Singulisphaera rosea sp. nov., a planctomycete from acidic *Sphagnum* peat, and emended description of the genus *Singulisphaera*

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An aerobic, pink-pigmented, budding bacterium, designated strain S26^T, was isolated from an acidic *Sphagnum* peat bog of north-western Russia. Cells were non-motile and spherical, occurring singly, in pairs or in short chains, and were able to attach to surfaces by means of a holdfast material. Strain S26^T was a moderately acidophilic, mesophilic organism capable of growth at pH 3.2–7.1 (optimum at pH 4.8–5.0) and at 4–33 °C (optimum at 20–26 °C). Most sugars, several organic acids and polyalcohols were the preferred growth substrates. The major fatty acids were C_{16:0}, C_{18:1ω9c} and C_{18:2ω6c,12c}. The major neutral lipids were n-C_{31:9} hydrocarbon and squalene; the polar lipids were phosphatidylglycerol, phosphatidylcholine and components with an unknown structure. The DNA G + C content of strain S26^T was 62.2 mol%. 16S rRNA gene sequence analysis showed that strain S26^T is a member of the order *Planctomycetales*. Among taxonomically characterized representatives of this order, highest levels of 16S rRNA gene sequence similarity (95.1–95.2%) were observed with strains of the non-filamentous, peat-inhabiting planctomycete *Singulisphaera acidiphila*. Strain S26^T could be differentiated from *Singulisphaera acidiphila* based on pigmentation, significant differences in substrate utilization patterns, greater tolerance of acidic conditions and the presence of C_{16:1ω9c}. Based on the data presented, strain S26^T is considered to represent a novel species of the genus *Singulisphaera*, for which the name *Singulisphaera rosea* sp. nov. is proposed; the type strain is S26^T (=DSM 23044^T=VKM B-2599^T).

Members of the order *Planctomycetales* are budding bacteria that possess highly distinctive cell morphology, peptidoglycan-less cell walls and a unique cell organization (Staley *et al.*, 1992; Fuerst, 1995, 2005; Ward *et al.*, 2006). Planctomycetes represent a numerically abundant bacterial population in northern *Sphagnum*-dominated wetlands (Dedysh *et al.*, 2006; Kulichevskaya *et al.*, 2006). Several peat-inhabiting planctomycetes have been isolated in pure culture and described as representatives of three novel species of new genera, namely *Schlesneria paludicola*, *Singulisphaera acidiphila* and *Zavarzinella formosa* (Kulichevskaya *et al.*, 2007, 2008, 2009). These planctomycetes are aerobic, moderately acidophilic and mesophilic budding bacteria,

which utilize a wide range of sugars and several polysaccharides as growth substrates.

Differential detection of planctomycetes in acidic peat bogs using newly developed group-specific 16S rRNA gene-targeted fluorescent probes revealed that *Singulisphaera*-like micro-organisms were numerically the largest group of indigenous planctomycetes (A. O. Ivanova and S. N. Dedysh, unpublished data). They were represented by spherical cells that occurred singly, in short chains or in shapeless aggregates. We isolated a novel peat-inhabiting planctomycete of similar morphology, designated strain S26^T. Despite significant morphological similarity to *Singulisphaera acidiphila*, cells of the novel isolate were slightly larger and colonies were pink-pigmented. Preliminary substrate utilization tests revealed that strain S26^T was capable of growth on several organic acids, a feature lacking in *Singulisphaera acidiphila*. Finally, a partial (approx. 500 bp) 16S rRNA gene sequence of the novel isolate showed only 95% similarity to the

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain S26^T is FN391026.

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

corresponding gene fragment in *Singulisphaera acidiphila* MOB10^T. Therefore, this study was initiated to characterize strain S26^T in more detail and to determine its taxonomic position.

Strain S26^T was obtained from a peat sample collected from the top layer (5–10 cm) of the acidic (pH 3.8) *Sphagnum* peat bog Staroselskoye, located in the Central Forest Reserve, Tver region, north-west Russia (56° 58' N 32° 30' E). The isolation procedure and the culture conditions used were as described previously (Dedysh *et al.*, 2006; Kulichevskaya *et al.*, 2006). The isolate was maintained on agar medium M31 (modification of medium 31 described by Staley *et al.*, 1992) containing (per litre of distilled water): 0.1 g KH₂PO₄, 20 ml Hutner's basal salts, 1.0 g *N*-acetylglucosamine, 0.1 g peptone, 0.1 g yeast extract and 15 g agar-agar (Difco), pH 5.8. *Singulisphaera acidiphila* MOB10^T (Kulichevskaya *et al.*, 2008) was used as a reference strain in our study and was maintained on the same medium.

Morphological observations and cell size measurements were made with a Zeiss Axioplan 2 microscope and Axio-vision 4.2 software (Zeiss). Physiological tests were performed in liquid medium M31. Growth of strain S26^T was monitored by nephelometry at 600 nm in an Eppendorf BioPhotometer for 7–14 days under a variety of conditions, including 4–37 °C, pH 3.0–8.0 and NaCl concentrations of 0–4.0 % (w/v). Variations in pH were achieved by adding 0.1 M solutions of H₂SO₄ and KOH. Carbon source utilization was determined by using liquid mineral medium (MM) supplemented with respective carbon sources (0.05 %, w/v). MM contained (per litre distilled water): 0.1 g KH₂PO₄, 0.1 g (NH₄)₂SO₄, 0.1 g MgSO₄·7H₂O and 1 ml of metal salt solution '44' (Staley *et al.*, 1992), pH adjusted to 5.5. Cultivation was done in 100 ml flasks containing 10 ml medium. Cultures were incubated at 25 °C for 2–3 weeks on a shaker. Nitrogen sources were tested by using liquid MM with 0.05 % glucose in which (NH₄)₂SO₄ was replaced with one of the following compounds at a concentration of 0.05 % (w/v): KNO₃, KNO₂, urea or one of the amino acids listed in the species description below. The ability to degrade different biopolymers was determined as described for *Singulisphaera acidiphila* (Kulichevskaya *et al.*, 2008). Oxidative and fermentative utilization of carbohydrates was determined as described for the Hugh–Leifson test (Gerhardt, 1981) and by using the API 20NE kit (bioMérieux). Enzyme activities were examined by using the API ZYM kit (bioMérieux). Cultures were tested for growth under anaerobic conditions in anaerobic jars by using AnaeroGen anaerobic system envelopes (Oxoid). Susceptibility to antibiotics was determined on M31 agar plates by using discs containing the following compounds: 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) (Oxoid).

For analyses of fatty acids, and neutral and polar lipids, strain S26^T and *Singulisphaera acidiphila* MOB10^T were grown in parallel in liquid medium M31 under identical

conditions. Phospholipid fatty acids were analysed as described for *Schlesneria paludicola* (Kulichevskaya *et al.*, 2007). The positions of unsaturation in monoenoic and dienoic fatty acids were determined as described by Bodelier *et al.* (2009). Batches of cell material were subjected to base and acid hydrolysis and, subsequently, were analysed for fatty acids and neutral lipids. Acid hydrolysis was performed in 5 % (v/v) HCl/methanol by refluxing for 3 h. Base hydrolysis was performed by treatment with 1 M KOH in 96 % methanol by refluxing for 1 h. For neutral lipid analysis, the procedures described by Sinninghe Damsté *et al.* (2004) were used. Intact polar lipids were analysed by LC/MS according to Sturt *et al.* (2004) with some modifications. An Agilent 1200 series liquid chromatograph, equipped with a thermostatted auto-injector and column oven, was used, coupled to a Thermo LTQ XL linear ion trap with Ion Max source with electrospray ionization probe (Thermo Scientific).

Genomic DNA of strain S26^T was extracted as described by Marmur (1961). The G+C content of the DNA was determined by means of thermal denaturation by using a Unicam SP1800 spectrophotometer at a heating rate of 0.5 °C min⁻¹ and was calculated according to Owen *et al.* (1969). DNA of *Escherichia coli* K-12 (G+C content 51.7 mol%) was used as the standard. PCR-mediated amplification of the 16S rRNA gene from positions 28 to 1491 (numbering according to the International Union of Biochemistry nomenclature for the *E. coli* 16S rRNA gene) was performed by using primers 9f and 1492r and the reaction conditions described by Weisburg *et al.* (1991). 16S rRNA gene amplicons were purified by using QIAquick spin columns (Qiagen) and sequenced on an ABI Prism 377 DNA sequencer with BigDye terminator chemistry, as specified by the manufacturer (PE Applied Biosystems). Phylogenetic analysis was carried out by using the ARB program package (Ludwig *et al.*, 2004).

On agar medium M31, strain S26^T formed raised, pink-pigmented, circular colonies with an entire edge and a smooth surface. No gliding motility was observed in cultures of the novel isolate. Liquid cultures displayed light-pink turbidity. Mature cells of strain S26^T were spherical and typically varied in size from 1.8 to 3.2 µm, although some cells in old cultures were up to 3.5 µm in diameter (Fig. 1a). Cells were non-motile and encapsulated. They reproduced by budding and occurred singly, in pairs or in short chains containing up to 4–5 cells. However, these cell chains were unstable and no filaments were observed in cultures of strain S26^T. Examination of old cultures grown on agar plates revealed the presence of an amorphous holdfast substance excreted from the poles of many cells (Fig. 1b). Similar holdfast production has been reported for *Pirellula*-like bacteria (Schlesner, 1994) as well as for *Singulisphaera acidiphila* and *Z. formosa* (Kulichevskaya *et al.*, 2008, 2009). This holdfast substance enables the cells to attach to surfaces or to form aggregates by attaching to each other.

Fig. 1. Phase-contrast micrographs of cells of strain S26^T grown for 7 days in liquid culture (a) and for 2 weeks on agar medium (b); white arrows point to an amorphous holdfast substance excreted by the cells grown on agar medium. Bars, 10 µm.

Strain S26^T was an obligately aerobic chemoheterotroph. Similar to *Singulisphaera acidiphila*, it was not capable of fermenting carbohydrates. However, strain S26^T grew well under microaerobic conditions.

The list of carbon substrates tested in our experiments and the results are given in the species description below. Most sugars, several organic acids and sugar alcohols were the preferable growth substrates. Strain S26^T was capable of hydrolysing starch, laminarin, chondroitin sulfate, aesculin, gelatin and pullulan but not xylan, pectin, chitin or cellulose. It was positive for catalase activity, but negative for cytochrome oxidase and urease activities and dissimilatory nitrate reduction. Growth factors were not required. Ammonia, nitrate, *N*-acetylglucosamine, Bacto peptone, Bacto yeast extract, alanine, aspartate, arginine, glutamine, threonine, tryptophan, glycine, isoleucine, lysine, phenylalanine, proline and valine were utilized as nitrogen sources. Nitrite was not utilized. The following enzyme activities (API ZYM) were detected in strain S26^T: alkaline phosphatase, acid phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, phosphohydrolase, *N*-acetyl- β -glucosaminidase and β -glucosidase. The following enzyme activities were absent: arginine hydrolase, lipase, trypsin, chymotrypsin, cystine arylamidase, α -galactosidase,

β -glucuronidase, α -glucosidase, α -mannosidase and α -fucosidase. The isolate was resistant to ampicillin, streptomycin, chloramphenicol, neomycin and novobiocin, but sensitive to lincomycin, kanamycin and gentamicin.

Strain S26^T grew in the pH range 3.2–7.1, with optimum growth at pH 4.8–5.0 (see Supplementary Fig. S1 in IJSEM Online), and was therefore more acidophilic than *Singulisphaera acidiphila*. The temperature range for growth of strain S26^T was 4–33 °C, with optimum growth at 20–26 °C. Compared with *Singulisphaera acidiphila*, strain S26^T was slightly less sensitive to salt stress. Growth inhibition of 50–80 % was observed in the presence of NaCl in the medium at concentrations of 0.8–1.0 % (w/v), whereas complete inhibition of growth occurred at NaCl concentrations above 1.0 % (w/v).

The major fatty acids of strain S26^T recovered after both base and acid hydrolysis were C_{16:0}, C_{18:1 ω 9c} and C_{18:2 ω 6c,12c} (Table 1, Supplementary Fig. S2, available in IJSEM Online). High levels of these fatty acids were also characteristic for *Singulisphaera acidiphila* (Kulichevskaya *et al.*, 2008). However, the presence of C_{16:1 ω 9c} in the fatty acid profile of strain S26^T contrasted with that of *Singulisphaera acidiphila*. Analysis of the intact polar lipids by LC/MS (Sturt *et al.*, 2004) revealed the presence of phosphatidylglycerol, phosphatidylcholine and polar lipids of unknown structure with similar distributions for strain S26^T and *Singulisphaera acidiphila* MOB10^T (Supplementary Fig. S3, available in IJSEM Online). Major neutral lipids of strain S26^T were an n-C_{31:9} hydrocarbon and squalene (Supplementary Fig. S2), which are also the dominant neutral lipids of *Singulisphaera*

Table 1. Fatty acid composition (%) of strain S26^T and *Singulisphaera acidiphila* MOB10^T

Major fatty acids are shown in bold after base and acid hydrolysis of cell material. The phospholipid fatty acid composition (i.e. after Bligh–Dyer extraction, separation and hydrolysis of the methanol fraction) was nearly identical. Fatty acids comprising <0.1 % of the total were not included. –, Not detected.

Fatty acid	Strain S26 ^T		<i>Singulisphaera acidiphila</i> MOB10 ^T	
	Base	Acid	Base	Acid
C _{14:0}	5.7	5.2	1.8	1.8
C _{15:0}	0.3	0.3	0.4	0.4
iso-C _{16:0}	–	–	0.4	0.5
C _{16:1ω9c}	5.4	4.5	0.1	0.1
C _{16:1ω7c}	0.7	0.6	1.4	1.3
C_{16:0}	27.4	28.3	33.6	33.1
C _{17:1ω7c}	0.3	0.4	0.4	0.5
C _{17:0}	0.2	0.3	0.2	0.2
C_{18:2ω6c,12c}	11.6	13.3	11.6	11.9
C_{18:1ω9c}	41.8	40.2	42.7	42.8
C _{18:1ω9t}	0.5	0.5	0.9	0.8
C _{18:0}	6.3	6.5	6.5	6.5

acidiphila (Kulichevskaya *et al.*, 2008). Base hydrolysis of the residue following extraction released, in addition to the characteristic suite of fatty acids, the uncommon 27-hydroxy-octacosanoic acid at a relatively high abundance. Again, this was also observed for *Singulisphaera acidiphila* (Kulichevskaya *et al.*, 2008).

Comparative sequence analysis of the 16S rRNA gene showed that strain S26^T is a member of the order *Planctomycetales* and belongs to a phylogenetic cluster defined by the genera *Isosphaera*, *Aquisphaera* and *Singulisphaera* (Fig. 2). At the time of writing, this cluster contained three recognized representatives, namely *Isosphaera pallida* (a thermophilic filamentous neutrophile from hot springs; Giovannoni *et al.*, 1987), *Aquisphaera giovannonii* (a mesophilic non-filamentous neutrophile from the sediments of a freshwater aquarium; Bondoso *et al.*, 2011) and *Singulisphaera acidiphila* (a non-filamentous acidophile from northern peatlands; Kulichevskaya *et al.*, 2008). Levels of 16S rRNA gene sequence similarity between strain S26^T and strains of *I. pallida* and *A. giovannonii* were 89.0 and 92.0–92.4%, respectively, whereas the corresponding values between strain S26^T and strains of *Singulisphaera acidiphila* were 95.1–95.2%. Among taxonomically uncharacterized planctomycetes, the highest level of 16S rRNA gene sequence similarity (96.8%) was observed with *Isosphaera*-like isolate C2-3 from a laboratory ampicillin solution (Wang *et al.*, 2002). In a maximum-likelihood analysis, this latter isolate clustered together with strain S26^T. The use of other algorithms, however, failed to produce a consistent affiliation of isolate C2-3 to strain S26^T. Given that isolate C2-3 is non-pigmented (Wang *et al.*, 2002), it is unlikely to be a member of the same species as strain S26^T. The DNA G + C content of strain S26^T was 62.2 mol%.

In summary, our novel isolate from acidic peat was morphologically, phenotypically and phylogenetically most similar to *Singulisphaera acidiphila*. However, in contrast to the latter planctomycete, strain S26^T was pigmented, was able to utilize organic acids and starch, had greater tolerance to acidic conditions and contained C_{16:1}ω9c. We therefore suggest that strain S26^T be classified as a representative of a novel species of the genus *Singulisphaera*, for which the name *Singulisphaera rosea* sp. nov. is proposed. The characteristics that differentiate this novel species from *Singulisphaera acidiphila* are summarized in Table 2.

Emended description of the genus *Singulisphaera* Kulichevskaya *et al.* 2008

Singulisphaera (Sin.gu.li.spha.e'ra. L. adj. *singuli* single, separate; L. fem. n. *sphaera* sphere; N.L. fem. n. *Singulisphaera* a single spherical cell).

Spherical cells that occur singly, in pairs, in short chains or as shapeless aggregates. Non-motile. Reproduce by budding. Stalk-like structures are absent. Attach to surfaces by means of amorphous holdfast material. Encapsulated. Colonies are colourless or pink-coloured. Chemo-organotrophic aerobes. Capable of growth under microaerobic conditions. Catalase-positive. Cytochrome oxidase- and urease-variable. Dissimilatory nitrate reduction is negative. Moderately acidophilic and mesophilic. Sensitive to NaCl. The major quinone is MK-6. The major fatty acids are C_{16:0}, C_{18:1}ω9c and C_{18:2}ω6c,12c, with C_{18:2}ω6c,12c being characteristic of the genus. The major neutral lipids are n-C_{31:9} hydrocarbon and squalene. The polar lipids are phosphatidylglycerol, phosphatidylcholine and polar lipids with an unknown structure. The genus is a member of the phylum *Planctomycetes*, order *Planctomycetales*, family

Fig. 2. 16S rRNA gene-based maximum-likelihood tree showing the phylogenetic position of strain S26^T in relation to *Singulisphaera acidiphila*, *Isosphaera pallida*, *Aquisphaera giovannonii* and other representatives of the order *Planctomycetales*. Five anammox-planctomycetes, i.e. '*Candidatus Scalindua brodae*' (AY254883), '*Candidatus Scalindua sorokinii*' (AY257181), '*Candidatus Scalindua wagneri*' (AY254882), '*Candidatus Brocadia anammoxidans*' (AF375994) and '*Candidatus Kuenenia stuttgartiensis*' (AF375995), were used as an outgroup (not shown). Bar, 0.1 substitutions per nucleotide position.

Table 2. Differential characteristics of strain S26^T and *Singulisphaera acidiphila*

Taxa: 1, S26^T; 2, *Singulisphaera acidiphila* [data from Kulichevskaya *et al.* (2008)].

Characteristic	1	2
Cell diameter (µm)*	1.8–3.2	1.6–2.6
Arrangement of cells	Single, in pairs or in short chains	Single or in pairs
Colony pigmentation	Pink	Unpigmented
Salinity tolerance (%)	<1.0	<0.2–0.5
pH growth range (optimum)	3.2–7.1 (4.8–5.0)	4.2–7.5 (5.0–6.2)
Utilization of:		
Raffinose	+	–
Succinate	+	–
Fumarate	+	–
Malate	+	–
Citrate	+	–
Starch	+	–
Pectin	–	+
Dulcitol	+	–
Sorbitol	+	–
Mannitol	+	–
Presence of C _{16:1} ω9c	+	–
DNA G + C content (mol%)	62.2	57.7–59.9

*Cell sizes refer to cells in exponential growth phase.

Planctomycetaceae, and includes two species, *Singulisphaera acidiphila* and *Singulisphaera rosea*. The type species is *Singulisphaera acidiphila*.

Description of *Singulisphaera rosea* sp. nov.

Singulisphaera rosea (ro'se.a. L. fem. adj. *rosea* pink-coloured, rosy).

Mature cells are 1.8–3.2 µm in diameter. Utilizes the following carbon sources (0.05 %, w/v): D-glucose, D-fructose, D-galactose, lactose, D-arabinose, cellobiose, maltose, raffinose, L-sorbose, D-mannose, melibiose, L-rhamnose, D-ribose, DL-sucrose, D-xylose, N-acetylglucosamine, salicin, citrate, fumarate, lactate, malate, pyruvate, succinate, dulcitol, mannitol, sorbitol, L-arginine and L-glutamine. Unable to utilize glycerol, methanol, ethanol, glucuronic acid, acetate, benzoate, caproate, formate, formaldehyde, galacturonate, propionate, L-alanine, L-asparagine, aspartate, L-cysteine, L-cystine, L-glycine, L-histidine, L-isoleucine, L-leucine, DL-lysine, L-methionine, L-norleucine, DL-ornithine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine and DL-valine. Hydrolyses starch, laminarin, chondroitin sulfate, aesculin, gelatin and pullulan, but not pectin, xylan, chitin or cellulose. Positive for the following enzyme activities: alkaline phosphatase, acid phosphatase, esterase, esterase lipase, leucine arylamidase, valine

arylamidase, phosphohydrolase, N-acetyl-β-glucosaminidase and β-glucosidase (API ZYM). Utilizes the following nitrogen sources (0.05 %, w/v): ammonia, nitrate, N-acetylglucosamine, Bacto peptone, Bacto yeast extract, L-alanine, aspartate, L-arginine, L-glutamine, L-threonine, L-tryptophan, L-glycine, L-isoleucine, DL-lysine, L-phenylalanine, L-proline and DL-valine. Nitrite is not utilized. Vitamins are not required. Resistant to ampicillin, streptomycin, chloramphenicol, neomycin and novobiocin, but sensitive to lincomycin, kanamycin and gentamicin. Grows at pH 3.2–7.1 (optimum at pH 4.8–5.0) and at 4–33 °C (optimum at 20–26 °C). NaCl inhibits growth at concentrations above 1 % (w/v). The predominant fatty acid is C_{16:1}ω9c.

The type strain is S26^T (=DSM 23044^T=VKM B-2599^T), isolated from the *Sphagnum* peat bog Staroselskoye, Tver region, north-west Russia. The DNA G + C content of the type strain is 62.2 mol%.

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