Methanobacterium paludis sp. nov. and a novel strain of Methanobacterium lacus isolated from northern peatlands

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Two mesophilic, hydrogenotrophic methanogens, designated strains SWAN1T and AL-21, were isolated from two contrasting peatlands: a near circumneutral temperate minerotrophic fen in New York State, USA, and an acidic boreal poor fen site in Alaska, USA, respectively. Cells of the two strains were rod-shaped, non-motile, stained Gram-negative and resisted lysis with 0.1 % SDS. Cell size was 0.6×1.5–2.8 μm for strain SWAN1T and 0.45–0.85×1.5–35 μm for strain AL-21. The strains used H2/CO2 but not formate or other substrates for methanogenesis, grew optimally around 32–37 °C, and their growth spanned through a slightly low to neutral pH range (4.7–7.1). Strain AL-21 grew optimally closer to neutrality at pH 6.2, whereas strain SWAN1T showed a lower optimal pH at 5.4–5.7. The two strains were sensitive to NaCl with a maximal tolerance at 160 mM for strain SWAN1T and 50 mM for strain AL-21. Na2S was toxic at very low concentrations (0.01–0.8 mM), resulting in growth inhibition above these values. The DNA G+C content of the genomes was 35.7 mol% for strain SWAN1T and 35.8 mol% for strain AL-21. Phylogenetic analysis of the 16S rRNA gene sequences showed that the strains are members of the genus Methanobacterium. Strain SWAN1T shared 94–97 % similarity with the type strains of recognized species of the genus Methanobacterium, whereas strain AL-21 shared 99 % similarity with Methanobacterium lacus 17A1T. On the basis of phenotypic, genomic and phylogenetic characteristics, strain SWAN1T (DSM 25820T = JCM 18151T) is proposed as the type strain of a novel species, Methanobacterium paludis sp. nov., while strain AL-21 is proposed as a second strain of Methanobacterium lacus.

Peatlands cover about $5 \times 10^6$ km$^2$, are concentrated in northern latitudes (temperate to arctic regions), contain nearly one-third of all soil carbon (Gorham, 1991) and contribute about 20 % of the total annual CH4 emissions into the atmosphere (Cicerone & Oremland, 1988). Thus, methanogenic Archaea that inhabit peatlands are important in global carbon and atmospheric CH4 cycles. Peatlands include numerous habitats, such as acidic moss-dominated bogs and grass-dominated fens with near neutral pH (Gorham, 1991). Studies assessing the methanogenic Archaea from northern sites have shown that in addition to new uncultured archaeal lineages, phyotypes associated with the orders Methanomicrobiales, Methanosarcinales, Methanobacterales and Methanocellales are the common inhabitants of these ecosystems (Basiliko et al., 2003; Bridgham et al., 2013; Galand et al., 2003). The importance of each group varies depending on the peatland type, as observed in comparisons of the archaeal community of minerotrophic fens and bogs (Cadillo-Quiroz et al., 2006, 2008; Juottonen et al., 2005; Yavitt et al., 2012). For example, the order Methanosarcinales seems to have the greatest diversity in fens, whereas Methanomicrobiales dominate in bogs. In contrast, the net contribution of members of the order Methanobacterales to peatland methanogenesis is poorly understood.

Most of the Methanobacterales sequences recovered from peatlands are phylogenetically related to the genus Methanobacterium, and have been found in boreal (Kotsyurbenko et al., 2004) and temperate (Basiliko et al., 2003). The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains SWAN1T and AL-21 are CP002772 and CP002551, respectively.

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

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2003; Cadillo-Quiroz et al., 2006; Yavitt et al., 2012) sites. Although the contribution of Methanobacterium species to peatland methanogenesis is not clear, in vitro peat soil manipulations showed that lower pH conditions increased the presence of Methanobacterium as well as the contribution of hydrogenotrophic methanogenesis (Kotsyurbenko et al., 2007), suggesting a potential role of this group in some low pH peatlands.

The genus Methanobacterium contained eight formally accepted species in the most recent edition of Bergey’s Manual of Systematic Bacteriology (Boone et al., 2001). Since then, 12 other species have been formally described within the genus: Methanobacterium lacus (Borrel et al., 2012), Methanobacterium congelense (Cuzzin et al., 2001), Methanobacterium oryzae (Joulian et al., 2000), Methanobacterium kanagiense (Kitamura et al., 2011), Methanobacterium arcticum (Shcherbakova et al., 2011), Methanobacterium veterum (Krivushin et al., 2010), Methanobacterium aarhusense (Shilmon et al., 2004), Methanobacterium petrolearium and Methanobacterium ferruginis (Mori & Harayama, 2011), Methanobacterium beijingense (Ma et al., 2005), Methanobacterium movens and Methanobacterium flexile (Zhu et al., 2011). Members of the genus have been isolated from various sources, including marine and terrestrial environments (Garcia et al., 2000; König, 1984). All species are capable of growth by reduction of CO2 with H2, but they present a broad range and variation in phenotypic and genomic characteristics, including catabolism of substrates and DNA G+C content (30–57%) (Boone et al., 2001).

Only Methanobacterium palustre (Zellner et al., 1988) and Methanobacterium sp. strains MB2–MB4 (Kotsyurbenko et al., 2007) have been isolated from peatlands. It is unlikely that these strains contain all the potential genetic and physiological diversity of the class Methanobacteria in peatlands. Thus, more isolates are necessary to assess their properties and their potential role in peatlands. The present study describes the isolation and characterization of two novel Methanobacterium strains from two contrasting sites: strain SWAN1T, isolated from a minerotrophic fen in a cool temperate climate, is proposed as the type strain of a novel species of the genus Methanobacterium, and strain AL-21, isolated from a poor fen under a boreal climate, is proposed as a strain of Methanobacterium lacus (Borrel et al., 2012).

Anaerobically sampled peat soil was collected in 2005 and 2006 from the shallow anaerobic layers (approx. 20 cm below the surface) of two peatlands with the following locations and characteristics. Michigan Hollow is a minerotrophic temperate fen in New York State located near Danby (42° 19’ N 76° 29’ W), a sedge-dominated site with near neutral pH (~6.5) (Cadillo-Quiroz et al., 2008). Baxter fen is located near Anchorage, Alaska (61° 32’ N 150° 27’ W), and is an acidic poor fen with pH near 5.4 and is dominated by Sphagnum mosses and ericaceous shrubs. All samples were incubated as peat slurries supplemented with H2/CO2 and rifampicin as previously described (Bräuer et al., 2006; Cadillo-Quiroz et al., 2008). Tubes that showed high methane accumulation in their headspace were selected for subsequent transfer into anaerobic peat medium 2 (PM2).

PM2 composition and preparation were as previously described (Cadillo-Quiroz et al., 2008) using the modified anaerobic technique of Hungate (Sowers & Noll, 1995). Several hours before inoculation, the following additions were made to the basal medium under sterile and anaerobic conditions: 1.0 mM titanium (III) nitrilotriacetate (7.2 ml 1 M Tris/HCl adjusted to pH 8, 4.8 ml 0.5 M sodium nitrilotriacetate and 0.55 ml 15% titanium III chloride from Riedel-de-Haen), 10 mM MES (pKs=6.2 at 28 °C, filter-sterilized 1 M stock solution adjusted to pH 7.8), 0.5 mM coenzyme M (2-mercaptoethanesulfonic acid), 0.4 mM sodium acetate, 1% (v/v) vitamin solution (Balch et al., 1979) and 0.4 mM Na2S. The final liquid volumes in the tubes were approximately 5 ml, and 70.7 kPa H2/CO2 (80%/20%) was added to the headspaces unless otherwise specified.

Isolates were obtained after several dilutions to extinction (10^-8 or 10^-9) in PM2 adjusted to pH 5.6. Purity was evaluated by microscopy, and growth with organic substrates such as yeast extract (0.2 g l^-1), glucose or lactate (20 mM), each in the absence of rifampicin, to test for heterotrophic contaminants.

Cell morphology and motility were examined under phase-contrast microscopy with a Nikon Eclipse E600 microscope equipped with a Hamamatsu CCD digital camera. Gram staining and susceptibility to SDS lysis were determined as described by Boone & Whitman (1988) with SDS final concentrations ranging from 0.1 to 2% (w/v), and cell lysis determined by microscopic observations. Negative staining transmission electron microscopy was performed as described by Firtel et al. (1995) using 2% uranyl acetate (pH 6.5) and a Phillips Technai 12 Biotwin electron microscope equipped with a Gatan Multiscan model 791 camera and Digital Micrograph software. Micrographs were taken at 100 kV.

For experiments on the effect of pH, the pH was adjusted by addition of 30 mM MES adjusted to pH 4.5–7.6. The pH of the cultures was assessed at the end of incubations at 10 days of growth. For the Na2S sensitivity test, filter-sterilized anaerobic solutions of Na2S, 9H2O were prepared with different concentrations so that the same volume of reagent (0.05 ml) was added to replicate 5 ml cultures. To determine the effect of sodium on the growth of strains SWAN1T and AL-21, additions from sterile anaerobic NaCl stock solutions of different concentrations were made to achieve values of up to 250 mM. The background sodium content with no addition, from the different components of the medium, was approximately 15 mM. The following methanogenic substrates were individually tested: sodium formate and sodium acetate (2 mM), and methanol, 2-propanol, ethanol, 1-butanol, 2-butanol and trimethylamine.
(all at 5 mM). Cultures were tested under (i) an N₂/CO₂ and (ii) an N₂ atmosphere with 10 p.s.i. (~69 kPa) H₂ over-pressure to test for substrate utilization. In addition, parallel tubes were simultaneously grown (iii) under an H₂/CO₂ atmosphere to verify that amounts of substrates were not inhibitory. Treatments were monitored for CH₄ production using a gas chromatograph with a flame ionization gas detector as previously described (Cadillo-Quiroz et al., 2006). Experiments were conducted in triplicate tubes and incubated for at least 29 days using a gyratory shaker at 28–30 °C and 200 r.p.m., except that temperature tests were performed at static conditions at the corresponding temperatures (4, 10, 16, 22, 28, 32, 37, 40 or 45 °C).

Full 16S rRNA and mcrA (alpha subunit of methyl-coenzyme M reductase) gene sequences were obtained from genome sequencing of strains SWAN1T and AL-21 by the Joint Genome Institute under accession numbers NC_015574 and NC_015216, respectively. Phylogenetic analyses of 16S rRNA gene sequences were completed using ARB software (Ludwig et al., 2004) and the ‘Silva 111’ database release (July 2012) (Quast et al., 2013). The 16S rRNA gene sequence alignment was exported from ARB and phylogenetic trees were reconstructed by Bayesian analyses. The approximation of posterior probabilities was accomplished with MrBayes version 3.2.1 (Ronquist & Huelsenbeck, 2003) using four-chain Metropolis-coupled Markov chain Monte Carlo analysis. Bayesian consensus trees were built with a burnout of 500 and posterior probabilities were calculated. Tree topology was confirmed using the maximum-likelihood and neighbour joining methods (implemented in ARB) with Olsen evolutionary distance correction. Complementary analysis of the mcrA gene was done using nearly complete fragments, available at NCBI, translated to their predicted amino acid sequences (378 aa positions), aligned with CLUSTAL X and followed by tree reconstruction with the neighbour-joining method and 1000 bootstrap trees. DNA G+C measurements were obtained from the genome sequences.

Cells from the two isolates had a rod-shaped morphology, with somewhat variable dimensions (Fig. 1). Cell sizes were 0.6 × 1.5–2.8 and 0.45–0.85 × 1.5–3.5 μm for strains SWAN1T and AL-21, respectively. Strain AL-21 showed significant variability in cell length, with early growing cultures commonly dominated by 'small' (up to 3 μm long) cells (Fig. 1d) while 'long' cells increased in frequency at late stages of growth (Fig. 1e and f). Cells from the two strains stained Gram-negative and resisted lysis with 0.1 or 2 % SDS. Cells of both strains were non-motile, which corresponded to the absence of flagella. However, fimbriae were observed by electron microscopy. In fact, strain SWAN1T showed a high abundance of fimbriae and its cell surface had a rugose or coarse configuration (Fig. 1a–c).

Both strains were slightly acidiphilic, with differences in pH optima and range (Table 1 and Fig. S1 available in the online Supplementary Material). Strain SWAN1T had a narrower pH range (4.8–6.6) with an optimum near pH 5.4–5.7, whereas strain AL-21 had broader pH range from 4.7 to 7.1 with an optimum near pH 6.2. Most members of the genus Methanobacterium have been found to grow well near neutral pH with a few alkaliphilic or

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**Fig. 1.** Negative stain electron microscopy of cells of strains SWAN1T (a–c) and AL-21 (d–f). F, fimbriae. Bars: (a, b, e), 0.5 μm; (c, f), 0.2 μm; (d), 2 μm.
Table 1. Physiological characteristics of the novel strains as compared with other species of the genus *Methanobacterium*

<table>
<thead>
<tr>
<th>Characteristic</th>
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<th>7</th>
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<th>11</th>
<th>12</th>
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<tbody>
<tr>
<td>Source</td>
<td>Minerotrophic fen</td>
<td>Poor fen</td>
<td>Deep lake sediment</td>
<td>Anaerobic digester</td>
<td>Peat bog</td>
<td>Anaerobic digester</td>
<td>Permamvrost</td>
<td>Marshy soil</td>
<td>Rock core</td>
<td>Anaerobic digester</td>
<td>Rice field</td>
<td>Granitic groundwater</td>
<td>Lake</td>
<td>Alkaline lake</td>
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<td>Cell size (width × length, μm)</td>
<td>0.6 × 1.5–2.8</td>
<td>0.45–0.85 × 15</td>
<td>0.2–0.4 × 2–15</td>
<td>0.4–0.5 × 2–10</td>
<td>0.5 × 2.5–5</td>
<td>0.4–0.5 × 3–5</td>
<td>0.4–0.45 × 2–8</td>
<td>0.2–0.6 × 1–15</td>
<td>0.5–0.8 × 1–15</td>
<td>0.5–1 × 3–10</td>
<td>0.3–0.4 × 2–10</td>
<td>0.1 × 0.6–1.2</td>
<td>0.4–0.5 × 2–5</td>
<td>0.5–0.6 × 2–25</td>
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<tr>
<td>Substrates</td>
<td>F</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
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<td>M + H₂</td>
<td>–</td>
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<td>iP, iB</td>
<td>–</td>
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<tr>
<td>pH for growth</td>
<td>Range</td>
<td>4.8–6.6</td>
<td>4.7–7.1</td>
<td>5–8.5</td>
<td>5.9–8.2</td>
<td>ND</td>
<td>6.5–8</td>
<td>5.2–9.4</td>
<td>6–8.5</td>
<td>6.5–8.5</td>
<td>ND</td>
<td>6.0–8.5</td>
<td>6.5–9.2</td>
<td>6–9</td>
</tr>
<tr>
<td>Optimum</td>
<td>5.4–5.7</td>
<td>6.2</td>
<td>6.5</td>
<td>7.2</td>
<td>7</td>
<td>7.2–7.4</td>
<td>ND</td>
<td>7–7.4</td>
<td>6–7.2</td>
<td>ND</td>
<td>6–7.8</td>
<td>7</td>
<td>7.8–8.8</td>
<td>7.2–7.5</td>
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<tr>
<td>NaCl range (M)</td>
<td>0–0.25</td>
<td>0–0.06</td>
<td>0–0.4</td>
<td>0–0.3</td>
<td>0–0.5</td>
<td>0–0.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0–0.4</td>
<td>ND</td>
<td>0.2–1.2</td>
<td>0–1.7</td>
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<tr>
<td>DNA G + C content (mol%)</td>
<td>35.7 (Gw)</td>
<td>35.8 (Gw)</td>
<td>37 (Lw)</td>
<td>39.5 (Lw)</td>
<td>34 (Tm)</td>
<td>38.9 (Tm)</td>
<td>22.8 (Tm)</td>
<td>29 (Tm)</td>
<td>36.6 (Tm)</td>
<td>33–38 (Bd)</td>
<td>31 (Lw)</td>
<td>54.5 (Tm)</td>
<td>39.1 (Tm)</td>
<td>57 (Bd)</td>
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<td>NaS range (mM)</td>
<td>0–0.4</td>
<td>0–0.1</td>
<td>ND</td>
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*Measured by Tm (Ma et al., 2005).
Low amounts of NaCl (10–60 mM) added to the approximately 15 mM Na⁺ already present in the medium slightly improved methanogenesis. Concentrations above 160 and 50 mM inhibited the growth of strains SWAN⁴ and AL-21, respectively (Fig. S2). Also, strains AL-21 and SWAN⁴ were susceptible to inhibition by Na₂S concentrations above 0.4 mM, in agreement with results of our previous studies, which have shown a wide variability of optimal and inhibitory Na₂S additions among methanogenic cultures (Bräuer et al., 2011, 2004; Cadillo-Quiroz et al., 2009). However, many cultured strains are tolerant to Na₂S concentrations around or above 1 mM, as is typically used for reducing anaerobic media (Jarrell & Kalmokoff, 1988). Na₂S sensitivity has been observed in other isolates from peatlands (Bräuer et al., 2011; Cadillo-Quiroz et al., 2009). Thus, for some environments, inhibitory effects may limit the culturability of methanogens in sulfide-containing media.

The two strains were mesophiles with optimal growth between 32 and 37 ℃ and a range of growth of 16–40 and 10–37 ℃ for strains SWAN⁴ and AL-21, respectively. In terms of methanogenic substrates, both strains demonstrated growth exclusively with H₂/CO₂ and no significant growth or methanogenesis was observed with the other tested substrates under an N₂/CO₂ or N₂/H₂ atmosphere, and none of the substrates inhibited growth under H₂/CO₂. Coenzyme M and acetate (4 mM) were found to stimulate growth. Acetate is used by hydrogenotrophic methanogens as a carbon source stimulating growth (Borrel et al., 2012; Bräuer et al., 2011; Krivushin et al., 2010; Ma et al., 2005). Under optimal conditions the estimated doubling time for strains SWAN⁴ and AL-21 was approximately 35 h, as calculated from the methane production rate.

In terms of 16S rRNA gene sequence phylogeny, the two strains were found to be in the order Methanobacteriales within the genus Methanobacterium (Fig. 2). Strain SWAN⁴ was related closely to Methanobacterium sp. strain MB-4, Methanobacterium congolense C⁴ and Methanobacterium curvum at 99, 97 and 96 % sequence similarity. Levels of 16S rRNA gene sequence similarity with the type strains of other species of the genus Methanobacterium were between 95 and 96 %. Strain MB-4 was isolated from an acidic Siberian bog (Kotsyurbenko et al., 2007), and no formal taxonomic description has been published up to the time of completion of this report. Strain AL-21 was closely related (99 % 16S rRNA gene sequence similarity) to

![Fig. 2. 16S rRNA gene sequence phylogenetic analysis of strains SWAN⁴ and AL-21 and members of the order Methanobacteriales. Strain numbers are given following the species name, and accession numbers for 16S rRNA gene sequences are given in parentheses. Posterior probabilities above 89 % are indicated at corresponding nodes in the tree. Bar, 0.1 changes per nucleotide position.](http://ijs.sgmjournals.org)
Methanobacterium lacus 17A1{T} (Borrel et al., 2012), and to clones from a pH 4.5 H2/CO2 enrichment culture derived from a German acidic bog (98 % similarity) (Horn et al., 2003). The mcrA gene sequence tree (Fig. S3) showed a similar topology for the close relatives of strain SWAN-1{T} with nucleotide sequence similarity of 97, 87 and 79 to strain MB-4, Methanobacterium congolense C{T} and strain AL-21, respectively. The mcrA gene tree supported strain AL-21 as an independent lineage although bootstrap support for this was low and the mcrA gene sequence of Methanobacterium lacus 17A1{T} was not available for comparison. The DNA G + C content from full genome sequencing was 35.7 and 35.8 mol% for strains SWAN1{T} and AL-21, respectively.

The differences in sequence similarity between strain SWAN1{T} and other species in this group were as low as or lower than among the recognized species, suggesting that strain SWAN1{T} represents a novel species in the genus Methanobacterium. The high sequence similarity (99 %) between strain AL-21 and Methanobacterium lacus 17A1{T} (Borrel et al., 2012) suggests the two strains are members of the same species. However, note that recent reports on Methanobacterium species taxonomy have found that even strains sharing 99 % 16S rRNA gene sequence similarity can be considered to represent different species in conjunction with other genomic assessments including DNA G+C content and DNA–DNA hybridization (Shcherbakova et al., 2011). Differences in DNA G+C content between strain AL-21 (35.8 mol%) and Methanobacterium lacus 17A1{T} (37 mol%) are within limits previously found between different species of the genus Methanobacterium with 99 % 16S rRNA gene sequence similarity (Shcherbakova et al., 2011). However, DNA–DNA hybridization tests between strain AL-21 and Methanobacterium lacus 17A1{T} have been precluded by the lack of growth of strain 17A1{T} to densities high enough to provide sufficient DNA yields for those tests (H. Cadillo-Quiroz, personal observation; DSMZ, personal communication). A more fine-scale genomic approach with less DNA demand such as high-throughput sequencing was not available at the time of completion of this work.

Based on morphological, physiological (Table 1) and phylogenetic characteristics, these two novel isolates are considered to be members of the genus Methanobacterium. Several lines of physiological evidence were found differentiating these strains from their closest formally described relative. Shorter cells, a lower optimal pH and range, lower optimal temperature and range, and variable DNA G+C content (3–9 % difference) differentiated strain SWAN1{T} from Methanobacterium congolense C{T}. Strain AL-21 also showed several physiological characteristics differentiating it from Methanobacterium lacus 17A1{T}, including a lack of growth on methanol in the presence of H2, a lower optimal pH and range, a narrower temperature range and higher optimal temperature, and a nearly ten times higher sensitivity to NaCl. However, strain AL-21 and Methanobacterium lacus 17A1{T} also showed a high genetic similarity (99 %), and sufficient genomic evidence is not available for their separation as independent groups. Therefore, we suggest that strain SWAN1{T} represents a novel species of the genus Methanobacterium, for which the name Methanobacterium paludis sp. nov. is proposed, and provisionally propose that strain AL-21 represents a second strain of Methanobacterium lacus.

**Description of Methanobacterium paludis sp. nov.**

Methanobacterium paludis (pa.lu’dis. L. gen. n. paludis of a marsh).

Cells are Gram-stain-negative, non-motile rods (1.5–2.8 μm) that are resistant to lysis by 0.1 % (w/v) SDS. The cell surface has a rugose appearance when observed by electron microscopy. Cells are obligately hydrogenotrophic and do not consume formate or other substrates in the absence or presence of H2. Growth is observed over a pH range of 4.8–6.6 with an optimum near pH 5.4–5.7, and over a temperature range of 16–40 °C with optimal growth around 32–37 °C. Sensitive to sodium ions and the reducing agent Na2S, with inhibition occurring above 160 and 0.4 mM, respectively.

The type strain, SWAN1{T} (=DSM 25820{T} =JCM 18151{T}), was isolated from Michigan Hollow fen, a minerotrophic circumneutral (~pH 6.5) peatland in New York State, USA. The genomic G+C content of the type strain is 35.7 mol%, as determined by genome sequencing.

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**References**


Novel methanobacteria from northern peatlands


