Taxonomic investigation of representatives of the genus *Sphaerotilus*: descriptions of *Sphaerotilus montanus* sp. nov., *Sphaerotilus hippei* sp. nov., *Sphaerotilus natans* subsp. *natans* subsp. nov. and *Sphaerotilus natans* subsp. *sulfidivorans* subsp. nov., and an emended description of the genus *Sphaerotilus*

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Seven strains of the genus *Sphaerotilus* were obtained from natural thermal sulfide (strains D-501^T, D-502, D-504, D-505 and D-507) and low-temperature ferrous (strain HS^T) springs and from an activated sludge system (strain D-380). These *Sphaerotilus* isolates and strains of *Sphaerotilus natans* obtained from the DSMZ (*S. natans* DSM 6575^T, DSM 565 and DSM 566) were studied using a polyphasic taxonomic approach. All strains had Q-8 as the major quinone and C_{16:1ω7}, C_{16:0} and C_{18:1ω7} as the major fatty acids. The DNA–DNA hybridization results and 16S rRNA, *hsp60* and *gyrB* gene sequencing experiments showed that isolates D-501^T, D-502, D-504, D-505, D-507 and D-380 were closely related to the type strain of *S. natans* DSM 6575^T. However, strains D-501^T, D-502, D-504, D-505 and D-507 significantly differed from the heterotrophic strain *S. natans* DSM 6575^T by their capability for lithotrophic growth with reduced sulfur compounds as an electron donor for energy conservation and some other phenotypic features. For this reason, strains D-501^T, D-502, D-504, D-505 and D-507 merit a separate taxonomic classification at the subspecies level. The name *Sphaerotilus natans* subsp. *sulfidivorans* subsp. nov. (type strain D-501^T=DSM 22545^T=VKM B-2573^T) is proposed. The subspecies *Sphaerotilus natans* subsp. *natans* subsp. nov. is automatically created as a result of this proposal. Strain D-380 was phenotypically closely related to *S. natans* DSM 6575^T. Strains D-380 and *S. natans* DSM 6575^T were assigned to the subspecies *Sphaerotilus natans* subsp. *natans* subsp. nov. (type strain DSM 6575^T=ATCC 13338^T). The 16S rRNA, *hsp60* and *gyrB* gene sequences obtained for strains HS^T and DSM 565 showed very low sequence similarity values of 97.3%, 89.7% and 88.4%, respectively, with *S. natans* DSM 6575^T. Strain HS^T shared 99% DNA–DNA relatedness with strain

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains HS^T, *Sphaerotilus natans* DSM 6575^T, DSM 566^T, DSM 565, D-501^T, D-507 and D-380 are EU636006, EU642571, GU591795, GU591794, FJ871054, FJ871053 and GU591793, respectively. The accession numbers for the *gyrB* gene sequences of strains HS^T, *Sphaerotilus natans* DSM 6575^T, DSM 566^T, DSM 565, D-501^T, D-507 and D-380 are FJ161075, FJ032203, GU591797, GU591798, GU734705, GU734704 and GU734706, respectively. The accession numbers for the *hsp60* gene sequences of strains HS^T, *Sphaerotilus natans* DSM 6575^T, DSM 566^T, DSM 565, D-501^T, D-507 and D-380 are FJ167405, FJ167404, GU591796, GU591799, FJ871056, FJ871057 and GU734707, respectively.

Supplementary figures and tables are available with the online version of this paper.

DSM 565 and 48 % with *S. natans* DSM 6575^T. The 16S rRNA, *hsp60* and *gyrB* gene sequence similarities between strain DSM 566^T and *S. natans* DSM 6575^T were 97.5 %, 91.5 % and 87.0 %, respectively. Strain DSM 566^T had 52 % DNA–DNA relatedness to *S. natans* DSM 6575^T and shared 44 % DNA–DNA similarity with strain HS^T. The creation of two novel species is proposed, *Sphaerotilus montanus* sp. nov. for strains HS^T and DSM 565 (type strain HS^T=DSM 21226^T=VKM B-2519^T) and *Sphaerotilus hippei* sp. nov. for strain DSM 566^T (type strain DSM 566^T=ATCC 29330^T). Emended descriptions of the genus *Sphaerotilus* and of *Sphaerotilus natans* are presented.

The filamentous organoheterotrophic bacteria, which are affiliated with the genus *Sphaerotilus* within the class *Betaproteobacteria*, are widespread in various shallow natural freshwater habitats such as marshy waters, rivers and ponds (Stokes, 1954; Dondero, 1961; van Veen *et al.*, 1978) and anthropogenic ecosystems such as domestic and industrial sewage and activated sludge systems (Dondero, 1961; Eikelboom, 1975; Kämpfer, 1997, 1998; Pellegrin *et al.*, 1999). Numerous isolates of members of the genus *Sphaerotilus* obtained from various ecosystems and geographical regions represent a taxonomically and phylogenetically homogeneous group. Comparative sequence analysis of 16S rRNA genes from the majority of *Sphaerotilus* isolates has demonstrated that they have a high, similarity to each other and to the 16S rRNA gene sequence of the single recognized species within the genus *Sphaerotilus*, *Sphaerotilus natans* DSM 6575^T (99.6–99.9 % similarity) (Corstjens & Muyzer, 1993; Siering & Ghiorse, 1996; Ludwig *et al.*, 1998; Pellegrin *et al.*, 1999; Chernousova *et al.*, 2008). The similarity of the cellular fatty acid composition and quinone types of isolates of the genus *Sphaerotilus* has also been noted. Although the chemotaxonomic and phylogenetic properties of a number of *Sphaerotilus* isolates were similar, significant physiological differences between the *Sphaerotilus* strains isolated from different habitats and *Sphaerotilus natans* DSM 6575^T have been detected (Kämpfer, 1997, 1998; Pellegrin *et al.*, 1999; Gridneva *et al.*, 2009). Members of the genus *Sphaerotilus* obtained from paper mill slimes were capable of metabolizing complex polysaccharides, polyalcohols or organic acids as carbon sources (Pellegrin *et al.*, 1999). In natural habitats, *S. natans* forms mats with iron oxide depositions in sheaths and therefore it is considered a typical representative of the heterotrophic iron bacteria (Stokes, 1954; Dondero, 1961; van Veen *et al.*, 1978). Recently, stable bacterial communities with a clear domination of representatives of the genus *Sphaerotilus* have been found in the sulfur mats of various sulfide springs of the North Caucasus, Russia (Chernousova *et al.*, 2008; Gridneva *et al.*, 2009). In contrast to known *S. natans* strains, these novel *Sphaerotilus* strains presented atypical characteristics such as the ability for both organoheterotrophic and lithotrophic growth with reduced sulfur compounds as electron donor for energy conservation (Gridneva *et al.*, 2009). These *Sphaerotilus* strains were closely related to *S. natans* DSM 6575^T (99.9 % 16S rRNA

gene sequence similarity in the 500 nt region sequenced) (Chernousova *et al.*, 2008). Earlier phylogenetic investigations of numerous isolates of the genus *Sphaerotilus* were performed using analysis of only the 16S rRNA gene sequences. It is well known that 16S rRNA gene sequence similarity alone is not sufficient to guarantee species discrimination (Fox *et al.*, 1992). Protein-encoding genes are more variable. In particular, the functional genes *hsp60* (coding the 60 kDa chaperonin) and *gyrB* (coding the subunit B protein of DNA gyrase) can be used as additional phylogenetic markers because they allow better discrimination of taxa, especially at the species and strain level (Yamamoto & Harayama, 1998; Kwok *et al.*, 1999; Blackwood *et al.*, 2000). Taking into account the above-mentioned metabolic heterogeneity, it is possible to speculate that the genus *Sphaerotilus* may encompass further diverse novel species and subspecies.

In this study, a comparative analysis of seven novel strains of the genus *Sphaerotilus* is presented. The novel strains were obtained from various habitats and strains of *S. natans* were obtained from the DSMZ. The study was conducted by means of a polyphasic approach including 16S rRNA, *hsp60* and *gyrB* gene sequencing, DNA–DNA hybridization experiments and extensive phenotypic characterization.

Pure cultures of iron-oxidizing bacteria, which were morphologically similar to representatives of the genus *Sphaerotilus*, were isolated from natural habitats and a wastewater treatment plant. Strains D-501^T, D-502, D-504, D-505 and D-507 were isolated from a sulfur mat of a sulfide spring, Petushok spring. The temperature was 34–37 °C. The sulfide content at different times of observation varied from 2 to 11 mg S²⁻ l⁻¹. The oxygen content varied from 0.1 to 0.5 mg l⁻¹ and the pH was 7.5–7.6. Strain HS^T was obtained from a friable deposit of iron oxides that had accumulated on the outflow of the ferriferous spring. The iron content was 0.6–1.8 mg l⁻¹, the pH was 7.2–7.5 and the water temperature was 11 °C. The total water mineral content did not exceed 1.6–2.0 g l⁻¹. Both springs were located in the discharge region of the deep underground waters of the Psekups mineral water deposit of the North Caucasus. Strain D-380 was isolated from activated sludge in a wastewater treatment plant in Voronezh, Russia. The temperature was 25 °C and the pH was 7.5. The type strain of *S. natans*, DSM 6575^T, and strains DSM 565 and DSM

566^T were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). The full list of the *Sphaerotilus* strains investigated and their origin is presented in Table 1.

The cultures were maintained and their physiological and biochemical characteristics were investigated in a medium containing the following (mg l⁻¹): (NH₄)₂SO₄, 100; CaCl₂, 50; MgSO₄·7H₂O, 100; sodium lactate, 200; Na₂S₂O₃, 1000 or Na₂S·9H₂O, 100 and 10% phosphate buffer, 10 ml; distilled water, 1 l, plus vitamins and trace elements (Pfennig & Lippert, 1966) at pH 7.5. In the medium used for biomass accumulation, the lactate content was increased to 0.5 g l⁻¹ and peptone (1 g l⁻¹) was added. Strain HS^T was isolated on Veen medium, which contained complexed organic Fe²⁺ as ammonia citric acid iron (van Veen *et al.*, 1978). Colonies of all strains in Veen medium were rusty coloured with fibrous edges and were 2–5 mm in size. Cell morphology was observed with a light microscope (CX 4; Olympus) equipped with a phase-contrast device and by transmission electron microscopy (JEM-100C; JEOL) with an accelerating voltage of 60 kV. Cells were straight Gram-negative rods, arranged in chains within sheaths. Swimmers released from one end of the filaments were motile by means of bundles of polar flagella. Sheaths were encrusted by ferric oxide Fe³⁺ when the cultures were grown in the media containing Fe²⁺. Cells of strains D-501^T, D-502, D-504, D-505 and D-507 were able to accumulate sulfur granules intracellularly when the cultures were grown in media containing sulfide (see Supplementary Fig. S1 in IJSEM Online). The filaments were not mobile. The filaments

could be attached to a solid substrate by means of holdfasts. Cells contained granules of poly-β-hydroxy-alkanoates as reserve material.

The novel strains were examined for a range of phenotypic and biochemical properties using standard methods (Gerhardt *et al.*, 1981). The physiological properties of the novel *Sphaerotilus* strains and the strains of *S. natans* from the DSMZ collection were determined by cultivation in the mineral media described above containing a single compound as the carbon source. Comparisons of the morphological and physiological properties of the novel *Sphaerotilus* strains and *S. natans* DSM 6575^T are shown in Table 2 and Table 3. All strains had a respiratory type of metabolism and were not able to use fumarate or nitrate as electron acceptor under anaerobic conditions. All of the strains investigated, except for strain DSM 566^T, reduced nitrate to nitrite. Strain DSM 566^T was not capable of assimilatory nitrate reduction. Casein and starch were not hydrolysed. Gelatin was not liquefied. No formation of pigments was observed during growth on aromatic amino acids. Indole was not formed. No growth took place in the presence of 3% (w/v) NaCl. Strains D-501^T, D-502, D-504, D-505 and D-507 were capable of lithoheterotrophic growth with utilization of thiosulfate or sulfide as electron donor for energy metabolism. The differences in the end products, rates of thiosulfate oxidation and activities of the key enzymes of oxidative sulfur metabolism were determined among the strains. All strains except D-507 were capable of lithoheterotrophic growth with reduced sulfur compounds used as electron donors for energy metabolism only under microaerobic conditions. In order

Table 1. List of strains used in this study and their origin

ND, No data.

Organism	Strain	New designation	GenBank accession no.	Source	Reference
<i>Sphaerotilus natans</i>	DSM 6575 ^T	<i>S. natans</i> subsp. <i>natans</i> subsp. nov.	EU642571	Polluted waters	Kützing (1833)
<i>Sphaerotilus</i> sp.	D-380	<i>S. natans</i> subsp. <i>natans</i> subsp. nov.	GU591793	Activated sludge system, Voronezh, Russia	This study
<i>Sphaerotilus</i> sp.	D-501 ^T	<i>S. natans</i> subsp. <i>sulfidivorans</i> subsp. nov.	FJ871054	Thermal sulfide spring, North Caucasus, Russia	Chernousova <i>et al.</i> (2008); Gridneva <i>et al.</i> (2009)
<i>Sphaerotilus</i> sp.	D-502	<i>S. natans</i> subsp. <i>sulfidivorans</i> subsp. nov.	ND	Thermal sulfide spring, North Caucasus, Russia	Chernousova <i>et al.</i> (2008); Gridneva <i>et al.</i> (2009)
<i>Sphaerotilus</i> sp.	D-504	<i>S. natans</i> subsp. <i>sulfidivorans</i> subsp. nov.	ND	Thermal sulfide spring, North Caucasus, Russia	Chernousova <i>et al.</i> (2008); Gridneva <i>et al.</i> (2009)
<i>Sphaerotilus</i> sp.	D-505	<i>S. natans</i> subsp. <i>sulfidivorans</i> subsp. nov.	ND	Thermal sulfide spring, North Caucasus, Russia	Chernousova <i>et al.</i> (2008); Gridneva <i>et al.</i> (2009)
<i>Sphaerotilus</i> sp.	D-507	<i>S. natans</i> subsp. <i>sulfidivorans</i> subsp. nov.	FJ871053	Thermal sulfide spring, North Caucasus, Russia	Chernousova <i>et al.</i> (2008); Gridneva <i>et al.</i> (2009)
<i>Sphaerotilus</i> sp.	HS ^T	<i>S. montanus</i> sp. nov.	EU636006	Low-temperature ferrous spring, North Caucasus, Russia	This study
<i>Sphaerotilus natans</i>	DSM 565	<i>S. montanus</i> sp. nov.	GU591794	Pond water, Germany	ND
<i>Sphaerotilus natans</i>	DSM 566 ^T	<i>S. hippei</i> sp. nov.	GU591795	Pond water, Germany	ND

Table 2. Differential characteristics of *Sphaerotilus natans*

Strains: 1, DSM 6575^T; 2, D-380 (=VKM B-2579); 3, D-501^T (=DSM 22545); 4, D-502 (=VKM B-2575); 5, D-504 (=VKM B-2576); 6, D-505 (=VKM B-2577); 7, D-507 (=DSM 22546). All strains utilize the following as sole carbon and energy sources: succinate, lactate, pyruvate, malate, oxaloacetate, oxalate, sorbitol, 2-oxoglutarate, malonate, glucose, sucrose, galactose, maltose, fructose, glycerol, ethanol, glutamate, proline, aspartate, alanine, phenylalanine, asparagine, ornithine, peptone, yeast extract and casein hydrolysate. Formate, glycolate, glyoxylate, salicylate, lysine and methionine are not utilized. Ammonium salts, nitrates, aspartate, glutamate, cysteine, peptone, yeast extract and casein hydrolysate may be used as a nitrogen source; methionine is not used. +, Positive; –, negative; ND, no data available.

Characteristic	<i>S. natans</i> subsp. <i>natans</i> subsp. nov.		<i>S. natans</i> subsp. <i>sulfidivorans</i> subsp. nov.				
	1	2	3	4	5	6	7
Cell size (μm)	1.2–2.0 × 2–6.0	1.2–1.9 × 2–6.0	1.0–2.0 × 3.9–6.0	1.0–2.0 × 3.9–6.0	1.0–2.0 × 3.9–6.0	1.0–2.0 × 3.9–6.0	1.0–2.0 × 3.9–6.0
Optimal pH	6.5–7.5	7.5	7.5	7.5	7.5	7.5	7.5
Optimal temperature (°C)	25	25	32	32	32	32	32
DNA G + C content (mol%; <i>T_m</i>)	70.8	ND	69.2	ND	ND	ND	69.2
Carbon source:							
Acetate	–	+	+	+	+	+	+
Aconitate	+	–	–	–	+	+	–
Arabinose	–	+	+	+	+	+	+
Benzoate	–	+	+	+	–	+	+
Butanol	–	+	+	+	+	+	+
Citrate	–	–	–	+	+	+	+
Cysteine	–	–	+	+	+	+	+
Cystine	–	+	+	+	+	+	+
Fumarate	+	–	–	+	+	+	+
Histidine	–	–	–	–	+	+	–
iso-Butanol	–	+	+	+	+	+	+
iso-Citrate	–	–	–	–	+	+	–
Lactose	–	+	–	+	+	+	+
Mannitol	–	+	+	+	+	+	+
Mannose	–	–	–	+	+	+	–
Propanol	–	+	–	+	+	+	+
Raffinose	–	+	+	+	+	+	+
Serine	–	–	–	–	+	+	+
Sorbose	–	+	+	+	+	+	+
Tryptophan	+	–	–	+	+	+	–
Tyrosine	+	+	–	+	–	–	+
Thiosulfate used as electron donor for energetic metabolism†	–	–	+	+	+	+	+

*Poor growth.

†Only under microaerobic conditions.

to create microaerobic conditions, rubber-stoppered 50 ml vials with 10 ml medium were flushed with filter-sterilized argon. A calculated volume of sterile air was then injected into the vials to create a final oxygen concentration of 5 % in the gas phase. Strain D-507 was capable of thiosulfate oxidation regardless of the oxygenation regime. The ability of the strains to conduct lithotrophic growth was confirmed by using enzymic methods and polarographic

analysis (Gridneva *et al.*, 2009). Oxidation of sulfide was accompanied by the deposit of sulfur granules within the cells.

Analysis of fatty acids was conducted on a specialized chromatographic Microbial Identification System (Sherlock) (MIDI Inc.) according to standard protocols (Stead *et al.*, 1992). The fatty acid composition of the cell

Table 3. Differential characteristics of the type strains of species of the genus *Sphaerotilus*

Strains: 1, *S. natans* DSM 6575^T; 2, *S. montanus* sp. nov. HS^T (=DSM 21226^T; the properties of strains HS^T and DSM 565 were highly similar; therefore, only data for strain HS^T are presented); 3, *S. hippei* sp. nov. DSM 566^T. Succinate, lactate, pyruvate, malate, oxaloacetate, 2-oxoglutarate, malonate, glucose, sucrose, maltose, fructose, glycerol, ethanol, glutamate, proline, peptone, yeast extract and casein hydrolysate are utilized as sole carbon and energy sources by all strains. Formate, glycolate, salicylate, iso-citrate, raffinose, cysteine, cystine, lysine, serine and methionine are not utilized. Ammonium salts, nitrates, aspartate, glutamate, cysteine, peptone, yeast extract and casein hydrolysate may be used as a nitrogen source; methionine is not used. +, Positive; –, negative.

Characteristic	1	2	3
Cell size (µm)	1.2–2.0 × 2–6.0	0.8–1.4 × 3.3–6.0	0.7–1.5 × 2.0–6.2
Optimal pH	6.5–7.5	7.5	7.0
Optimal temperature (°C)	25	26–27	26–27
DNA G + C content (mol%; T _m)	70.8	67.8	69.0
Carbon source:			
Acetate	–	–	+
Aconitate	+	+	–
Alanine	+	+	–
Arabinose	–	+	+
Asparagine	+	–	–
Aspartate	+	+	–
Benzoate	–	+	–
Butanol	–	+	+
Citrate	–	+	+
Fumarate	+*	+	–
Galactose	+	–	–
Glyoxylate	–	+	+
Histidine	–	–	+
Iso-butanol	–	–	+
Lactose	–	+	–
Mannitol	–	+	–
Mannose	–	+	+
Ornithine	+	+	–
Oxalate	+*	+	–
Petroleum products†	+	–	–
Phenylalanine	+	+	–
Propanol	–	+	+
Sorbitol	+	+	–
Sorbose	–	+	+
Tryptophan	+	+	–
Tyrosine	+	–	–

*Poor growth.

†Petroleum products are oil and petroleum.

membranes of the novel strains is shown in Table 4. All strains possessed C_{16:1}ω7, C_{16:0} and C_{18:1}ω7 as the predominant fatty acids.

Genomic DNA was extracted from cells of the test strains according to the protocol of Ausubel *et al.* (1994), with slight modifications, and by the freeze–thaw method (Bej *et al.*, 1991). The 16S rRNA gene of all the strains and *S. natans* DSM 6575^T was amplified using the primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') (Lane, 1991; Medlin *et al.*, 1988). The PCR products were sequenced by using an

automatic sequencer (CEQ2000 XL; Beckman Coulter). Alignment of the 16S rRNA gene sequences (1400 nt) was carried out using the CLUSTAL_X software package (Thompson *et al.*, 1997). An evolutionary-distance matrix was calculated using the correction of Jukes & Cantor (1969). The phylogenetic trees were constructed by using the neighbour-joining (Saitou & Nei, 1987) and the maximum-likelihood (Guindon & Gascuel, 2003) methods, with the 16S rRNA gene sequence of *Alcaligenes faecalis* subsp. *faecalis* ATCC 8750^T as an outgroup. Bootstrap analyses of the neighbour-joining and the maximum-likelihood methods were based on 1000 and 100 resamplings,

Table 4. Comparison of the cellular fatty acid contents of the type strains of species and subspecies of the genus *Sphaerotilus*

Strains: 1, *S. natans* subsp. *natans* subsp. nov. DSM 6575^T; 2, *S. natans* subsp. *sulfidivorans* subsp. nov. D-501^T (=DSM 22545^T); 3, *S. montanus* sp. nov. HS^T (=DSM 21226^T); 4, *S. hippei* sp. nov. DSM 566^T. –, Not detected; tr, trace peak (area<0.5 %).

Fatty acid	1	2	3	4
C _{12:0}	1.4	0.9	1.0	2.0
C _{10:0} 3-OH	1.2	1.2	0.6	0.6
C _{14:1} ω5	0.7	0.6	5.4	tr
C _{14:1} ω3	–	–	–	tr
C _{14:0}	tr	tr	–	1.8
C _{15:1} ω6	tr	–	–	tr
C _{15:0}	tr	–	0.8	tr
C _{16:1} ω7	58.2	68.6	52.2	56.2
C _{16:1} ω5	tr	0.8	0.8	tr
C _{16:0}	18.6	12.0	30.7	32.0
C _{17:1} ω6	–	–	tr	–
C _{18:1} ω9	tr	tr	tr	tr
C _{18:1} ω7	18.6	14.2	5.6	6.0
C _{18:0}	tr	0.8	tr	0.6
11-Me-C _{18:1}	0.7	0.5	2.6	tr

respectively. The TREECON software package was used for the analysis (Van de Peer & De Wachter, 1994) in combination with the PHYL software package (Felsenstein, 1993). The phylogenetic trees based on 16S rRNA gene sequences constructed by using the neighbour-joining and the maximum-likelihood methods are shown in Fig. 1 and Supplementary Fig. S2 (available in IJSEM Online), respectively. Protein-encoding genes are regarded as more useful for species discrimination. Therefore, the functional genes

hsp60 and *gyrB* were used as additional phylogenetic markers to discriminate the taxa at the species and strain level (Yamamoto & Harayama, 1998; Kwok *et al.*, 1999; Blackwood *et al.*, 2000). The *hsp60* gene was amplified from bacterial genomic DNA of all investigated strains using the universal *hsp60* degenerate primers H1612 (5'-GAIHIGC-IGGYGACGGYACSAAC-3') and H1613 (5'-CGRCGRT-CRCCGAAGCCSGGIGCCTT-3') and PCR conditions as described by Hill *et al.* (2006). The *gyrB* gene was amplified by PCR using universal *gyrB* degenerate primers Up1 (5'-GAAGTCATCATGACCGTTCTGCAYGCNNGGNGNAA-RTTYGA-3') and Up2r (5'-AGCAGGGTACGGATGT-GCGAGCCRTCNACRTCNCRCTCNGTCAT-3') and the conditions as described by Yamamoto and Harayama (1995). The partial *hsp60* (550 bp) and *gyrB* (1200 bp) gene sequences obtained for the *Sphaerotilus* strains were aligned as described for the 16S rRNA gene sequences.

Strains D-501^T, D-502, D-504, D-505 and D-507 have previously been compared by ERIC-PCR (Chernousova *et al.*, 2008). The ERIC profiles of all the *Sphaerotilus* strains were identical, indicating the absence of population heterogeneity among the strains. However, strain D-507 was found to be physiologically different from the other strains of its group. Therefore, the use of strain D-501^T (as the reference strain of the group) and strain D-507 for the comparative analysis of the 16S rRNA, *hsp60* and *gyrB* genes and for the performance of the DNA–DNA hybridization experiments seemed appropriate. *S. natans* strains IF4, IF5, IF15 and IF9 isolated from paper mill slimes and *S. natans* strain ATCC 15291 were also included in the comparative analysis of the 16S rRNA genes. The nearly full-length 16S rRNA gene sequence for *S. natans* strain ATCC 15291 (=NBRC 13543) was available only from the database of the NITE Biological Research Center (NBRC). The nearly full-length 16S rRNA gene sequences for the *S.*

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of the strains of the genus *Sphaerotilus* among their closest relatives. Numbers at the branch nodes are bootstrap values, expressed as a percentage of 1000 replicates (only values >70 % are shown). The tree was rooted using *Alcaligenes faecalis* subsp. *faecalis* ATCC 8750^T as an outgroup. Bar, 0.02 substitutions per nucleotide position.

natans strains from the paper mill slimes were retrieved from GenBank.

Analysis of 16S rRNA, *hsp60* and *gyrB* gene sequences of isolates D-501^T and D-380 revealed an extremely close phylogenetic relationship to *S. natans* DSM 6575^T (see Supplementary Table S1). Strains D-501^T and D-507 were identical based on analysis of these phylogenetic markers. The 16S rRNA gene sequence similarities between strains D-501^T, D-380 and the reference strains of *S. natans* (ATCC 15291 and the strains from paper mill slimes) ranged from 99.7 to 99.9%. This suggested that strains D-501^T, D-507 and D-380 were members of the same species, *S. natans*, within the genus *Sphaerotilus*.

The 16S rRNA, *hsp60* and *gyrB* gene sequences obtained for strains HS^T, DSM 565 and DSM 566^T showed very low sequence similarity values when compared with those of *S. natans* DSM 6575^T (Supplementary Table S1). Strain HS^T and DSM 565 were found to be identical based on analysis of these phylogenetic markers. Strain DSM 566^T shared 98.6, 91.6 and 91.7% similarity in the 16S rRNA, *hsp60* and *gyrB* gene sequences with strain HS^T. The noticeable phylogenetic differences between strains DSM 566^T and HS^T and between these novel strains and *S. natans* DSM 6575^T suggested that strains DSM 566^T and HS^T may represent two novel species of the genus *Sphaerotilus*.

DNA G+C content was determined by the thermal denaturation method as described previously (Owen & Lapage, 1976). The DNA G+C content of the novel strains was 67.8–70.8 mol%. Levels of DNA–DNA relatedness were determined by measuring the renaturation rates of the denatured DNAs at the optimal renaturation temperatures as recommended by De Ley *et al.* (1970). A spectrophotometer (SP 1800; Pye Unicam) equipped with a thermo-programmer and hermetically sealed thermocuvettes was used. DNA–DNA hybridization studies of strains D-380, D-501^T, D-504, D-507 and *S. natans* DSM 6575^T revealed a similarity value of 80–98%, indicating a clear affiliation to the species *S. natans* according to the taxonomic guidelines proposed by Wayne *et al.* (1987). Strains HS^T and DSM 566^T showed 44% DNA–DNA relatedness and had relatedness values of 48% and 52%, respectively, with *S. natans* DSM 6575^T (see Supplementary Table S2 in IJSEM Online).

Based on phenotypic, chemotaxonomic and phylogenetic characteristics, strains HS^T and DSM 565 represent a novel species within the genus *Sphaerotilus* for which the name *Sphaerotilus montanus* sp. nov. is proposed. The type strain is HS^T. Strain DSM 566^T also represents a separate novel species, for which the name *Sphaerotilus hippei* sp. nov. is proposed.

The DNA–DNA hybridization results, the 16S rRNA, *hsp60* and *gyrB* gene sequencing studies and the phenotypic data revealed that the sulfur spring isolates, strains D-501^T, D-502, D-504, D-505 and D-507, were closely related to *S.*

natans DSM 6575^T, but they significantly differed from earlier known strains of *S. natans* by their capability for lithotrophic growth with reduced sulfur compounds and their ability to thrive in thermal sulfide habitats, and in some additional phenotypic characteristics. For this reason, strains D-501^T, D-502, D-504, D-505 and D-507 represent a separate taxon at the subspecies level and thus the name *Sphaerotilus natans* subsp. *sulfidivorans* subsp. nov. is proposed, with strain D-501^T as the type strain. The proposal of the novel subspecies automatically creates the subspecies *Sphaerotilus natans* subsp. *natans* subsp. nov., with strain DSM 6575^T as the type strain and strain D-380 as an additional member of this subspecies. Strain ATCC 15291 and the IF group of *Sphaerotilus* strains isolated from paper mill slimes have been previously assigned to the species *S. natans* (Kämpfer, 1997; Pellegrin *et al.*, 1999), but additional physiological and phylogenetic studies will be required to clarify the subspecies affiliation of these strains.

Emended description of the genus *Sphaerotilus* Kützing, 1833 (Approved Lists 1980)

In addition to organoheterotrophic growth, some strains (D-501^T, D-502, D-504, D-505 and D-507) are capable of both mixotrophic growth, with simultaneous utilization of organic substrates and sulfur compounds as electron donors for energy metabolism, and lithoheterotrophic growth using reduced sulfur compounds in energy metabolism (Gridneva *et al.*, 2009). Oxidation of sulfide is accompanied by deposition of sulfur granules within the cells. Able to develop in natural sulfide thermal springs. The temperature range for growth is 10–40 °C.

Description of *Sphaerotilus natans* subsp. *natans* subsp. nov.

Sphaerotilus natans subsp. *natans* (na'tans. L. part. adj. *natans* swimming).

The description is as given by Kützing (1833) (Kämpfer & Spring, 2005). The type strain is DSM 6575^T (=ATCC 13338^T=LMG 7172^T).

Description of *Sphaerotilus natans* subsp. *sulfidivorans* subsp. nov.

Sphaerotilus natans subsp. *sulfidivorans* subsp. nov. (sul.fi. di.vo'rans. N.L. n. *sulfidum* sulphide; L. part. adj. *vorans* consuming; N.L. part. adj. *sulfidivorans* sulfide-consuming).

Cell morphology is the same as that given for the genus (Kämpfer & Spring, 2005). Cells are 1.0–2.0 × 3.9–6.0 µm. Capable of developing in natural sulfide springs. The temperature range for growth is 15–40 °C, with optimum growth occurring at 32 °C. The pH range for growth is 6.3–8.5, with optimum growth at pH 7.5. Chemoorgano-heterotrophic; chemolithoheterotrophic; metabolism is respiratory and aerobic. Cells are able to use reduced

sulfur compounds as electron donors for energy conservation. When grown in a medium with sulfide and under the natural conditions of a sulfide spring, granules of elemental sulfur are accumulated. Acetate, lactate, succinate, pyruvate, malate, oxalate, oxaloacetate, 2-oxoglutarate, malonate, glucose, arabinose, galactose, sorbose, raffinose, sucrose, maltose, fructose, glycerol, ethanol, butanol, iso-butanol, mannitol, sorbitol, glutamate, aspartate, cysteine, cystine, alanine, phenylalanine, asparagine, proline, ornithine, peptone, yeast extract, casein hydrolysate and petroleum products are utilized as carbon and energy sources. Formate, salicylate, glyoxylate, glycolate, lysine and methionine are not utilized. Peptone, yeast extract, aspartate, glutamate, cysteine, casein hydrolysate, ammonium and nitrate are used as nitrogen sources. Methionine is not used as a nitrogen source. Nitrate is reduced to nitrite. Casein and starch are not hydrolysed; gelatin is not liquefied. Nitrate and fumarate are not used as electron acceptors. Indole is not formed. No growth in the presence of 3 % (w/v) NaCl. Positive in tests for oxidase and catalase activities. Pigments are not formed when the organism is grown on a medium containing aromatic amino acids. Acids are not produced from sugars. The major quinone is Q-8. The major fatty acids are $C_{16:1\omega7}$, $C_{16:0}$ and $C_{18:1\omega7}$. The G+C content of the DNA is 69.2 mol% (T_m).

The type strain, D-501^T (=DSM 22545^T=VKM B-2573^T), was isolated from a sulfur mineral thermal spring, located in the foothills of the North Caucasus, Russia.

Description of *Sphaerotilus montanus* sp. nov.

Sphaerotilus montanus sp. nov. (mon.ta'nus. L. masc. adj. *montanus* referring to a mountain, pertaining to an iron spring, located in the foothills of the North Caucasus, Russia, the place from where the strain was isolated).

Straight rods, $0.8\text{--}1.4 \times 3.3\text{--}6.0$ μm , arranged in chains within sheaths. Gram-negative-staining. The swimmers that are released from one of the ends of the filaments are motile by means of bundles of polar flagella. Possess sheaths with encrustations of ferric oxides. Filaments are not mobile. Branching is absent. Filaments may be attached by means of a holdfast to solid substrates. Cells contain granules of poly- β -hydroxybutyrate as a reserve material. Colonies with fibrous edges are rusty yellow-coloured due to the deposition of ferric oxides and are 2–5 mm in size. The temperature range for growth is 7–36 °C, with optimum growth occurring at 28–30 °C. The pH range for growth is 5.5–8.5, with optimum growth at pH 7.5. Chemoorganotrophic; metabolism is respiratory and aerobic. Lactate, pyruvate, fumarate, oxaloacetate, oxalate, 2-oxoglutarate, malate, malonate, citrate, benzoate, succinate, aconitate, glyoxylate; aspartate, glutamate, phenylalanine, tryptophan, proline, ornithine, alanine; glycerol, ethanol, butanol, propanol, mannitol, sorbitol; lactose, glucose, sorbose, mannose, sucrose, maltose, fructose, arabinose, peptone, yeast extract and casein hydrolysate

are utilized as carbon and energy sources. Acetate, formate, salicylate, glycolate, iso-citrate, iso-butanol, galactose, raffinose, cysteine, cystine, lysine, serine, methionine, histidine, asparagine, tyrosine and petroleum products are not utilized. Peptone, yeast extract, aspartate, glutamate, cysteine, casein hydrolysate, ammonium and nitrate are used as nitrogen sources. Methionine is not used as a nitrogen source. Nitrate is reduced to nitrite. Casein and starch are not hydrolysed; gelatin is not liquefied. Nitrate and fumarate are not used as electron acceptors. Indole is not formed. No growth in the presence of 3 % (w/v) NaCl is found. Positive in tests for oxidase and catalase activities. Pigments are not formed when the organism is grown on a medium containing aromatic amino acids. Acids are not formed in the presence of sugars. The major quinone is Q-8. The major fatty acids are $C_{16:1\omega7}$, $C_{16:0}$ and $C_{18:1\omega7}$. The G+C content of the DNA is 67.8 mol% (T_m).

The type strain, HS^T (=DSM 21226^T=VKM B-2519^T), was isolated from a ferrous iron-containing cold mountainous spring located in the foothills of the North Caucasus, Russia.

Description of *Sphaerotilus hippei* sp. nov.

Sphaerotilus hippei sp. nov. (hip'pe.i. N.L. gen. masc. n. *hippei* of Hippe, named after H. Hippe, a German microbiologist, who isolated this strain).

Cell morphology is the same as that given for the genus (Kämpfer & Spring, 2005). Cells are $0.7\text{--}1.5 \times 2.0\text{--}6.2$ μm . The temperature range for growth is 7–35 °C, with optimum growth occurring at 26–27 °C. The pH range for growth is 6.3–8.5, with optimum growth at pH 7.5. Chemoorganotrophic; metabolism is respiratory and aerobic. Acetate, lactate, succinate, pyruvate, malate, oxaloacetate, citrate, 2-oxoglutarate, malonate, glyoxylate, glucose, arabinose, sorbose, mannose, sucrose, maltose, fructose, glycerol, ethanol, propanol, butanol, iso-butanol, glutamate, histidine, proline, peptone, yeast extract and casein hydrolysate are utilized as carbon and energy sources. Formate, fumarate, oxalate, iso-citrate, benzoate, aconitate, mannitol, sorbitol, galactose, raffinose, lactose, serine, aspartate, cysteine, cystine, tryptophan, alanine, phenylalanine, asparagine, tyrosine, ornithine, glycolate, salicylate, lysine, methionine and petroleum products are not utilized. Peptone, yeast extract, aspartate, glutamate, cysteine, casein hydrolysate and ammonium are used as nitrogen sources. Methionine is not used as a nitrogen source. Nitrate is not reduced to nitrite. Casein and starch are not hydrolysed; gelatin is not liquefied. Nitrate and fumarate are not used as electron acceptors. Indole is not formed. No growth in the presence of 3 % (w/v) NaCl is found. Positive in tests for oxidase and catalase activities. Pigments are not formed when the organism is grown on a medium containing aromatic amino acids. Acids are not produced from sugars. The major quinone is Q-8. The major fatty acids are $C_{16:1\omega7}$, $C_{16:0}$ and $C_{18:1\omega7}$. The G+C content of the DNA is 69 mol% (T_m).

The type strain, DSM 566^T (=ATCC 29330^T), was isolated by H. Hippe from pond water, Guttingen, Germany.

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