with entire margins. Colonies were visible to the naked eye only after approximately 3–4 weeks of incubation. Increasing the incubation time to 6–8 weeks did not result in a significant increase in colony size. Liquid cultures were non-homogeneous due to the formation of cellular conglomerates.

Cells of the isolates were non-motile coccoids or short rods measuring 0.5–0.8 × 0.8–1.3 μm (Fig. 1a). They multiplied by normal cell division and formed irregularly shaped cell aggregates. Cells were Gram-negative according to the Gram-staining and KOH test. Thin-sectioned cells of strain MPL3$^T$ displayed a cell-wall structure similar to that in Gram-negative bacteria. The outer membrane, the cytoplasmic membrane and an extensive periplasmic space filled with an electron-dense substance were evident in ultrathin sections (Fig. 1b, c). A highly distinctive feature, however, was the absence of a visible peptidoglycan layer, which could not be revealed using the standard protocol for cell fixation and staining. This feature has not been described for other taxonomically characterized acidobac-

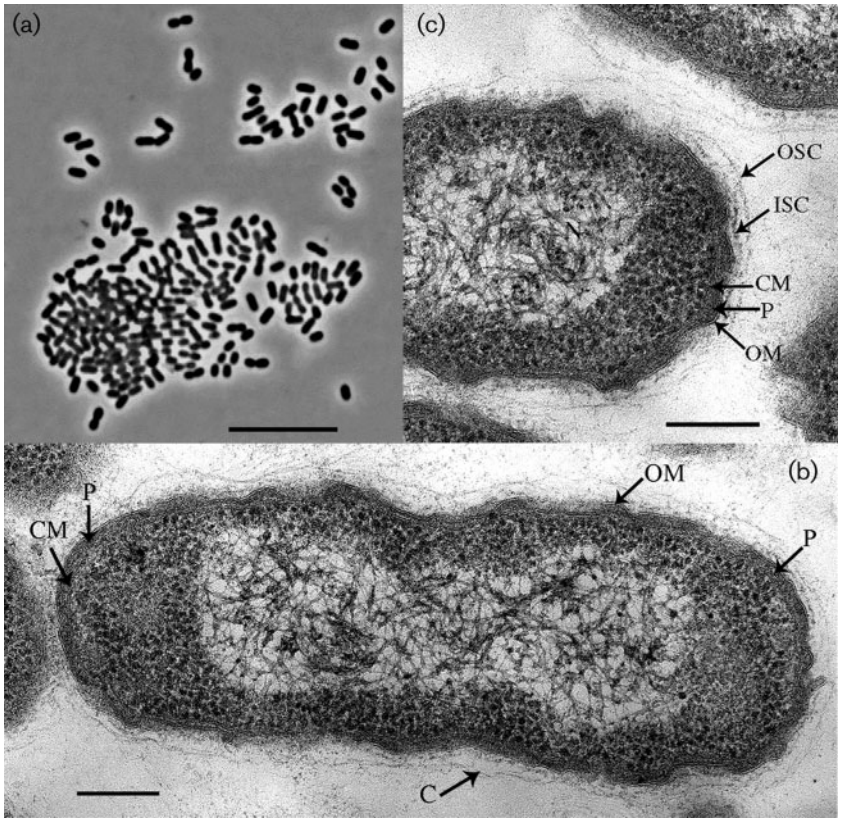


**Fig. 1.** Phase-contrast micrographs (a) and electron micrographs of ultrathin sections (b, c) of cells of strain MPL3$^T$. OM, Outer membrane; CM, cytoplasmic membrane; P, periplasm; OSC, outer surface of a capsule; ISC, inner surface of a capsule; C, capsule. Bars, 10 μm (a) and 0.2 μm (b, c).

teria. Internal membranes or other specialized structures were not observed in cells of strains MPL3$^T$, MPL1011 and MOB76. As revealed by contrasting the acid mucopolysaccharides with ruthenium red, the cells were covered with 40 nm thick capsules of unusual structure. Two electron-dense layers representing the inner and the outer surfaces of this capsule were separated by an electron-transparent layer; the inner surface of this capsule was close to the outer membrane.

Phenotypic characteristics of the peat-inhabiting strains MPL3$^T$, MPL1011 and MOB76 are summarized in Table 1. These isolates were strictly aerobic chemo-organotrophs that grew best on media containing sugars. Most organic acids were utilized either poorly or not at all. However, the isolates were capable of utilizing galacturonic and glucuronic acids, two characteristic components of the cell wall in *Sphagnum* spp. (Clymo, 1963, 1964), which are released during moss debris decomposition. Results of carbon and nitrogen source utilization tests are given in the species description (see below) and in Table 1. Growth factors were required. The isolates were incapable of nitrate respiration. Strains MPL3$^T$, MPL1011 and MOB76 were oxidase- and urease-negative, but catalase-positive. They had the following enzyme activities: β-galactosidase, alkaline and acid phosphatases, esterases (C4 and C8), leucyl and valyl arylamidases, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Lipase, cystine arylamidase, α-galactosidase, β-glucuronidase and α-glucosidase activities were absent (API ZYM test). Strains MPL3$^T$, MPL1011 and MOB76 were resistant to ampicillin, chloramphenicol, lincomycin, kanamycin and neomycin, but sensitive to streptomycin, novobiocin and gentamicin.

The three strains grew at pH 4.5–7.2, with optimum growth at pH 5.5–6.5. The temperature range for growth was 4–33 °C, with optimum growth at 22–28 °C. Growth was inhibited completely at NaCl concentrations above 1.5 % (w/v). After a few years of maintenance in laboratory media, the doubling time of these bacteria under optimal growth conditions was 18–20 h.

Strains MPL3$^T$, MPL1011 and MOB76 contained MK-9 and MK-10 as the predominant isoprenoid quinones. The major cellular fatty acids were iso-$C_{15:0}$, $C_{16:0}$ and summed feature 3 (iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$) (Table 2). Large proportions of these fatty acids were also characteristic for previously described acidobacteria.

Strains MPL3$^T$, MPL1011 and MOB76 possessed nearly identical 16S rRNA gene sequences and belonged to subdivision 3 of the *Acidobacteria* (Fig. 2), being only distantly related (90 % sequence similarity) to several acidobacterial isolates (Ellin342, Ellin371, Ellin6071, Ellin6115 and '*Solibacter usitatus*' Ellin6076) obtained from soils (Sait *et al.*, 2002; Joseph *et al.*, 2003). Sequence similarities of strains MPL3$^T$, MPL1011 and MOB76 to taxonomically characterized acidobacteria were 85.7–

**Table 1.** Phenotypic characteristics of strains MPL3$^T$, MPL1011 and MOB76

All strains utilized glucose, fructose, galactose, lactose, cellobiose, maltose, mannose, melibiose, melezitose, rhamnose, ribose, trehalose, sucrose, xylose, raffinose, lactulose, *N*-acetylglucosamine, salicin, arbutin, starch, galacturonic acid and glucuronic acid. None of the strains utilized acetate, benzoate, caproate, citrate, ethanol, formate, formaldehyde, fumarate, glycerol, malate, mannitol, methanol, oxalate, propionate, succinate or tartrate. All strains were capable of hydrolysing aesculin, alginate, casein, chondroitin sulphate, gelatin, pectin, peptone and protein hydrolysate. None of the strains were capable of hydrolysing laminarin, fucoidan, pullulan, xylan, cellulose, CM-cellulose, chitin or chitosan. All strains utilized ammonia, nitrate, peptone, yeast extract, alanine, valine, asparagine, isoleucine, lysine, ornithine and methionine as nitrogen sources. None of the strains utilized nitrite, urea, arginine, glycine or serine. w, Weak growth.

| Characteristic | MPL3$^T$ | MPL1011 | MOB76 |
|---|---|---|---|
| Cell size (μm) | 0.5–0.8 × 0.8–1.1 | 0.6–0.8 × 0.8–1.3 | 0.6–0.8 × 0.9–1.0 |
| Temperature range (°C) | 6–33 | 4–33 | 6–33 |
| Temperature optimum (°C) | 22–28 | 15–26 | 22–28 |
| Carbon source utilization | | | |
| Arabinose | + | + | − |
| Sorbose | + | + | − |
| Dulcitol | w | + | − |
| Sorbitol | + | − | w |
| Fucose | + | w | w |
| Lactate | w | + | − |
| Pyruvate | + | + | w |
| DNA G+C content (mol%) | 55.6 | 55.5 | 56.5 |

**Table 2.** Cellular fatty acid compositions of strains MPL3$^T$, MPL1011 and MOB76

Values are percentages of total fatty acids. −, Not detected. Major fatty acids are highlighted in bold.

| Fatty acid | MPL3$^T$ | MPL1011 | MOB76 |
|---|---|---|---|
| $C_{14:1}\omega5c$ | 0.2 | 0.1 | − |
| $C_{14:0}$ | 1.3 | 0.9 | 0.6 |
| **iso-$C_{15:0}$** | **27.8** | **28.8** | **25.1** |
| anteiso-$C_{15:0}$ | 0.5 | − | 0.9 |
| $C_{15:1}\omega6c$ | 0.4 | 0.4 | 0.4 |
| $C_{15:0}$ | 1.3 | 0.4 | − |
| **Summed feature 3*** | **36.1** | **37.3** | **34.9** |
| $C_{16:0}$ N alcohol | 1.1 | 0.5 | 1.0 |
| **$C_{16:0}$** | **17.3** | **19.2** | **24.2** |
| $C_{15:0}$ 2-OH | − | − | 0.4 |
| $C_{15:0}$ 3-OH | 1.0 | − | − |
| iso-$C_{17:1}\omega9c$ | 0.9 | 0.5 | 0.4 |
| iso-$C_{17:0}$ | 3.9 | 6.7 | 2.2 |
| anteiso-$C_{17:0}$ | 0.3 | − | 0.5 |
| $C_{17:1}\omega6c$ | − | − | 0.4 |
| $C_{17:0}$ | 0.6 | 0.4 | 0.9 |
| $C_{16:0}$ 3-OH | 0.4 | 0.3 | 0.7 |
| $C_{18:1}\omega9c$ | 1.0 | 0.7 | 1.0 |
| $C_{18:1}\omega7c$ | 1.1 | 0.7 | 4.9 |
| $C_{18:1}\omega5c$ | 1.1 | 0.9 | − |
| $C_{18:0}$ | 1.0 | 1.1 | 0.8 |
| iso-$C_{17:0}$ 3-OH | 0.4 | 0.4 | 0.7 |

*Summed feature 3 comprises $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH.

86.7 % for members of the genus *Edaphobacter*, 85.8–86.0 % for members of the genus *Terriglobus*, 84.7 % for the type strain of *Acidobacterium capsulatum* and 81.7–83.7 % for subdivision 8 acidobacteria, i.e. the type strains of *Geothrix fermentans*, *Holophaga foetida* and *Acanthopleuribacter pedis*. The DNA G+C contents of the novel strains ranged between 55.5 and 56.5 mol%.

In summary, our isolates from *Sphagnum* peat bogs are the first taxonomically characterized members of subdivision 3 of the *Acidobacteria*. It is therefore proposed that strains MPL3$^T$, MPL1011 and MOB76 should be classified as representatives of a novel species in a new genus, *Bryobacter aggregatus* gen. nov., sp. nov.

## Description of *Bryobacter* gen. nov.

*Bryobacter* [Bryo.bac′ter. Gr. neut. n. *bryon* moss; N.L. masc. n. *bacter* (equivalent of Gr. neut. n. *baktron*) a short rod; N.L. masc. n. *Bryobacter* rod-shaped moss-associated bacterium].

Cells are non-motile, Gram-negative coccoids or short rods. Encapsulated. Reproduce by normal cell division. Colonies are very small and non-pigmented. Strictly aerobic chemo-organotrophs. Various sugars are the preferred growth substrates. Capable of hydrolysing several heteropolysaccharides. With the exception of glucuronate and galacturonate, most organic acids are utilized poorly or not at all. Catalase-positive, but cytochrome oxidase- and urease-negative. Acidotolerant and mesophilic. The major quinones are MK-9 and MK-10. Major cellular fatty acids are iso-$C_{15:0}$, $C_{16:0}$ and summed feature 3 (iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$). The DNA G+C content is 55.5–56.5 mol%. Member of subdivision 3 of the phylum *Acidobacteria*. The type species is *Bryobacter aggregatus*.

## Description of *Bryobacter aggregatus* sp. nov.

*Bryobacter aggregatus* (ag.gre.ga′tus. L. masc. adj. *aggregatus* joined together, referring to the frequent formation of cell aggregates).

Description is as for the genus with the following additional information. Cells are 0.5–0.8 μm wide by 0.8–1.3 μm long and form irregularly shaped cell aggregates. Carbon sources utilized (0.05 %, w/v) include glucose, fructose, galactose, lactose, cellobiose, maltose, mannose, melibiose, melezitose, rhamnose, ribose, trehalose, sucrose, xylose, raffinose, lactulose, *N*-acetylglucosamine, salicin, arbutin, alginate, galacturonic acid and glucuronic acid. The ability to utilize arabinose, sorbose, dulcitol, sorbitol, fucose, lactate and pyruvate is variable across strains.

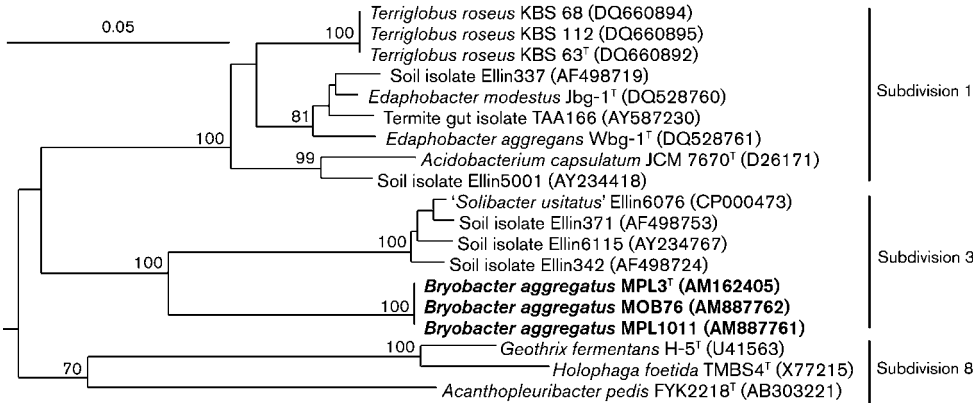


**Fig. 2.** 16S rRNA gene-based neighbour-joining tree (Jukes–Cantor correction) showing the phylogenetic relationship between the novel peatland isolates, taxonomically characterized representatives and some non-described members of the phylum *Acidobacteria*. The tree was constructed based on 1254 nt positions. Bootstrap values (1000 data resamplings) >50 % are shown. Six members of the *Planctomycetes*, *Isosphaera pallida* strain 563 (GenBank accession no. AJ231193), *Gemmata obscuriglobus* UQM 2246$^T$ (X54522), *Planctomyces brasiliensis* DSM 5305$^T$ (AJ231190), *Planctomyces maris* DSM 8797$^T$ (AJ231184), *Schlesneria paludicola* MPL7$^T$ (AM162407) and *Singulisphaera acidiphila* ATCC BAA-1392$^T$ (AM850678), were used as an outgroup (not shown). Bar, 0.1 substitutions per nucleotide position. Similar tree topology was obtained using maximum-likelihood method.

Unable to utilize acetate, benzoate, caproate, citrate, ethanol, formate, formaldehyde, fumarate, glycerol, malate, mannitol, methanol, oxalate, propionate, succinate or tartrate. Capable of hydrolysing aesculin, casein, chondroitin sulphate, gelatin, pectin, peptone, protein hydrolysate and starch. Unable to hydrolyse laminarin, fucoidan, pullulan, xylan, cellulose, CM-cellulose, chitin or chitosan. Cells show the following enzyme activities (API ZYM test): $\beta$-galactosidase, alkaline and acid phosphatases, esterases (C4 and C8), leucyl and valyl arylamidases, $\alpha$-chymotrypsin, naphthol-AS-BI-phosphohydrolase, $\beta$-glucosidase, *N*-acetyl-$\beta$-glucosaminidase, $\alpha$-mannosidase and $\alpha$-fucosidase. Able to utilize the following nitrogen sources: ammonia, nitrate, Bacto peptone, Bacto yeast extract, alanine, valine, asparagine, isoleucine, lysine, ornithine and methionine. Nitrite, urea, arginine, glycine and serine are not utilized. Growth factors are required. Resistant to ampicillin, chloramphenicol, lincomycin, kanamycin and neomycin, but sensitive to streptomycin, novobiocin and gentamicin. Growth occurs at pH 4.5–7.2 and between 4 and 33 °C (optimum growth at pH 5.5–6.5 and 22–28 °C). NaCl inhibits growth at concentrations above 1.5 % (w/v).

The type strain is MPL3$^T$ (=ATCC BAA-1390$^T$ =DSM 18758$^T$), isolated from the *Sphagnum* peat bog Bakchar, Tomsk region, West Siberia. Reference strains MPL1011 and MOB76 were also isolated from *Sphagnum*-dominated acidic wetlands.

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