**Thermogutta terrifontis** gen. nov., sp. nov. and **Thermogutta hypogea** sp. nov., thermophilic anaerobic representatives of the phylum **Planctomycetes**

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Two novel strains of thermophilic planctomycetes were recovered from terrestrial and subterranean habitats. Strain R1T was isolated from a hot spring (Kunashir Island, Russia) and strain SBP2T was isolated from a deep gold mine (South Africa). Both isolates grew in the temperature range 30–60 °C and pH range 5.0–8.0. Strain R1T grew optimally at 60 °C and pH 6.0–6.5; for SBP2T optimal conditions were at 52 °C and pH 7.5–8.0. Both strains were capable of anaerobic respiration with nitrate and nitrite as electron acceptors as well as microaerobic growth. They also could grow by fermentation of mono-, di- and polysaccharides.

Based on their phylogenetic position and phenotypic features we suggest that the new isolates represent two novel species belonging to a new genus in the order **Planctomycetales**, for which the names **Thermogutta terrifontis** gen. nov., sp. nov. and **Thermogutta hypogea** sp. nov. are proposed. The type strain of **Thermogutta terrifontis**, the type species of the genus, is R1T (=DSM 26237T =VKM B-2805T), and the type strain of **Thermogutta hypogea** is SBP2T (=JCM 19991T =VKM B-2782T).

**Planctomycetes** represents a deep distinct phylum in the domain **Bacteria** and includes a large group of microorganisms with unique features not found in other bacteria: they reproduce by budding, have characteristic intracytoplasmic membranes dividing the cell into compartments and lack peptidoglycan in the cell wall (Fuerst, 2005). Although the phylum has long been known, only a few representatives have been taxonomically characterized (Fuerst & Sagulenko, 2011). At the time of writing, validly named planctomycetes comprised two orders: the **Phycisphaerales** (Fukunaga et al., 2009) represented by two genera with a sole species in each, and the **Planctomycetales** (Schlesner & Stackebrandt, 1986), which has 11 genera that accommodate 19 species. Metabolically, planctomycetes are divided into two separate groups: aerobic organotrophs utilizing diverse mono-, di- and polysaccharides and obligately anaerobic lithoautotrophic anammox bacteria. The latter group is represented by many genera, but none of these micro-organisms so far has been isolated in pure culture (Ward et al., 2006). Isolates of the phylum **Planctomycetes** maintained in laboratory cultures are mesophiles and neutrophiles. At the same time, the 16S rRNA genes of planctomycetes have been detected in different thermal natural and industrial environments: terrestrial hot springs, oilfields and waste-degrading reactors (Hugenholtz et al., 1998; Lau et al., 2009; Nazina et al., 2006; Sekiguchi et al., 1998). Yet to date, no thermophilic members of the **Planctomycetes** with a growth optimum above 50 °C have been described. In this study we report the isolation and characterization of two novel thermophilic planctomycetes from terrestrial and subterranean habitats.

Strain R1T was isolated from a terrestrial sample of water and microbial mat (pH 7.3, 46 °C) that was collected in 2012 from a hot spring (Kunashir Island, Kurils, Russia). Strain SBP2T was isolated from a subterranean sample (pH 8.8, 32 °C) that was collected in 2011 from fracture water in an existing borehole at 1375 m below the shaft collar of the Beatrix gold mine (SibanyeGold, Free State,

**Abbreviation**: CFA, cellular fatty acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains R1T and SBP2T are KC867694 and KC867696, respectively.
South Africa). The samples were collected in sterile pre-evacuated N2-filled serum vials allowing for anaerobic sampling, transported to the laboratory and stored at 6–10°C until used to initiate anaerobic enrichments. Enrichment and isolation were performed in a liquid medium of the following composition (per litre distilled water): 0.33 g NH4Cl, 0.33 g KCl, 0.33 g MgCl2.6H2O, 0.33 g CaCl2, 0.33 g KH2PO4, 2.0 g NaHCO3, 1 ml trace element solution (Slobodkin et al., 1997), 1 ml vitamin solution (Wolin et al., 1963) and 0.2 g yeast extract (Sigma). The medium was boiled and cooled under the flow of oxygen-free gases (CO2 or N2) to render it anaerobic and finally heat-sterilized at 121°C for 60 min. No reducing agents were added. The isolation medium for the terrestrial sample had a pH of 6.0–6.5 and the head space was filled with N2. Xanthan gum (Kelco) (5 g l\(^{-1}\)) and KNO\(_3\) (2 g l\(^{-1}\)) were used as electron donor and acceptor, respectively. The medium for the subterranean sample had a pH of 7.6–8.0 [adjusted with sterile 5% (w/v) NaOH solution]. The head space was filled with CO2; peptone and glucose (2 g l\(^{-1}\)) each were added as growth substrates. Unless otherwise noted, enrichments and pure cultures were grown in 10 ml medium in 17 ml Hungate tubes. All incubations were kept in the dark with the terrestrial sample at 65°C and the subterranean sample at 50°C. Enrichment cultures were initiated by inoculation of 10% (w/v) of the mixed water and mat (terrestrial sample) or 50% (v/v) of the fracture water (subterranean sample) to the respective liquid media described above. After 10–14 days of incubation, cells of two or three morphological types were observed, including oval-shaped cells. For isolation of these oval cells, vancomycin (100 μg ml\(^{-1}\)) or ampicillin (200 μg ml\(^{-1}\)) were added to the medium for terrestrial and subterranean samples, respectively, as a selection factor. After three subsequent transfers followed by serial 10-fold dilutions in the presence of antibiotic, only the coccoid cells were observed in the highest dilution (10\(^{-7}\)). Attempts to obtain separate colonies at 50 or 65°C, either anaerobically in agar blocks or aerobically on the surface of the medium solidified with 1.5% agar or phytagel as solidifying agent, were unsuccessful. Pure cultures of the two strains were obtained by a dilution-to-extinction technique in the liquid antibiotic-containing medium. Culture purity was both confirmed by microscopic observations and 16S rRNA gene sequencing. The isolates obtained were designated strains R1\(^T\) (terrestrial isolate) and SBP2\(^T\) (subterranean isolate). Light and electron microscopy, tests for temperature, pH and salinity ranges for growth, tests for microaerobic growth, analytical techniques for determination of metabolic products, DNA extraction, determination of G+C content and determination of cellular fatty acid (CFA) composition were performed as described previously (Slobodkina et al., 2013). For CFA analysis cells of strains R1\(^T\) and SBP2\(^T\) were grown in the presence of xanthan gum or glucose at 2.0 g l\(^{-1}\), respectively, and with nitrate as electron acceptor. For determination of the nitrate reduction products, a medium free of nitrogen-containing compounds was used. Nitrate and nitrite were determined using Nitrate-Test strips (MQuant; Merck); ammonium was determined with the use of Nessler’s reagent. Sulfide was measured colorimetrically with dimethyl-p-phenylendiamine (Trueper & Schlegel, 1964). Oxidase and catalase activities were determined using standard methods (Gerhardt et al., 1994). Genomic DNA isolation, 16S rRNA gene amplification, sequencing, evolutionary analysis and phylogenetic tree reconstruction were performed as described previously (Slobodkina et al., 2013). Pairwise similarity values were calculated by means of the EzTaxon server (Kim et al., 2012).

Cells of the two new isolates were non-motile ellipsoids of very similar morphology, 1.0–1.5 μm in diameter (Fig. 1a). Cells reproduced by budding (Fig. 1b). Young cultures contained highly motile cells that were smaller (0.5–0.8 μm) and apparently represented buds separated from the mother cells. In most cases, one thick polar flagellum per cell was observed (Fig. 1c). Upon ageing, these young cells transformed into non-motile cells that occurred singly or in pairs or were arranged in shapeless aggregates (Fig. 1d). Examination of thin sections revealed characteristic cell organization in which a single intracytoplasmic membrane divided the cell into two major compartments (Fig. 2) with the nucleoid located in one of these compartments. This type of cell compartmentalization is characteristic of Pirellula-like planctomycetes (Fuerst, 2005; Fuerst & Sagulenko, 2011). Strain R1\(^T\) grew at temperatures between 25 and 67°C with optimum growth at 55–60°C. No growth was detected at 70°C or above. The pH range for growth of strain R1\(^T\) was 4.0–8.0 with the optimum at pH 6.0–6.5. No growth was detected at pH 3.5 or below or at pH 9.0 and above. The temperature range for growth of strain SBP2\(^T\) was 30–60°C with an optimum at 52°C. No growth of strain SBP2\(^T\) was detected at 65°C or above as well as at 28°C or below. The pH range for growth of SBP2\(^T\) was 5.0–9.0, with an optimum at pH 7.5–8.0. No growth was observed at pH 4.5 or

![Fig. 1. Morphological characteristics of strain SBP2\(^T\).](http://ijs.sgmjournals.org)
Carbohydrates (Table 1). The end products of glucose fermentation were hydrogen, acetate and lactate. Production of hydrogen was not detected in the presence of nitrate, but occurred at low O2 concentrations and ceased at O2 concentrations of 5% (in the gas phase) and above. Strain R1T tested positive for catalase but negative for oxidase; strain SBP2T was negative for catalase and oxidase reactions. Both isolates were resistant to ampicillin, penicillin (200 µg ml\(^{-1}\) each) and vancomycin (100 µg ml\(^{-1}\)), but sensitive to kanamycin, streptomycin and chloramphenicol (100 µg ml\(^{-1}\) each). CFAs of both strains were represented by straight-chain saturated compounds including long-chain fatty acids; in addition, branched fatty acids were detected in cells of strain R1T (Table 1). No unsaturated or hydroxy fatty acids were detected. The major components of the CFA profile of the two strains were: C\(_{16:0}\) C\(_{18:0}\) and C\(_{20:1}\). Strain SBP2T also contained a significant amount of C\(_{19:0}\) (9.2%), whereas strain R1T contained iso-C\(_{19:0}\) (7.6%). Other fatty acids were present in low amounts (<2% of the total fatty acid content). The G+C content of the genomic DNA of strains R1T and SBP2T was 57.3 and 66.6 mol%, respectively. A comparison of nearly complete 16S rRNA gene sequences of the new isolates with those available in the GenBank database revealed that they belonged to the order Planctomycetales (Fig. 3). All of the 16S rRNA gene sequences available in GenBank closely related to strains R1T and SBP2T (93–100% similarity) were recovered from thermal environments. Strains R1T and SBP2T displayed high 16S rRNA gene sequence similarity (95.63 and 98.73%, respectively) to ‘Thermopirellula anaerolimosa’ VM20-7 (Liu et al., 2012). The 16S rRNA gene similarity between strains R1T and SBP2T was 96.7%. The closest match to a recognized species was to Blastopirellula marina DSM 3645T (Schlesner et al., 2004) with a 16S rRNA gene sequence similarity of 87.3%.

The Planctomycetes isolates obtained in this study are thermophilic (growth optima above 50 °C), facultatively anaerobic and microaerophilic organisms. Thus far, only two Planctomycetes have been reported to be able to grow in the temperature range 40–50 °C, namely Isophphaera pallida (isolated from a hot spring in Oregon) and ‘Thermopirellula anaerolimosa’ VM20-7 (isolated from a thermophilic waste treatment plant). However, I. pallida grows optimally at 41 °C and has a temperature maximum for growth of 52 °C (Giovannoni et al., 1987a) and ‘T. anaerolimosa’ grows optimally at 45 °C with an upper temperature limit of 50 °C (Liu et al., 2012). In contrast, the new isolates can grow up to 65–67 °C with the optimum at 52–60 °C, and thus represent the first genuine thermophiles among the phylum Planctomycetes. Both isolates are able to perform diverse catabolic processes, namely fermentation of organic substrates as well as aerobic and anaerobic respiration. The vast majority of characterized planctomycetes are strict aerobes; only three species have the ability for growth via fermentation of organic substrates, namely Schlesneria paludicola, Physicphaera mikurensis and ‘Thermopirellula anaerolimosa’ (Fukunaga et al., 2009; Kulichevskaya et al., 2007; Liu et al., 2012).
Nitrate reduction has been demonstrated for a few species in the phylum *Planctomycetes*, but production of ammonium was not previously reported (Fukunaga *et al.*, 2009; Kulichevskaya *et al.*, 2007). Strain SBP2<sup>T</sup> is also capable of anaerobic growth using sulfur as electron acceptor with production of hydrogen sulfide. This ability has previously been reported for only one *Planctomycetes* strain, *Zi62*, isolated from a sulfide-saturated spring (Elshahed *et al.*, 2007). The level of 16S rRNA gene sequence similarity between strains SBP2<sup>T</sup> and R1<sup>T</sup> is below 97%, indicating that these strains represent different species of the same genus. The distinct position of this genus is indicated by the low level (<87%) of 16S rRNA gene sequence similarity with validly described species (Fig. 3). It is also supported by the considerable difference in CFA profiles between the new isolates and other representatives of the phylum *Planctomycetes*. C<sub>18:1</sub>ω9c and C<sub>16:0</sub> are the two most abundant fatty acids in the *Pirellula*—*Rhodopirellula*—*Blastopirellula* group and in some strains of *Planctomyces* (Kerger *et al.*, 1988) as well as in *Singulisphaera acidiphila* (Kulichevskaya *et al.*, 2008). Several planctomycetes contain 3-OH fatty acids (Giovannoni *et al.*, 1987b; Sittig & Schlesner, 1993). C<sub>16:0</sub> is also a major component of the new, thermophilic strains, but their CFA profiles lack unsaturated and hydroxy fatty acids. Some phenotypic differences exist between strains R1<sup>T</sup> and SBP2<sup>T</sup>. Strain R1<sup>T</sup> is more thermophilic and utilizes a narrower spectrum of substrates; it is incapable of sulfur reduction and contains branched fatty acids among its major CFAs (Tables 1 and 2).

Thus, this work demonstrates the existence of thermophilic planctomycetes belonging to a separate genus in the family *Planctomycetaceae*. Sharing the main morphological and nutritional features with mesophilic organotrophic members of the order *Planctomycetales*, the new isolates differ from them by the capacity for anaerobic growth in both the absence and the presence of electron acceptors. Thermophilic planctomycetes occur in various natural and anthropogenic thermal habitats, sharing this ecological niche with many moderately thermophilic chemo-organotrophic bacteria participating in destruction of complex organic substrates in these environments. On the basis of their phylogenetic position and phenotypic and physiological properties we suggest that the new isolates represent two novel species belonging to a new genus. Strains R1<sup>T</sup> and SBP2<sup>T</sup> are closely related to ‘*Thermopirellula anaerolimosa*’ VM20-7 (Liu *et al.*, 2012). However, because the genus ‘*Thermopirellula*’ has not been formally described, we propose strain R1<sup>T</sup> as the type strain of the type species of a new genus, *Thermogutta terrifontis* gen. nov., sp. nov. We also propose that strain SBP2<sup>T</sup> should be placed in the genus *Thermogutta* as the type strain of a second species, *Thermogutta hypogea* sp. nov.

### Table 2. CFA composition (%) of strains R1<sup>T</sup> and SBP2<sup>T</sup>

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>R1&lt;sup&gt;T&lt;/sup&gt;</th>
<th>SBP2&lt;sup&gt;T&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;14:0&lt;/sub&gt;</td>
<td>1.4</td>
<td>0.8</td>
</tr>
<tr>
<td>iso-C&lt;sub&gt;15:0&lt;/sub&gt;</td>
<td>3.0</td>
<td>0.1</td>
</tr>
<tr>
<td>anteiso-C&lt;sub&gt;15:0&lt;/sub&gt;</td>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>C&lt;sub&gt;15:0&lt;/sub&gt;</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>iso-C&lt;sub&gt;16:0&lt;/sub&gt;</td>
<td>0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>C&lt;sub&gt;16:0&lt;/sub&gt;</td>
<td>48.5</td>
<td>61.2</td>
</tr>
<tr>
<td>iso-C&lt;sub&gt;17:0&lt;/sub&gt;</td>
<td>1.6</td>
<td>0.4</td>
</tr>
<tr>
<td>C&lt;sub&gt;18:0&lt;/sub&gt;</td>
<td>27.9</td>
<td>18.7</td>
</tr>
<tr>
<td>C&lt;sub&gt;19:0&lt;/sub&gt;</td>
<td>0.5</td>
<td>9.2</td>
</tr>
<tr>
<td>iso-C&lt;sub&gt;19:0&lt;/sub&gt;</td>
<td>7.6</td>
<td>–</td>
</tr>
<tr>
<td>C&lt;sub&gt;20:0&lt;/sub&gt;</td>
<td>6.9</td>
<td>8.2</td>
</tr>
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</table>
Description of Thermogutta terrifontis sp. nov.

Thermogutta terrifontis (ter.ri fon’ tis. L. n. terra earth, soil; L. n. fons, fons spring, fountain; N.L. gen. n. terrifontis of a terrestrial spring or fountain).

Has the following properties in addition to those listed in the genus description. Cells are ovoid or drop-shaped, 1.0–1.5 μm in diameter. The temperature range for growth is 25–67 °C with the optimum at 55–60 °C. The pH range for growth is 4.0–8.0 with the optimum at pH 6.0–6.5. Able to grow in the presence of up to 3.0 % (w/v) NaCl. Capable of microaerobic growth. Grows under anaerobic conditions with glucose, cellobiose, trehalose, sucrose, starch, xylan and xanthan gum as electron donors and nitrate or nitrite as electron acceptors. Ammonium is the only product of nitrate reduction. Does not use sulfur, sulfate, sulfite, thiosulfate, fumarate, cerierydrite or Fe(III) citrate as electron acceptors. Capable of growth with external electron acceptors by fermentation of glucose, cellobiose, trehalose, sucrose, starch, xylan and xanthan gum. The end products of glucose fermentation are hydrogen, lactate and acetate. Catalase-positive, oxidase-negative. Resistant to penicillin, vancomycin and ampicillin, but sensitive to kanamycin, streptomycin and chloramphenicol.

The type strain, R1T (=DSM 26237T=VKM B-2805T), was isolated from a terrestrial hot spring at Kunashir Island, Kurils, Russia. The DNA G+C content of the type strain is 57.3 mol% (Tm).

Description of Thermogutta hypogea sp. nov.

Thermogutta hypogea (hy.po.ge’a. Gr. adj. hypogeus -on underground, subterranean; N.L. fem. adj. hypogea under the earth, referring to the site of isolation).

Has the following properties in addition to those listed in the genus description. Cells are ovoid or drop-shaped, 1.0–1.5 μm in diameter. The temperature range for growth is 30–60 °C with the optimum at 52 °C. The pH range for growth is 5.0–9.0 with the optimum at pH 7.5–8.0. Able to grow in the presence of up to 3.0 % (w/v) NaCl. Capable of microaerobic growth. Grows under anaerobic conditions with glucose, fructose, lactose, galactose, mannose, trehalose, xylose, agarose, cellobiose, sucrose, pectin, starch, xanthan gum, xylan and lactate as electron donors and nitrate, nitrite or elemental sulfur as electron acceptors. Nitrate is reduced to ammonium, and sulfur is reduced to sulfide. Does not use sulfate, sulfite, thiosulfate, fumarate, cerierydrite or Fe(III) citrate as electron acceptors. Capable of growth with external electron acceptors by fermentation of glucose, cellobiose, trehalose, sucrose, starch, xylan and xanthan gum. The end products of glucose fermentation are hydrogen, lactate and acetate. Catalase-positive, oxidase-negative. Resistant to penicillin, vancomycin and ampicillin, but sensitive to kanamycin, streptomycin and chloramphenicol.

The type strain, SBP2T (KC867695), was isolated from a terrestrial spring at Kunashir Island, Kurils, Russia. The DNA G+C content of the type strain is 57.3 mol% (Tm).
of growth without external electron acceptors by fermentation of glucose, fructose, lactose, galactose, trehalose, agarose, sucrose, pectin, starch and xanthan gum. The end products of glucose fermentation are hydrogen, lactate and acetate. Catalase- and oxidase-negative. Resistant to penicillin, vancomycin and ampicillin, but sensitive to kanamycin, streptomycin and chloramphenicol.

The type strain, SBPZT (=JCM 19991T=VKM B-2782T), was isolated from deep-subsurface gold mine fracture water (Beatrix mine, Free State, South Africa). The DNA G+C content of the type strain is 66.6 mol% (Tm).

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References


