Tepidisphaera mucosa gen. nov., sp. nov., a moderately thermophilic member of the class Phycisphaerae in the phylum Planctomycetes, and proposal of a new family, Tepidisphaeraceae fam. nov., and a new order, Tepidisphaerales ord. nov.

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Three strains of facultatively aerobic, moderately thermophilic bacteria were isolated from terrestrial hot springs in Baikal Lake region and Kamchatka (Russia). Cells of the new isolates were cocci reproducing by binary fission. The temperature range for growth was between 20 and 56 °C and the pH range for growth from pH 4.5 to 8.5, with optimal growth at 47–50 °C and pH 7.0–7.5. The organisms were chemoheterotrophs preferring sugars and polysaccharides as growth substrates. 16S rRNA gene sequences of strains 2842, 2813 and 2918Kr were nearly identical (99.7–100 % similarity) and indicated that the strains belonged to the phylum Planctomycetes. The phylogenetically closest cultivated relatives were Algisphaera agarlytica 06SJIR6-2T and Phycisphaera mikurensis FYK2301M01T with 16S rRNA gene sequence similarity values of 82.4 and 80.3 %, respectively. The novel strains differed from them by higher growth temperature, sensitivity to NaCl concentration above 3.0 % and by their cellular fatty acids profile. On the basis of phylogenetic and physiological data, strains 2842T, 2813 and 2918Kr represent a novel genus and species for which we propose the name Tepidisphaera mucosa sp. nov. The type strain is 2842T (=VKM B-2832T =JCM 19875T). We also propose that Tepidisphaera gen. nov. is the type genus of a novel family, Tepidisphaeraceae fam. nov. and a novel order, Tepidisphaerales ord. nov.

Thermophilic micro-organisms represent diverse phylogenetic lineages of prokaryotes, including new deep ones (Cole et al., 2013; Kawaichi et al., 2013; Podosokorskaya et al., 2013). Some phylogenetic groups inhabiting thermal environments contain only thermophiles, while others contain organisms with different temperature characteristics (Lebedinsky et al., 2007). Thermophilic representatives are now found even in bacterial phyla previously considered to contain only mesophilic prokaryotes, such as the phylum Acidobacteria (Losey et al., 2013). Another example is the phylum Planctomycetes. Analysis of the diversity and distribution of this group in thermal environments based on environmental molecular data revealed several phylogenetic groups of the phylum Planctomycetes most frequently detected in these habitats (A. Yu. Merkel and others, unpublished results). Several isolates from different terrestrial and subsurface thermal habitats were obtained, enriched and/or isolated in pure cultures and identified as members of the new genus ‘Thermogutta’ (Slobodkina et al., 2014) which is part of the Pirellula–Blastopirellula–Rhodopirellula cluster. These organisms, together with a representative of the genus ‘Thermopirellula’ (name not validly published; Liu et al., 2012), are to our knowledge the first thermophilic representatives of the phylum Planctomycetes.

Abbreviation: ECL, equivalent chain length.
The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 2842T, 2813 and 2918Kr are KM036168, KM052380 and KM052379, respectively.

A supplementary figure is available with the online Supplementary Material.
Phylogenetically, the phylum Planctomycetes comprises three large groups at the class level: (i) ‘Planctomycetia’, (ii) Phycisphaerae, and (iii) deep-branching planctomycetes of the order ‘Brocadiales’ represented by ‘anammox’ (anaerobic ammonium oxidation) bacteria which have not been isolated in pure culture to date. Most cultivated species belong to the class ‘Planctomycetia’ whereas the class Phycisphaerae includes only two genera, both represented by marine isolates, Phycisphaera mikurensis FYK2301M01^T (Fukunaga et al., 2009) and Algisphaera agarlytica 06SJ6R6-2^T (Yoon et al., 2014). Both organisms were isolated from marine algae and turned out to be mesophilic marine bacteria.

Here we report on three strains of moderately thermophiliic planctomycetes belonging to a deep branch in the class Phycisphaerae.

The strains were isolated from different terrestrial hot springs in Russia (Table 1). Samples of water and surface sediment layer with microbial mat were collected in tightly stoppered sterile 50 ml plastic tubes during expeditions in the summers of 2012 and 2013. The tubes were stored under environmental conditions during transportation and after that at 4 °C until being used.

Initial enrichment cultures were obtained on basal medium containing (g l^−1): NH₄Cl, 0.33; KCl, 0.33; MgCl₂·6H₂O, 0.33; CaCl₂, 0.33; KH₂PO₄, 0.33; NaHCO₃, 0.5; yeast extract (Helicon), 0.05; trace element solution (Kevbrin & Zavarzin, 1992) 1.0 ml; vitamin solution (Wolin et al., 1963), 1.0 ml. To achieve anaerobic conditions the basal medium was boiled and degassed in N₂ atmosphere. No reducing agents were added. pH was adjusted to pH 7.5. Xanthan gum (0.5 g l^−1; Kelco) was used as substrate. Penicillin at a final concentration of 0.2 g l^−1 was added in order to provide selective conditions for the growth of planctomycetes. Enrichments were grown in 50 ml glass vials filled with 10 ml of basal medium and inoculated with about 1 ml of water/sediment mixture. The headspace was filled with N₂. Vials were incubated at 47 °C for 14–18 days. At the end of the incubation time cells of 2–3 morphological types were revealed, among them coccioid cells of different size. Several subsequent transfers following serial 10-fold dilutions on the same media resulted in isolation of organisms with cocoid cells. According to the designations of sampling sites the strains were designated 2842^T, 2813 and 2918Kr. Routine morphological observations were made with an Olympus CX-41 light microscope. Cell size measurement was made with a Zeiss Axioplan 2 microscope and Axiovision 4.2 software (Zeiss).

Cells of the new isolates shared the same morphology. They were motile and non-motile cocoid cells, 0.5 to 1.2 μm in size. Sometimes the cells were in pairs or formed short chains (up to 4–5 cells) or shape-less aggregates (Fig. 1a). 16S rRNA gene sequence analysis revealed 99.7–100 % similarity among the new isolates, thus revealing their belonging to the same species. Strain 2842^T was selected as the type strain and was characterized in detail.

The ability to grow aerobically was tested in 100 ml glass vials filled with 10 ml basal medium prepared under aerobic conditions. In these conditions, the growth of the isolate was much better. Thus, strain 2842^T was found to be a facultative anaerobe. Yeast extract did not influence the growth of the strain. Unless mentioned otherwise, all the tests described below were performed under aerobic conditions in the absence of yeast extract in the medium.

Colonies were formed on basal medium supplemented with 1.0 % (w/v) agar and 1 g raffinose l^−1 as a substrate. On the surface of agar plates, strain 2842^T formed circular, 1 mm in diameter colonies raised above the agar surface. The colonies were opaque and white to rosy in colour.

Culture for electron microscopy was grown on basal medium containing raffinose (1 g l^−1) and collected during late exponential phase. Negative staining and ultrathin section preparation were performed as described previously (Bonch-Osmolovskaya et al., 1990). The cells possessed a single flagellum and divided by binary fission (Fig. 1b). No intracytoplasmic membranes were observed (Fig. 2).

The basal medium was used for all physiological tests and for chemotaxonomic analyses. Experiments were carried out in triplicate in 100 ml vials filled with 15 ml medium. Growth was tested at different temperatures (between 14 and 65 °C), pH (from pH 4.0 to 9.0) and NaCl concentrations (0–3.5 %, w/v) by observing cell growth for up to 10–14 days. Optimal growth was defined as the fastest growth rate observed by measuring protein concentration using a Qubit 2.0 Fluorometer with the Qubit Protein assay kit (Invitrogen). The novel isolates grew at temperatures between 20 and 56 °C and pH ranging from pH 4.5 to 8.5. No growth was detected at 14 or 60 °C or at pH 4.0 or 9.0. The optimal conditions for growth of strain 2842^T were 47–50 °C at pH 7.0–7.5.

**Table 1. Sampling sites characteristics**

<table>
<thead>
<tr>
<th>Sample (designation and type)</th>
<th>Sampling site</th>
<th>Temp. (°C)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>2813 Sediment with microbial mat on it</td>
<td>Baikal Lake region (Russia) (54° 59’ 15.6″ N 111° 07’ 06.3″ E)</td>
<td>41</td>
<td>9.3</td>
</tr>
<tr>
<td>2842 Microbial mat</td>
<td>Baikal Lake region (Russia) (52° 59’ 14.3″ N 108° 18’ 28.1″ E)</td>
<td>51</td>
<td>8.8</td>
</tr>
<tr>
<td>2918Kr Sediment with microbial mat on it</td>
<td>Bank of Karymsha river (52° 48.622’ N 158° 05.509′ E)</td>
<td>52</td>
<td>5.2</td>
</tr>
</tbody>
</table>
Catalase activity was determined with 5% (v/v) hydrogen peroxide solution and oxidase activity was determined using an oxidase reagent (bioMérieux). The strain revealed positive oxidase but negative catalase reactions.

For growth substrate studies, filter-sterilized carbon sources were added to a final concentration of 1.0 g l⁻¹. Strain 2842ᵀ was able to grow on xylose, sucrose, raffinose, trehalose, galactose, lactose, maltose, mannose, glucose, N-acetylglucosamine, starch, pectin, xanthan gum, locust bean gum, gum arabic and dextrin. Arabinose, fructose, xylan, chitin, yeast extract and peptone, as well as organic acids (acetate, formate, propionate, butyrate, lactate, citrate, malate, pyruvate, fumarate) were not utilized.

The ability to perform fermentative growth was tested in Hungate tubes filled with 8 ml of degassed basal medium reduced by adding Na₂S·9 H₂O (0.5 g l⁻¹) and supplemented with resazurin (1 mg l⁻¹) as redox indicator. The growth substrates tested were added at a final concentration of 1 g l⁻¹. Xanthan gum was used at a concentration of 0.5 g l⁻¹. The headspace was filled with N₂. Strain 2842ᵀ was checked for the ability to ferment xanthan gum, glucose, raffinose, trehalose, xylose, maltose and mannose. All these substrates supported anaerobic growth that was estimated by microscopy and organic acids production. Production of organic acids was analysed with a Stayer HPLC chromatograph (Aquilon) equipped with a refractometric detector (Knauer) and Aminex HPX-87H column (Bio-Rad), operated isocratically using 5 mM H₂SO₄ as eluent at 0.6 ml min⁻¹. Identification of gaseous products of metabolism was performed by GC with a Haye Sep N 80/100 mm column at 40°C and at flow rates of 20 ml min⁻¹ (carrier gas, argon). The main products of fermentation were acetate and propionate. Hydrogen production was not observed.

Sodium nitrate (10 mM), sodium nitrite (5 mM), sodium sulfate (10 mM), elemental sulfur or sodium fumarate supplemented to the medium as potential electron acceptors did not influence anaerobic growth and no reduced products were detected.

Cells for chemotaxonomic analysis were grown with raffinose (1 g l⁻¹) as growth substrate under optimal conditions and collected in late exponential phase. Cellular fatty acids were analysed according to the protocol of Sasser (1990) as fatty acid methyl esters but GC-MS signal strength was low, so we suspected that the large part of strain 2842ᵀ lipids was either unsaponifiable or destroyed during sample preparation. For this reason, cellular fatty acids were additionally analysed by derivatization of freeze-dried cells by a solution of BF₃ in methanol (Christie et al., 2001) and the signal strength was approximately 10 times higher compared with that when fatty acids were analysed as described by Sasser (1990). The fatty acids profile of strain 2842ᵀ differed significantly from that of other members of the phylum. Major fatty acids (>5%) extracted according to the method of Sasser (1990) included iso-C₁₆:₀ (66.8 %), anteiso-C₁₅:₀ (10.1 %) and an unidentified fatty acid [6.9 %, equivalent chain length (ECL) = 16.39, major peaks in 70 eV EI-MS spectrum were 88, 101, 143, 157, 199, 241, 284]. Major fatty acids (>5%) extracted according the method of Christie et al. (2001) included iso-C₁₆:₀ (74.2 %), anteiso-C₁₇:₀ (8.3 %) and anteiso-C₁₅:₀ (5.1 %) (Table 2). Both methods revealed iso-C₁₆:₀ as one of the dominating fatty acid and that is quite uncommon for bacteria.
Table 2. Cellular fatty acids of strain 2842<sup>T</sup>

Extraction method: 1, according to the protocol of Sasser (1990); 2, by methylation using BF<sub>3</sub> in methanol. Values represent percentages of total fatty acids. Major fatty acids are shown in bold type. tr, Trace amount (<1.0 %); ND, not detected.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated straight-chain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C&lt;sub&gt;14&lt;/sub&gt;:0</td>
<td>ND</td>
<td>tr</td>
</tr>
<tr>
<td>C&lt;sub&gt;15&lt;/sub&gt;:0</td>
<td>2.9</td>
<td>tr</td>
</tr>
<tr>
<td>C&lt;sub&gt;16&lt;/sub&gt;:0</td>
<td>tr</td>
<td>1.4</td>
</tr>
<tr>
<td>C&lt;sub&gt;17&lt;/sub&gt;:0</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>C&lt;sub&gt;18&lt;/sub&gt;:0</td>
<td>ND</td>
<td>tr</td>
</tr>
<tr>
<td>C&lt;sub&gt;19&lt;/sub&gt;:0</td>
<td>ND</td>
<td>tr</td>
</tr>
<tr>
<td>C&lt;sub&gt;20&lt;/sub&gt;:0</td>
<td>ND</td>
<td>1.5</td>
</tr>
<tr>
<td>Branched</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i-C&lt;sub&gt;14&lt;/sub&gt;:0</td>
<td>4.4</td>
<td>1.7</td>
</tr>
<tr>
<td>i-C&lt;sub&gt;15&lt;/sub&gt;:0</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>i-C&lt;sub&gt;16&lt;/sub&gt;:0</td>
<td><strong>66.8</strong></td>
<td><strong>74.2</strong></td>
</tr>
<tr>
<td>i-C&lt;sub&gt;18&lt;/sub&gt;:0</td>
<td>ND</td>
<td>0.8</td>
</tr>
<tr>
<td>ai-C&lt;sub&gt;15&lt;/sub&gt;:0</td>
<td>10.1</td>
<td>5.1</td>
</tr>
<tr>
<td>ai-C&lt;sub&gt;17&lt;/sub&gt;:0</td>
<td>3.7</td>
<td><strong>8.3</strong></td>
</tr>
<tr>
<td>Hydroxy substituted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C&lt;sub&gt;14&lt;/sub&gt;:0 2-OH</td>
<td>tr</td>
<td>ND</td>
</tr>
<tr>
<td>Unidentified</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECL 16.39</td>
<td><strong>6.9</strong></td>
<td>ND</td>
</tr>
<tr>
<td>ECL 17.38</td>
<td>tr</td>
<td>ND</td>
</tr>
<tr>
<td>ECL 20.61</td>
<td>tr</td>
<td>ND</td>
</tr>
</tbody>
</table>

The determination of polar lipids was performed as described previously (Slobodkina et al., 2013). Polar lipids of strain 2842<sup>T</sup> were phosphatidylethanolamine, phosphatidyserine and phosphatidylglycerol. In addition, three unidentified aminophospholipids were detected. Three unidentified lipids displayed no reaction with any reagents used for lipid identification (Fig. S1, available in the online Supplementary Material). The respiratory quinone fraction was analysed by HPLC (Jasco) on a C<sub>18</sub> reversed-phase column and by LC-MS. Strain 2842<sup>T</sup> contained menaquinone-6 (MK-6; structure was confirmed by LC-MS data) as the predominant quinone.

Antibiotic resistance was tested by adding antibiotic stock solutions to inoculated tubes. Strain 2842<sup>T</sup> was resistant to penicillin, streptomycin (both 200 μg ml<sup>-1</sup>), kanamycin and polymyxin B (both 100 μg ml<sup>-1</sup>) but sensitive to vancomycin (100 μg ml<sup>-1</sup>) and chloramphenicol (100 μg ml<sup>-1</sup>). DNA was extracted using a phenol/chloroform method (Marmur, 1961). The 16S rRNA gene was amplified using general bacterial 11F/1492R primers (Lane, 1991). The PCR products were sequenced using a Big Dye Terminator v.3.1 sequencing reaction kit with an ABI 3730 DNA automatic sequencer according to manufacturer’s protocol (Applied Biosystems). Preliminary phylogenetic analysis of the new sequences was performed with the NCBI blast server (http://www.ncbi.nlm.nih.gov/BLAST/); complete (approx. 1460 bp) sequences of 16S rRNA genes were compared with each other and with known sequences in the GenBank database. Two strains (2842 and 2813) showed 100 % similarity over the length of sequences with shared positions in the alignment while in the 16S rRNA sequence of strain 2918Kr several replacements had taken place and thus, the sequence was only 99.7 % similar to that of strain 2842<sup>T</sup>. The DNA G + C (mol%) content of strain 2842<sup>T</sup> was determined by direct counting of complete genome data and was 53.0 mol%.

Preliminary phylogenetic analysis of the 16S rRNA gene sequences placed the strains in the class Phycisphaerae within the phylum Planctomycetes. 16S rRNA gene sequence comparison among species with validly published names revealed 82.4 % similarity of strain 2842<sup>T</sup> to Algisphaera agarlytica 06SR6-2<sup>T</sup> (Yoon et al., 2014) and 80.3 % similarity to Phycisphaera mikurensis FYK2301M01<sup>T</sup> (Fukunaga et al., 2009). Both micro-organisms are obligatory marine planctomycetes isolated from marine alga.

To define the exact phylogenetic position of the new isolates, 16S rRNA gene sequences of all cultivated species of the phylum Planctomycetes including 'Candidatus' species of the order 'Brocadiales' were retrieved from the SILVA database (115 release; Quast et al., 2013) according to Greengenes taxonomy (DeSantis et al., 2006). Sequences of uncultured members of the class Phycisphaerae were also added to the analysis. The resulting alignment contained 228 sequences with minimal length of 1400 bp, minimal pintail quality of 90 % and minimal sequence quality of 90 %. This number of sequences was then reduced by their clustering into Operative Taxonomic units (OTUs) using the Cd-hit program (Li & Godzik, 2006) with 90 % identity as clustering threshold. The clustering resulted in 36 OTUs. The 16S rRNA gene sequence of Caldithrix abyssi was used as a root.

Phylogenetic trees were reconstructed by using the PhyML 3 program (Guindon et al., 2010) and the following parameters of phylogenetic reconstruction: GTR as a model of nucleotide substitution, optimized equilibrium frequencies, estimated proportion of invariable sites, 10 substitution rate categories, estimated gamma distribution parameter, SPR topological rearrangements and Bayesian-like transformation of approximate likelihood ratio test (Anisimova et al., 2011). arbo software (version 5.5) (Ludwig et al., 2004) was used for phylogenetic tree reconstruction using mrbays 3.2 software (Ronquist et al., 2012) and the following parameters of phylogenetic reconstruction: GTR as a model of nucleotide substitution, estimated proportion of invariant sites and estimated gamma distribution parameter.

On the phylogenetic tree all three strains clustered together and formed a deep branch in the class Phycisphaerae (Fig. 3). This was also confirmed by another method of phylogenetic analysis based on Bayesian inference of phylogeny (data not shown). This branch contained about 500 individual 16S rRNA gene sequences of uncultured
organisms deposited in GenBank and represents one of the deepest lineages within the class **Phycisphaerae**.

Based on differences in growth conditions, physiology, chemotaxonomic characteristics and distinct phylogenetic position we suggest that strains 2842T, 2813 and 2918Kr represent a novel genus and novel species, for which the name **Tepidisphaera mucosa** gen. nov., sp. nov. is proposed. The type strain is 2842T. We also propose that strains 2842T, 2813 and 2918Kr should be classified as representatives of a new family, **Tepidisphaeraceae** fam. nov., and a new order, **Tepidisphaerales** ord. nov., in the class **Phycisphaerae** of the phylum **Planctomycetes**.

In summary, our isolates are clearly distinct from other members of the class **Phycisphaerae**. For a long period the class **Phycisphaerae** was represented by a single isolate – a mesophilic marine bacterium. The second member of this group, obtained recently also from a marine environment, was phylogenetically distinct and belonged to a novel genus, but phenotypically resembled the first one. Our findings showed that the representatives of the class **Phycisphaerae** are much more diverse both phylogenetically and phenotypically: our isolates represent a novel order of the same class and are adapted to life in terrestrial hot springs. Inhabiting geographically remote thermal environments they seem to be an ubiquitous component of thermal springs with moderate temperature.

**Description of Tepidisphaera gen. nov.**

*Tepidisphaera* [Te.pi.di.spha’ra. L. adj. tepidus warm; L. fem. n. sphaera a globe, sphere; N.L. fem. n. Tepidisphaera a warm sphere, spherical bacterium growing at moderately high temperature (up to 50 °C)].


Members of the class **Phycisphaerae** within the phylum **Planctomycetes** of the domain **Bacteria**. The type species is **Tepidisphaera mucosa**.

**Description of Tepidisphaera mucosa sp. nov.**

*Tepidisphaera mucosa* (mu.co’sa. L. fem. adj. mucosa slimy, referring to the property of cells to produce exopolysaccharide during growth in liquid medium).
In addition to the properties given in the genus description, displays the following characteristics. Cells are cocci, 0.5–1.2 μm in diameter, presented alone, in pairs, in short chains or forming non-compact aggregates. Some cells are motile by means of a single flagellum. Stalk-like structures or forming non-compact aggregates. Some cells are not observed. Growth is observed at temperatures between 20 and 56 °C with the optimum at 47–50 °C. Optimum pH for growth is pH 7.0–7.5 (range is pH 4.5–8.5). During aerobic cultivation in liquid medium cells are rosy although under anaerobic conditions the culture is creamy or white. On agar medium, circular, smooth, white to rosy colonies are formed. Agar is not degraded. Catalase activity is negative and oxidase activity is positive. Yeast extract is not required for growth. Can utilize sucrose, raffinose, xylose, trehalose, galactose, lactose, maltose, mannose, glucose, N-acetylglucomamine, starch, pectin, xanthan gum, locust bean gum, gum arabic and dextrin under aerobic conditions. Arabinose, fructose, xylan, chitin, yeast extract and peptone do not support growth. Able to perform fermentative growth on glucose, raffinose, trehalose, xanthan gum, xylose and maltose with production of acetate and propionate. Nitrate and nitrite reduction is negative. Organic acids are not utilized. Major fatty acids are i-C16:0, ai-C15:0, ai-C17:0 and one unidentified fatty acid (ECL = 16.39). The major quinone is MK-6. The polar lipids are phosphatidylethanolamine, phosphatidylserine and phosphatidylglycerol.

The type strain is 2842T (=VKM B-2832T = JCM 19875T), which was isolated from a microbial mat surrounding a hot water outflow near Goryachinsk city in Baikal lake region. The G + C content of the genomic DNA of the type strain is 53.0 mol%.

**Description of Tepidisphaeraceae fam. nov.**

*Tepidisphaerae* (Te.pi.di.sphae.rae.‘ae. N.L. fem. n. *Tepidisphaera* type genus of the family; -‘aece ending to denote a family; N.L. fem. pl. n. *Tepidisphaeraceae* family of the genus *Tepidisphaera*).

The description of the family is the same as that of the genus *Tepidisphaera*. The type genus is *Tepidisphaera*.

**Description of Tepidisphaerales ord. nov.**

*Tepidisphaerales* (Te.pi.di.sphae.‘les. N.L. fem. n. *Tepidisphaera* type genus of the order; -‘ales ending to denote an order; N.L. fem. pl. n. *Tepidisphaerales* the order of genus *Tepidisphaera*).

The description is the same as that for the genus *Tepidisphaera*. The order contains the family *Tepidisphaeraceae*. The type genus is *Tepidisphaera*.

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