Spirochaeta perfilievii sp. nov., an oxygen-tolerant, sulfide-oxidizing, sulfur- and thiosulfate-reducing spirochaete isolated from a saline spring

Galina Dubinina,¹ Margarita Grabovich,² Natalia Leshcheva,¹ Frederick A. Rainey³ and Ekaterina Gavrish⁴

Correspondence
Ekaterina Gavrish
e.gavrish@neu.edu

¹Winogradsky Institute of Microbiology, Russian Academy of Sciences, Pr. 60-letiya Oktyabrya 7/2, Moscow 117312, Russia

²Faculty of Biology and Soil Science, Voronezh State University, Universitetskaya Pl., 1, Voronezh 394893, Russia

³Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, USA

⁴Department of Biology, Northeastern University, Boston, MA 02115, USA

A novel strain of fermenting, aerotolerant, chemo-organoheterotrophic spirochaete designated P^T was isolated from a sulfur 'Thiodendron' mat in a saline spring at the Staraya Russa resort (Novgorod Region, Russia). Cells of strain P^T exhibited a helical shape. The spirochaete required sulfide in the growth medium and was able to oxidize it non-enzymically to elemental sulfur via the interaction of H₂O₂ with sulfide and deposit it in the periplasmic space. Growth occurred at 4–32 °C (optimum at 28–30 °C), pH 6.0–8.5 (optimum pH 7.0–7.5), and in 0.1–1 M NaCl (optimum 0.35 M). The isolate used several sugars and polysaccharides as carbon or energy sources but did not use peptides, amino acids, organic acids or alcohols. The products of glucose fermentation were formate, acetate, ethanol, pyruvate, CO₂ and H₂. The genomic DNA G + C content was 41.7 mol%. 16S rRNA gene sequence analysis showed that strain P^T fell within a group of species in the genus *Spirochaeta*, including *Spirochaeta litoralis*, *S. isovalerica* and *S. cellobiosiphila*, with which it shared less than 89 % sequence similarity. On the basis of its morphology, physiology and other phenotypic properties, as well as its phylogenetic position, the new isolate is considered to represent a novel species of the genus *Spirochaeta*, for which the name *Spirochaeta perfilievii* sp. nov. is proposed. The type strain is P^T (=DSM 19205^T =VKM B-2514^T).

Introduction

Spirochaetes are free-living, protist- or animal-associated bacteria that are mainly helical-shaped Gram-negative and motile bacteria with a distinctive ultrastructural cell organization. Their periplasmic flagella follow the formula $n:2n:n$, where the first n is the number of flagella at one end, $2n$ stands for overlaps of flagella in the middle and the second n is the number of flagella at the opposite end (Margulis, 2002). Many spirochaetes are symbiotic in humans and animals. The order *Spirochaetales*, within the phylum *Spirochaetes*, contains three families: *Spirochaetaceae*, *Brachyspiraceae* and *Leptospiraceae* (Olsen *et al.*, 2000; Paster

& Dewhirst, 2000; Leschine *et al.*, 2006). The genus *Spirochaeta* (free-living species of the family *Spirochaetaceae*) comprises obligate and facultative anaerobes. Comparison of 16S rRNA gene sequences shows that some species of the genus *Spirochaeta* form clusters interspersed with symbiotic species of the genus *Treponema*; two clusters of freshwater *Spirochaeta* species (*Spirochaeta zuelzeriae*, *Spirochaeta caldaria*, *Spirochaeta stenostrepta*) are more closely related to the *Treponema* species *Treponema pallidum*, *Treponema putidum*, *Treponema pectinovorum*, *Treponema berlinense*, *Treponema azotonutricium* and *Treponema primitia* than to other free-living spirochaetes. Within the genus *Spirochaeta*, the majority of species share 16S rRNA gene sequence similarities in the range of 80–88 % (Paster *et al.*, 1996; Olsen *et al.*, 2000; Paster & Dewhirst, 2000; Leschine *et al.*, 2006), with the exception of some alkaliphilic species, *Spirochaeta asiatica*, *Spirochaeta dissipatitrophica*, *Spirochaeta alkalica* and *Spirochaeta americana*, which share >98 % sequence similarity (Zhilina *et al.*, 1996; Hoover *et al.*, 2003;

Abbreviation: EPS, exopolymeric substance.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain P^T is AY337318.

Three supplementary figures, two supplementary tables and a supplementary method describing the composition of growth medium for cultivation of strain P^T are available with the online version of this paper.

Pikuta *et al.*, 2009). In other bacterial phyla, low 16S rRNA gene sequence similarities are usually reported for organisms belonging to different families or genera. The taxonomic uncertainty between the genera *Spirochaeta* and *Treponema* partially results from the ecological/medical descriptions such as 'host-associated' versus 'free-living' in the definitions of these genera. However, such strict criteria cannot be applied to the members of the genus *Leptospira*; some isolates of the genus found in Ecuador alternate between freshwater and human tissue as habitats (Trueba, 2008; Margulis *et al.*, 2009). Many other spirochaetes, including the type species of the genus *Spirochaeta*, *Spirochaeta plicatilis* (Ehrenberg, 1835), have never been isolated into pure culture or even characterized by 16S rRNA gene sequence analysis in spite of persistent attempts and have been observed only by direct microscopy of thin cross-section samples.

For many years we have been studying 'Thiodendron' sulfur mats that are formed as a result of massive growth of chemo-organoheterotrophic spirochaetes fermenting sugars, recalcitrant carbohydrates and other organic compounds (Dubinina *et al.*, 1993b). This type of mat was first discovered in the late 1920s by Professor Boris Perfil'ev in the sulfur mineral springs of the Staraya Russa resort in the Novgorod Region, Russia (Perfil'ev, 1969). Microscopic observations led him to the conclusion that colourless sulfur-mat bacteria had a complex history. Perfil'ev named them 'Thiodendron'. He assumed that he had isolated into culture a sulfide-oxidizing sulfur-depositing bacterium, which had flagellated stages and grew into long thin filaments from which vibrioid cells were released. To study the developmental cycle, he cultivated 'Thiodendron' in a specially constructed flow-through chamber filled with 'curative mud' from the Staraya Russa resort. Mud bacteria were bathed in a constant flow of sulfide-rich water from the saline spring. Small, highly motile vibrioids were consistently observed along with the thin-filament stage bacteria, which accumulate intracellular elemental sulfur globules. We demonstrated that Perfil'ev's interpretation of '*Thiodendron latens*', *Thiodendron* meaning sulfur tree and *latens* meaning lazy, was erroneous, it being a sulfur syntroph and part of a symbiotic consortium of two different types of bacteria: anaerobic aerotolerant spirochaetes and sulfidogenic eubacteria such as species of the genera *Desulfovibrio*, *Desulfobacter* and *Dethiosulfovibrio* (Dubinina *et al.*, 1993a; Surkov *et al.*, 2001).

The spirochaetes that compose the extracellular matrix appear to be a major sulfide-oxidizing and mat-forming component of these sulfureta mats, which are invariably associated with sulfidogens. This assertion is based on the morphology of spirochaetes under presumed mat conditions. The filaments extend in response to the quantity of dissolved oxygen in the liquid phase, this being natural water or nutrient medium. Under strictly anaerobic conditions, the filaments shorten by cell division and assume the helical or undulate shape that is typical of most spirochaetes (Dubinina *et al.*, 1993a).

In this paper we present a taxonomic description of a novel spirochaete, which we were able to isolate in pure culture from the 'Thiodendron' mats, for which we propose the name *Spirochaeta perfilievii* sp. nov.

Strain P^T was isolated from a sulfur 'Thiodendron' mat in a mineral spring at the Staraya Russa resort, Novgorod Region, Russia. The anaerobic cultivation methods that led to the isolation of this anaerobic spirochaete in pure culture have been described previously (Dubinina *et al.*, 1993a). The cultivation medium contained the following (g l⁻¹): NaCl (20), NH₄Cl (0.3), CaCl₂·2H₂O (0.3), MgCl₂·6H₂O (3), Bacto yeast extract (1), Bacto peptone (1), glucose (1) or soluble starch (2), KH₂PO₄ (1) and K₂HPO₄ (1). The ingredients were added from sterile solutions. The medium was adjusted to pH 7.0 with 1 M H₂SO₄. The medium was supplemented with (l⁻¹) 1 ml microelement solution (Pfennig & Lippert, 1966), 5 µg vitamin B₁₂ and 10–50 mg rifampicin. Before inoculation, a 1 M Na₂S·9H₂O solution was added to achieve a final concentration of 0.5 mM. The detailed procedure for the preparation of the growth medium is described in Supplementary Materials, available in IJSEM Online. The cultures were inoculated with a syringe and cultivated anaerobically in Hungate tubes, or in 100–500 ml bottles with rubber stoppers, in an argon or N₂ gas phase (50 % volume) at 28 °C for 24–48 h. Glucose or soluble starch was replaced by other potential growth substrates at a concentration of 5 mM where mentioned. For some tests, 0.3 to 1.5 % Difco agar was added to solidify the liquid medium so that colony morphology was easily visualized. The spirochaetes were incubated at varying temperatures in 20–50 ml vials in the dark under anaerobic conditions. To create micro-oxic conditions, inoculated medium (25 or 50 ml) with 0.25 mM Na₂S·9H₂O as a reducing agent was added to the vials (250 and 500 ml, respectively) which were then filled with the gas mixture. Although growth was easily measured in the total absence of oxygen, the apparent growth was invariably stimulated by small quantities of oxygen (from 0 to 1.5 % of the gas phase) (Dubinina *et al.*, 2004). Depending on cultivation conditions, specifically those that affect the presence of intracellular elemental sulfur, the cell yield was determined either by protein measurement using the Lowry or the Bradford method with Coomassie blue G-250 (Serva) or by spectrophotometric readings at OD₆₀₀. The doubling time was calculated from the changes in optical density.

The morphology of the cells was observed using phase-contrast light (NU-2, Zeiss) and transmission electron (JEM-100C) microscopy with an accelerating voltage of 80 kV. The inclusions of elemental sulfur were observed by polarizing light microscopy. The sulfur globules displayed a typical light-refraction effect. Intracellular elemental sulfur inclusions in the samples of thin sections were stained with silver nitrate (2 %) followed by contrast enhancement with ammonium molybdate (Scheminzyk *et al.*, 1972). The Hovind-Hougen (1976) method was used to reveal the cytology of strain P^T. To visualize the exopolymeric

substance (EPS) matrix, whole-cell preparations were stained with ruthenium red (Luft, 1971). The detailed methods have been described previously (Dubinina *et al.*, 1993b).

The products of glucose fermentation were measured by GLC (Chrom-5). Gaseous metabolic products were measured by GC (LKhM80). Details of these analytical procedures were published previously by Dubinina *et al.* (2004). The protocol for measuring enzymic activity of cell-free preparations has been described previously (Dubinina *et al.*, 2004). All experiments were repeated three times.

Measurement of cytochromes was performed in cell-free extracts. Reduced (with sodium dithionite) minus oxidized (with air) difference absorption spectra were recorded on a Pye-Unicam SP 1800 spectrophotometer.

The fatty acid content was obtained from lyophilized cells after cell autolysis followed by extraction according to the Folch method (Stead *et al.*, 1992). The derivatives were analysed by GC-MS (X-5985Y; Hewlett Packard).

Genomic DNA isolation and purification were carried out according to Marmur (1961). The genomic DNA G+C content of strain P^T was determined by thermal denaturation (T_m) (Owen & Lapage, 1976). DNA–DNA hybridization was carried out by the optical reassociation method (De Ley, 1970) on a Pye-Unicam SP 1800 spectrophotometer. *Spirochaeta litoralis* DSM 2029 was used as a reference strain. The 16S rRNA gene sequence was determined as described previously (Rainey *et al.*, 1996) and an alignment was generated using CLUSTAL_X software (Thompson *et al.*, 1997). An evolutionary-distance matrix was calculated using the Jukes and Cantor algorithm (Jukes & Cantor, 1969). Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods. Bootstrap analyses were based on 1000 resamplings. The analyses were conducted using MEGA version 4 (Tamura *et al.*, 2007).

Cells of strain P^T had a typical Gram-negative-stained cell wall. Electron microscopy confirmed the presence of structural features typical of those seen in other spirochaetes. The protoplasmic cylinder was surrounded by the outer lipoprotein membrane. Flagella were revealed after protease treatment. The flagella arrangement within the periplasmic space was 1:2:1 and the flagella were attached to the protoplasmic cylinder subterminally at both ends of the cell by the basal discs of standard bacterial rotary motors (Fig. 1). Globules of elemental sulfur were observed in the periplasmic space after ultrathin sections were treated with silver nitrate (Fig. 2c); they appeared as dense inclusions. The morphology of cells varied significantly depending upon growth conditions (Table 1). Cells growing exponentially under anaerobic conditions in medium containing 2 mM sulfide showed motile helical shapes, typical of those found in other spirochaetes (Figs 1 and 2a). Cell diameter varied from 0.15 to 0.35 µm. Cell

length varied from 10–20 µm in the early exponential growth phase to 100–200 µm in the stationary growth phase. The long filaments appeared twisted and thus formed pseudobranches (true dichotomous branches require three growing points per cell) or threads and parallel filaments with birefringent sulfur globules along their lengths. Typical ‘Thiodendron’ cell morphology in the field presented as long filaments studded with sulfur-globules under micro-oxic conditions (Fig. 2c). During the stationary phase or under unfavourable growth conditions, round bodies or spheroplasts (L-forms) were formed (Fig. 2b). The cells often folded their ends into dense tangles. Round body formation was also observed on microscope slides after 1–2 min exposure to air. The cell morphologies of strain P^T and the environmental conditions they are dependent on are presented in detail in Table 1.

Colonies of strain P^T were white and spherical in soft agar (Supplementary Fig. S1). Under micro-oxic conditions (up to 1.5% O₂ in a gas phase) or in the presence of limited sulfide concentrations (less than 0.1–0.2 mM), the spirochaete grew as slimy mats at the bottom of the tube or flask. The spirochaete filaments that were immersed in the viscous matrix lost motility and elongated to over 10–100 µm. Some straight, or slightly curled, filaments retained the terminal helical structure at one terminus.

Strain P^T was catalase- and oxidase-negative and was unable to grow under aerobic conditions. The isolate required anaerobic or micro-oxic conditions with 0.5–1.5% O₂ in a gas phase. Under anaerobic conditions, no growth occurred in medium in the absence of a strong reducing agent such as sulfide, titanium(III) citrate or titanium(III) nitrilotriacetate. For the last reducing agent, the concentrations used did not exceed 0.5–1.0 mM. Growth of P^T was dependent on sulfide concentrations between 0.02 and 5.0 mM (E_h range from 230 to –160 mV). The highest yield of biomass under anaerobic conditions was observed at 2 mM Na₂S·9H₂O (E_h –80 mV). The doubling time for strain P^T at the optimal sulfide concentration was 6.3 h.

Under micro-oxic conditions (0.5–1.5% O₂ in a gas phase), no growth occurred in the absence of sulfide. Sulfide could not be replaced by any of the other reducing agents that were tested, indicating that the new isolate depends upon Na₂S under micro-oxic conditions. The highest cell yield was obtained under micro-oxic cultivation in medium containing 0.25 mM sulfide. It has been shown that the maximum cell yield (Y_{max}) for strain P^T at an oxygen concentration of 1.4% in the gas phase (about 0.5 mg O₂ per litre of medium, determined experimentally) was two times higher than that for cultures grown under anaerobic conditions (Dubinina *et al.*, 2004).

Strain P^T was not able to use nitrate, nitrite, sulfate or fumarate as terminal electron acceptors under any cultivation conditions. The presence of these electron acceptors in the medium with glucose did not have an effect on the composition of the metabolic end products and was not

Fig. 1. Micrographs of cells of strain P^T . (a) Electron micrograph of negative stained cells exhibiting 1:2:1 flagellation. Additional protease treatment and a rinse in phosphoglycerol solution was applied to remove the outer membrane and make visible the periplasmic flagella (Hovind-Hougen, 1976). (b) Sub-terminal basal disc insertion point to the rotary motor of the periplasmic flagella in these Gram-negative staining spirochaetes.

accompanied by the reduction of the above-mentioned electron acceptors. No growth occurred under micro-oxic or anaerobic conditions with the above electron acceptors or when glucose was replaced by organic acids, alcohols or amino acids.

Cytochromes were not detected in any cells or cell-free extracts of strain P^T when grown in the medium with glucose under micro-oxic or anaerobic conditions in the presence of nitrate, nitrite, sulfate or fumarate as potential electron acceptors.

The formation of sulfur mats by aerotolerant spirochaetes in saline ecosystems has been linked with the physiological role of sulfide during micro-oxic growth. Microaerobic metabolism of the new isolate resulted in the production of hydrogen peroxide (H_2O_2), which is catalysed by NADH oxidase. The removal of H_2O_2 from cells via interaction with sulfide is a non-enzymic process and results in the accumulation of sulfur globules in the periplasmic space. The removal of toxic H_2O_2 is the only reason for developing a dependence on ambient reduced sulfur compounds by the spirochaete in the presence of dissolved O_2 .

During the shift from sulfide-dependent micro-oxic growth to anaerobic conditions, periplasmic sulfur and thiosulfate could be used as external electron acceptors and were reduced to sulfide. This process was accompanied by the formation of 3–4 mM H_2S , while the rate of hydrogen production decreased threefold (Dubinina *et al.*, 2004). A similar process has been reported previously for *Spirochaeta smaragdinae* (Magot *et al.*, 1997).

Strain P^T appeared to be an aerotolerant anaerobe with fermentative metabolism. When grown under anaerobic conditions, the strain metabolized glucose to produce ethanol, formate, H_2 , acetate, pyruvate and CO_2 , ethanol and formate being the major metabolic products. Under micro-oxic cultivation (0.5–1.5% O_2 in a gas phase), the metabolic end-product composition was different. The content of other oxidized products (acetate and CO_2) increased three- to fivefold, while formate, ethanol and sometimes H_2 were also produced, but only in very small amounts. Pyruvate was not detected among fermentation products under micro-oxic conditions (up to 1.5% O_2 in a gas phase). EPS matrix was abundant in micro-oxically grown culture and the activity of phosphoglucomutase, the

Fig. 2. Round bodies of strain P^T , present *in situ* and in pure culture under conditions unfavourable for growth (see Margulis *et al.*, 2009 for the generality of this phenomenon). (a) Helical or uncurled spirochaetes were observed at low frequency in fields in which round bodies were formed due to starvation (induced by deficient or other unfavourable growth conditions). (b) Electron micrograph of thin sections of round bodies at high magnification showing spirochaete protoplasmic cylinders within the Gram-negatively stained outer membrane. (c) 'Thiodendron' spirochaete filaments with periplasmic sulfur globules from the surface of sulfur mats (stained with $AgNO_3$).

Table 1. Morphology of strain P^T as affected by environmental conditions of growth

Cultivation conditions	Cell morphology/growth yield
Anaerobic; sulfide, Ti(III)-NTA or other strong reducing agents are absent	No growth
Anaerobic; sulfide 0.025–0.05 mM	Non-motile, straight or slightly bent, long (>100 µm) filaments in viscous matrix at the bottom of flask. Typical spirochaete cells absent. Cell yield low, no more than 6–8 mg protein l ⁻¹
Anaerobic; sulfide 2 mM	Motile, short, spiral-shaped spirochaete cells prevail. Cell yield ~25 mg protein l ⁻¹
Micro-oxic; 0.5 % O ₂ , sulfide 0.25 mM	As above but with long filaments also present (non-motile, straight or slightly bent) in mucous matrix at the bottom of flasks. Cell yield low
Micro-oxic; 1.4 % O ₂ , sulfide 0.25 mM	Non-motile, straight or slightly bent, long filaments in mucous matrix at the bottom of flasks. Maximal cell yield (45–50 mg protein l ⁻¹)
Micro-oxic; 2 % O ₂ , sulfide 0.25 mM	A few non-motile, straight or bent filaments immersed in the abundant mucous matrix at the bottom of flasks. Cell yield 6–8 mg protein l ⁻¹
No NaCl or high (>7 % NaCl) salt concentration	No growth. Round bodies form and persist from cells of inoculum
Two-week old or older culture in storage in standard medium, at 28 °C	Round bodies ~2–6 µm in diameter appear due to low-nutrient medium and acidification
Low temperature (<4–5 °C); after 1–2 days of incubation at 28 °C; after storage at 4 °C >1 month	Helical shapes retained for about a month, after which round bodies accumulate

enzyme that facilitates the interconversion of glucose 1-phosphate to glucose 6-phosphate, increased significantly.

Some sugars and polysaccharides, including glucose, maltose, sucrose, trehalose, starch, cellobiose, laminarin and glycogen, supported growth as the sole carbon source, whereas no growth occurred in a medium supplemented with agar, agarose, alcohols, alginate, amino acids, arabinose, galactose, dextrin, D-xylose, melibiose, ribose, cellulose, dulcitol, *myo*-inositol, mannitol, sorbate, sorbitol, organic acids, peptone or yeast extract as the sole carbon source.

Ammonium salts, asparagine, peptone, yeast extract or casein hydrolysate were utilized as nitrogen sources whereas urea, nitrate and nitrite were not.

Strain P^T grew with 0.1–1 M NaCl (optimum 0.4 M NaCl), at 4–32 °C (optimum 28 °C) and at pH 6.0–8.5 (optimum pH 7.0–7.5). The strain was resistant to rifampicin up to 100 µg ml⁻¹ and neomycin up to 10 µg ml⁻¹. The novel isolate was found to be sensitive to the following antibiotics (10 µg ml⁻¹): penicillin G, chloramphenicol, erythromycin, tetracycline, lincomycin, kanamycin and ampicillin.

The predominant cellular fatty acids were iso-C_{14:0} (16.0 %), iso-C_{15:0} (17.6 %) and anteiso-C_{15:0} (31.5 %). Aldehyde C_{14:0} (16.3 %) was also present in high levels (Supplementary Table S2). Similar to *S. litoralis* (Livermore & Johnson, 1974), the majority of the cellular fatty acids were branched-chain fatty acids. In contrast to *S. litoralis*, strain P^T contained a significant amount of iso-C_{14:0}, anteiso-C_{15:0} and aldehydes (Supplementary Table S2). Fatty acid compositions of the two closest relatives, *Spirochaeta isovalerica* and *Spirochaeta cellobiosiphila*, were not available.

The genomic DNA G+C content of strain P^T was 41.7 mol%. This value and the value obtained for *S.*

cellobiosiphila SIP1^T (41.4 mol%) are the lowest reported values within the genus *Spirochaeta* (Table 2).

The 16S rRNA gene sequence (1479 nt) was obtained and a phylogenetic tree was constructed based on compared sequences of related species of the genus *Spirochaeta* (Fig. 3). Strain P^T formed a stable cluster together with *S. litoralis*, *S. isovalerica* and *S. cellobiosiphila* within the genus *Spirochaeta*, which was supported by a 99 % bootstrap value and sequence similarities of 88.0–88.9 %. Similarity to other species in the genus was less than 87 %. The only available 16S rRNA gene sequences with high similarity (99 %) to that of strain P^T belong to the unpublished environmental clone BI-6 (DQ218325) isolated from sulfur-rich blooms in microbial mats (Fig. 3) and to *Spirochaeta* sp. strain M6 (AY337319). Strain M6 was isolated from a microbial mat in the vicinity of a hydrothermally active region of Matupi Harbor Bay on New Britain, Papua New Guinea. The 16S rRNA gene sequence of strain M6 was obtained and deposited in GenBank but, after several years of maintaining the culture, the strain lost viability.

Isolated from bacterial 'Thiodendron' sulfur mats, novel strain P^T resembles members of the genus *Spirochaeta* in morphology, saccharolytic heterotrophy and rifampicin resistance, but differs from other species of the genus *Spirochaeta* by genetic, ecological, physiological and developmental criteria. It tolerates but does not grow above 1.5 % oxygen in the gas phase. At higher concentrations of ambient oxygen, the strain reversibly develops from helical to filamentous form and later to round bodies. The tolerance of oxygen and ability both to oxidize sulfide and to reduce elemental sulfur and thiosulfate are unique to strain P^T. Sulfide-mediated oxidation of cellularly produced H₂O₂ (leading to the accumulation of sulfur globules in the periplasmic space) helps to protect the

Table 2. Distinguishing characteristics of strain P^T and type strains of phylogenetically related species of the genus *Spirochaeta*

Strains: 1, *S. perfilievii* sp. nov. P^T (data from this study); 2, *S. litoralis* R1^T (Hespell & Canale-Parola, 1970); 3, *S. isoalerica* MA-2^T (Harwood & Canale-Parola, 1983); 4, *S. cellobiosiphila* SIP1^T (Breznak & Warnecke, 2008). All strains produce acetate, ethanol, H₂ and CO₂ as major products of carbohydrate fermentation. ND, No data.

Characteristic	1	2	3	4
Cell width (µm)	0.3–0.5	0.4–0.5	0.4	0.3–0.4
Cell length (µm)	5–20	6–7	10–15	10–12
Optimum pH	7.0–7.5	7.0–7.5	ND	7.5
Optimum temperature (°C)	28–30	30	30	30–37
Optimum NaCl (M)	0.35	0.35	0.2–0.3	0.3–0.4
Substrate utilization:				
Fructose	–	+	+	–
Lactose	–	+	–	ND
Mannitol	–	–	+	–
Raffinose	–	+	–	ND
L-Rhamnose	–	+	+	–
D-Xylose	–	+	+	+
Tolerance of O ₂ *	AT	OA	OA	FA
DNA G + C content (mol%)	41.7	44.2/50.5 [†]	63.6–65.6	41.4

*AT, Aerotolerant; OA, obligate anaerobe; FA, facultative anaerobe.

[†]Results obtained using the *T_m* method by Breznak & Canale-Parola (1975) and the buoyant density method by Hespell & Canale-Parola (1970), respectively.

catalase-negative cells against toxic H₂O₂. Strain P^T contributes to at least three natural forms of sulfur (sulfide, thiosulfate and elemental sulfur) in the biogeochemical sulfur cycle in highly productive saline littoral and subaqueous ecosystems, making strain P^T the first spirochaete of geochemical significance in the global sulfur cycle. The abundance of this type of spirochaete in sulfureta ecosystems, the removal of sulfide in natural

communities and the production/degradation of EPS are thought to stimulate the activity of other sulfate- and sulfur-reducing bacteria. The implications of its metabolism for use in geochemistry, specifically in the preservation in the microbial fossil record, have been noted previously (Stephens *et al.*, 2008).

The significant phenotypic differences, along with the data from phylogenetic analysis, suggest that strain P^T and its closest phylogenetic relatives *S. litoralis*, *S. isoalerica* and *S. cellobiosiphila* represent a new genus. However, the absence of culture characteristics and 16S rRNA gene sequence data for the type species of the genus *Spirochaeta*, *S. plicatilis*, makes it difficult to create a new genus without revising descriptions of the remaining members of the genus *Spirochaeta*. Unfortunately, this is beyond the scope of the present paper. As such, we suggest that the properties of strain P^T qualify it as representative of a novel species of the genus *Spirochaeta*, for which the name *Spirochaeta perfilievii* sp. nov. is proposed.

Description of *Spirochaeta perfilievii* sp. nov.

Spirochaeta perfilievii [per.fi.li.e'vi.i N.L. gen. masc. n. *perfilievii* named after Professor B. V. Perfil'ev (1893–1974), the Russian microbiologist who discovered and described the 'Thiodendron' microbial mat].

Colonies are white, spherical and diffuse, up to 10–15 mm in diameter. Cells are Gram-negative and show rotation, undulation and gliding motility typical of spirochaetes. Helical cells with irregular coils are 0.2–0.4 × 10–200 µm in size. The shortest cells are those grown under strictly anaerobic conditions. In media with low sulfide concentrations and in the presence of some dissolved oxygen, long (beyond 200 µm) non-motile filaments with periplasmic sulfur globules predominate. Round bodies occur under any unfavourable conditions. The three morphotypes are often present simultaneously and in different frequencies depending on ambient conditions. Aerotolerant anaerobe. Catalase- and oxidase-negative. Cytochromes were not detected. Mesophilic with a temperature range 4–32 °C (optimum at 28–30 °C). Grows at pH 6.5–8.5 (optimum pH 7.0–7.5). Obligate, moderate halophile; grows in

Fig. 3. Neighbour-joining phylogenetic tree of a *Spirochaeta* cluster, based on 16S rRNA gene sequences. Bootstrap values greater than 50% (1000 resamplings) are shown. A phylogenetic tree of the family *Spirochaetaceae* is available as Supplementary Fig. S3. Bar, 0.02 substitutions per nucleotide position.

0.1–1 M NaCl (optimum 0.35 M NaCl). As a chemo-organoheterotroph, glucose, maltose, sucrose, trehalose, cellobiose, glycogen, starch and laminarin are used as sole carbon or energy sources. Peptides, amino acids, organic acids and alcohols are not used as sole carbon or energy sources. Growth is not supported by alginate, arabinose, galacturonic acid, laevulose, L-rhamnose, fructose, lactose, raffinose, galactose, dextrin, D-xylose, melibiose, ribose, cellulose or any sugar alcohols. Ammonium salts, casein hydrolysate, yeast extract, peptone and asparagine can be used as sources of nitrogen, whereas urea, nitrate, nitrite, aspartate, arginine, valine, leucine, ornithine, threonine, tryptophan and cysteine can not. Formate, acetate, ethanol, CO₂ and H₂ are the major metabolic end products of glucose fermentation. Under anaerobic conditions, sulfur and thiosulfate are reduced to sulfide. Under micro-oxic conditions (0.5–1.5% O₂ in the gas phase), sulfide is oxidized to sulfur. Elemental sulfur accumulates in the periplasmic space as birefringent globules. Resistant to rifampicin and neomycin. The major fatty acids are iso-C_{14:0}, iso-C_{15:0}, anteiso-C_{15:0} and aldehyde C_{14:0}. The genomic DNA G+C content of the type strain is 41.7 mol% (T_m).

The type strain, P^T (=DSM 19205^T =VKM B-2514^T), was isolated from a 'Thiodendron' bacterial sulfur mat in a saline spring at the Staraya Russa resort (Novgorod Region, Russia).

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