**Methanospirillum stamsii** sp. nov., a psychrotolerant, hydrogenotrophic, methanogenic archaeon isolated from an anaerobic expanded granular sludge bed bioreactor operated at low temperature

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### Abbreviations:
- EGSB, expanded granular sludge bed; VFA, volatile fatty acid.
- The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and mcrA gene sequences of **Methanospirillum stamsii** Pt1T are HF569045 and KC951357, respectively.

Microbiological processes in cold environments are catalysed by psychrotolerant and psychrophilic micro-organisms (Cavicchioli, 2006; D’Amico *et al.*, 2006; Margesin & Miteva, 2011). Low-temperature anaerobic digestion is a very attractive option for wastewater treatment (Lettinga *et al.*, 2001; McKeown *et al.*, 2012). Therefore, it is important to investigate anaerobic microbiological processes occurring at low temperatures and to get insight into the phylogeny and physiology of psychrophilic and psychrotolerant anaerobes that have been poorly studied thus far.

Conversion of volatile fatty acids (VFAs), which are important intermediates in anaerobic degradation, is a slow process, especially at low temperatures. Syntrophic interactions of anaerobic bacteria and methanogenic archaea are essential for the anaerobic conversion of VFA to methane. In syntrophic communities species of the genus **Methanospirillum** are an important hydrogen-consuming partner in VFA-degrading co-cultures (Stams, 1994; Schink 1997; McNerney *et al.*, 2008; Stams & Pluge, 2009; Stams *et al.*, 2012).

At the time of writing, ten psychrophilic and psychrotolerant methanogenic archaea have been isolated and characterized (Franzmann *et al.*, 1992, 1997; Simankova *et al.*, 2001; Chong *et al.*, 2002; von Klein *et al.*, 2002; Singh *et al.*, 2005; Kendall *et al.*, 2007; Zhang *et al.*, 2008; Krivushin *et al.*, 2010; Wagner *et al.*, 2013). Seven of these are halophilic. Two methanogens, isolated from Siberian permafrost (Krivushin *et al.*, 2010; Wagner *et al.*, 2013), had an optimal NaCl requirement of 0.05 and 0.02 M, respectively. One methanogenic archaeon, **Methanosarcina**
lacustris ZS, which we isolated from freshwater lake sediment (Simankova et al., 2001), did not require any NaCl for growth. Considering that most methanogenic environments are limnetic, it is important to find more psychrotolerant, non-halophilic, methanogenic archaea, which is also of practical importance.

The initial source of the inoculum was mesophilic methanogenic granular sludge originating from a full-scale upflow anaerobic sludge bed (UASB) reactor treating brewery wastewater (in the Netherlands). It was cultivated at Wageningen University in a pilot-scale expanded granular sludge bed (EGSB) reactor at 13–20°C on the matting wastewater (Rebac et al., 1997). Later the granulated biomass from this reactor was used as an inoculum of the laboratory EGSB bioreactor to treat the mixture of VFAs (acetate, propionate and butyrate) at temperatures of 3–12°C (Lettinga et al., 1999). Then the latter biomass, able to grow at low temperatures on propionate and butyrate, was used in growth experiments performed in glass serum bottles (Parshina et al., 2011).

Subsequently, defined methanogenic syntrophic consortia that grow on propionate and butyrate were obtained and analysed by molecular methods (Parshina et al. unpublished). Both consortia contained Methanospirillum-like hydrogenotrophic methanogens. On propionate their number was higher because of differences in stehiometry of syntrophic propionate and butyrate oxidation (Stams, 1994).

A strain representing a member of the genus Methanospirillum was isolated from a propionate-oxidizing consortium. At the time of writing, two species within the genus Methanospirillum have been described: Methanospirillum hungatei JF1T (Ferry et al., 1974) and Methanospirillum lacunae Ki8-1T (Iino et al., 2010). Both strains are mesophilic with fastest growth at 30–37°C (Methanospirillum hungatei JF1T) and 30°C (Methanospirillum lacunae). They do not grow at temperatures below 20–25°C (Methanospirillum hungatei) and 15°C (Methanospirillum lacunae). Another strain Methanospirillum hungatei GP1 has an even higher temperature optimum at 45°C (Patel et al., 1976).

For isolation, an enrichment culture (2 ml) was inoculated into 120 ml serum bottles filled with 18 ml basal medium. Basal medium of the following composition was used for enrichment and further cultivation: 10 ml mineral solution l⁻¹ (Pfenning 1965), 0.05% Na₂S, 9H₂O and 0.05% cysteine as reducing agent, 0.25% NaHCO₃, 0.002% resazurin, 0.02% yeast extract, 2 ml microelement solution l⁻¹ (Pfenning & Lippert, 1966), 10 ml vitamin solution l⁻¹ (Wolin et al., 1963). To check the ability to grow with formate as a carbon source, microelements containing tungsten were used (Kevbrin & Zavarzin, 1992).

The final pH was 6.8–7.0. The medium was flushed with a mixture of H₂/CO₂ (4:1, v/v).

The strain was isolated using a series of dilutions in liquid medium flushed with H₂/CO₂, cultivation with the antibiotics ampicillin (1 g l⁻¹), penicillin (2 g l⁻¹), vancomycin (100–200 mg l⁻¹), cycloserine (100 mg l⁻¹), kanamycin (100 mg l⁻¹), erythromycin (100 mg l⁻¹), or rifampicin (100–200 mg l⁻¹), and growth of single colonies in roll-tubes with a thin layer of solid (2% agar) medium (with H₂/CO₂). After purification procedures an axenic culture of the strain designated strain Pt1 was obtained. The purity of the strain was checked by inoculation into test medium, supplied (1⁻¹) with yeast extract 2 g, glucose 4 g and peptone 2 g and determination the 16S rRNA sequence of extracted and amplified DNA.

Growth of strain Pt1 was followed by methane formation. Chromatographic analyses of gases and liquid products were performed on a Chrom-5 gas chromatograph (Prague, the Czech Republic) equipped with a kathometer and a flame-ionization detector, as described by Parshina et al. (1993).

Cells of strain Pt1 were curved or wavy rods with blunted ends and were generally 0.4–0.5 μm wide and 7–25 μm long, sometimes up to a few hundred μm in length (Fig. 1a, b). Motility was observed under phase-contrast microscopy on MBI-3 (Russia) and AxioImager DI (CarlZeiss, Germany). Tufted flagella were observed by electron microscopy on JEO100C XII (Japan) (Fig. 1c). Cells stained Gram-negative using conventional Gram staining. Colonies were diffuse light yellow in the agar roll tubes.

Strain Pt1 was strictly anaerobic; no growth was observed under microaerobic or aerobic conditions. The effect of oxygen on growth was studied by incubation of the culture after injection of different volumes of air into the bottles, from 2 to 20% air in addition to H₂/CO₂. Acetate (2–5 mmol l⁻¹) and/or yeast extract (0.5–1.0 g l⁻¹) stimulated growth. Strain Pt1 grew at 5–37°C, with optimum growth at 20–30°C. No growth was observed at 2 and 40°C (Fig. 2). The pH range for growth was 6.0–10, with optimum growth at pH 7.0–7.5. No growth was observed at pH 5.5 or 10.5. Growth of strain Pt1 was observed with NaCl concentrations below 0.3 M. Optimum growth of the strain was observed in basal medium without NaCl. Strain Pt1 used H₂/CO₂ (4:1, v/v) for growth and methane production. Its growth was very weak with formate (10–20 mmol l⁻¹) even when the medium was supplemented with tungsten (Kevbrin & Zavarzin, 1992). No growth was observed with 10 mM acetate, pyruvate, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol or trimethylamine. The generation time of strain Pt1 in the medium supplied with H₂/CO₂ at 30°C and pH 7.5 was 39.8 h. The SDS lysis was analyzed according to method described by Boone & Whitman (1988). Lysis of the cells was not observed in an SDS solution up to 2% (w/v). During isolation procedures, it was established that strain Pt1 was susceptible to cycloserine (100 mg l⁻¹) and kanamycin (100 mg l⁻¹), but resistant to penicillin (2 g l⁻¹), ampicillin (1 g l⁻¹), erythromycin (100 mg l⁻¹), rifampicin (100–200 mg l⁻¹) and vancomycin (100–200 mg l⁻¹).

For amplification of the 16S rRNA gene and the mcrA gene, DNA was isolated by a modified alkaline extraction method.
procedure (Birnboim & Doly, 1979) and DNA purified using Promega Wizard technology. For amplification of the nearly full-sized 16S rRNA gene, primers 8fa (5'-TCCGGTTGATCCCTGGG-3') and 1492r (5'-TACG-GYTACCTTGTTAGCCTT-3') were used (Lane 1991). The PCR was conducted in 50 μl reactions with the following reagents: 1× PCR buffer [17 mM (NH₄)₂SO₄, 67 mM tris-HCl, pH 8.8, 2 mM MgCl₂]; 12.5 nmol of each dNTP, 50 ng of DNA template; 5 pmol of corresponding primers and 3 units of DNA polymerase BioTaq (Dialat). The temperature–time profile for the amplification was: initial denaturation at 94°C for 9 min followed by 30 cycles consisting of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and elongation at 72°C for 2 min and a final elongation at 72°C for 7 min. A Tetrad2 thermocycler (Bio-Rad) was used.

PCR products were analysed by electrophoresis in 2% agarose at 6 V cm⁻¹. The gels were photographed using the BioDocII video documentation system (Biometra). Isolation and purification of PCR products from low-melting-temperature agarose was performed using a Wizard PCR Preps kit (Promega) according to the manufacturer’s instructions. The amplification products were sequenced by the Sanger method (Sanger et al., 1977) on an ABI PRIZM 3730 automatic sequencer (Applied Biosystems) using the Big Dye Terminator v.3.1 kit (Applied Biosystems) according to the manufacturer’s instructions. The sequence chromatograms were edited using the Chromas software, version 1.45 (http://www.technelysium.com.au/chromas.html).

The obtained sequences were imported into the Geneious Pro software (v 6.1, Biomatters), were aligned with reference sequences obtained from the Ribosomal Database (RDP II, http://rdp.cme.msu.edu), using the MUSCLE alignment tool in Geneious Pro with default parameters (Edgar, 2004a, b). Maximum-likelihood analyses were performed with the PhyML tool (Guindon & Gascuel, 2003) in Geneious Pro using the Jukes–Cantor 69 substitution model (Jukes & Cantor, 1969). The reliability of the nodes of the phylogenetic tree was tested by

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**Fig. 1.** (a, b) Phase-contrast images of variations in cells morphology of strain Pt1 in different experiments. Bars, 10 μm. (c) Transmission electron micrograph of cells of strain Pt1 with tufted flagella (F). Bar, 0.4 μm.

**Fig. 2.** Influence of temperature (a), pH (b) and NaCl concentration (c) on the growth rate of strain Pt1.
bootstrap analysis at 1000 replicates. According to the 16S rRNA gene sequence phylogeny, strain Pt1 was affiliated with known species of the genus *Methanospirillum* and most closely with *Methanospirillum hungatei* JF1\(^T\) (Fig. 3a). It shared 97.5% and 94% sequence similarity with *Methanospirillum hungatei* JF1\(^T\) and *Methanospirillum lacunae* Ki8-1\(^T\), respectively.

The enzyme methyl-coenzyme M reductase (MCR) is essential to all methanogens, as it catalyses the final step in methanogenesis (Friedrich, 2005). It is highly conserved and has been used widely as a phylogenetic marker for methanogens (Luton et al., 2002; Friedrich, 2005). Here, a fraction of the gene encoding the \(\alpha\) subunit of MCR (mcrA) was also determined for further phylogenetic comparison between strain Pt1 and the other species of the genus *Methanospirillum*. This gene was partially amplified from strain Pt1 by PCR with primers mlas and mcrA-rev (Steinberg & Regan, 2008) and MLf and MLr (Luton et al., 2002) in separate reactions. The PCR was performed and the products were purified as described above except that the amplification was conducted as described by Steinberg & Reagan (2008). The mcrA gene sequences were imported into the Geneious Pro software and the amino acid sequence of strain Pt1 was aligned together with available sequences of relevant reference strains obtained from the NCBI database (http://www.ncbi.nlm.nih.gov). The mcrA gene sequences were tested by bootstrap analysis of 1000 replicates using the MUSCLE alignment tool in Geneious Pro with default parameters. A maximum-likelihood

![Fig. 3.](http://ijs.sgmjournals.org)
phylogenetic tree (Fig. 3b) was reconstructed using the PhyML tool in Geneious Pro using the Whelan and Goldman substitution model (Whelan & Goldman, 2001) and 1000 bootstrap repeats. In accordance with the 16S rRNA gene phylogeny, the mcrA sequence of strain Pt1 was also affiliated more closely with that of Methanospirillum hungatei JF1T (91 %) than with that of Methanospirillum lacunae Ki8-1T (86 %).

For DNA–DNA hybridization, a standard method of optic reassociation described by De Ley et al. (1970) was used. DNA was isolated and purified as described by Marmur (1961) and Marmur & Doty (1961). All measurements were performed on a Cary 100 Bio spectrophotometer (Varian). The G+C content of the DNA was determined by thermal denaturation of the DNA using a SP 1800 spectrophotometer (Pye Unicam). The G+C content (mol%) of the DNA of strain Pt1, calculated according to the method of Owen et al. (1969), was 40 mol%. The DNA–DNA hybridization between strain Pt1 and Methanospirillum hungatei JF1T revealed a relatedness of 39 %.

The morphological, biochemical and physiological properties that differentiate strain Pt1 from Methanospirillum lacunae and Methanospirillum hungatei are summarized in Table 1.

On the basis of its phylogenetic position, morphology and biochemical and physiological properties, we propose that strain Pt1 represents a novel species of the genus Methanospirillum, for which the name Methanospirillum stamsii sp. nov. is proposed.

**Description of Methanospirillum stamsii sp. nov.**

*Methanospirillum stamsii* (stams’i.i. N.L. gen. n. stamsii of Stams, named after Professor Alrons J. M. Stams, a Dutch microbiologist, for his great contribution to the understanding of syntrophic communities of bacteria and archaea).

Cells are curved rods, 0.4–0.5 to 7–25 µm in size, and sometimes can become wavy filaments a few hundred micrometres in length. Growth occurs at 5–37 °C, with optimum growth at 20–30 °C. The pH range for growth is 6.0–10, with optimum growth at pH 7.0–7.5. Growth occurs with less than 0.3 M NaCl, with optimum growth without the addition of NaCl. Methane is produced from H₂/CO₂ and very weakly from formate, but not from acetate, pyruvate, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol or trimethylamine. Acetate and yeast extract are stimulating for growth.

The type strain, Pt1T (DSM 26304T VKM B-2808T), was isolated from a propionate-degrading syntrophic consortium enriched previously from sludge from a laboratory-scale two-stage anaerobic EGSB bioreactor operated at low temperature and fed with VFA (acetate, propionate and butyrate). The G+C content of the genomic DNA of the type strain is 40 mol%.

**Acknowledgements**

This research was performed under financial support of the Russian Foundation for Basic Research (RFBR) (grant 07-04-01522). The authors thank Dr T. V. Kalganova and N. A. Kostrikina for their help.

**References**


**Table 1. Physiological characteristics of the type strains of species of the genus Methanospirillum**

<table>
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<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td>Cell shape</td>
<td>Curved rods</td>
<td>Curved rods</td>
<td>Curved rods</td>
</tr>
<tr>
<td>Cell width (µm)</td>
<td>0.4–0.5</td>
<td>0.5–0.6</td>
<td>0.4–0.5</td>
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<tr>
<td>Cell length (µm)</td>
<td>7–25 (sometimes 15 to &gt;100)</td>
<td>11–25 (often 8–26)</td>
<td>7.4–10 (often 15 to &gt;100)</td>
</tr>
<tr>
<td>Temperature range for growth</td>
<td>5–37</td>
<td>15–37</td>
<td>25–50</td>
</tr>
<tr>
<td>Optimum temperature (°C)</td>
<td>20–30</td>
<td>30</td>
<td>30–37</td>
</tr>
<tr>
<td>pH range for growth</td>
<td>6.0–10.0</td>
<td>6.0–9.5</td>
<td>6.5–10.0</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>7.0–7.5</td>
<td>7.5</td>
<td>6.6–7.4</td>
</tr>
<tr>
<td>NaCl range for growth (M)</td>
<td>0–0.3</td>
<td>0–0.2</td>
<td>0–0.2</td>
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<tr>
<td>Optimum NaCl</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
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<tr>
<td>Formate</td>
<td>±</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Genes time (h)</td>
<td>39.8 (at 30 °C)</td>
<td>32.3(at 30 °C)</td>
<td>20.7 (at 40 °C)</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>40 (Tm)</td>
<td>45.3 (HPLC)</td>
<td>45 (Tm)</td>
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<tr>
<td>Source</td>
<td>Sludge</td>
<td>Soil</td>
<td>Sewage sludge</td>
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